A High-Speed Liquid-Chromatographic Method for Measuring Urinary Porphyrins

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We describe a rapid quantitative and qualitative “high-performance” liquid-chromatographic (HPLC) method for measuring porphyrins in urine. Direct injection of acidified, filtered urine onto a 3-µm (particle size) 3-cm-long reversed-phase column fully resolves uroporphyrin, hepta-, hexa-, and pentacarboxylic acid porphyrins, and coproporphyrin. Instrument response is linearly related to concentration over the range 25 to 300 nmol/L. The method provides data essential for the differential diagnosis of porphyric states, including porphyria variegata and porphyria cutanea tarda. This relatively inexpensive method requires a run time of only 8 min per sample, making it particularly suitable for routine use in the clinical chemistry laboratory.

Additional Keyphrases: chromatography, reversed-phase, porphyria variegata, porphyria cutanea tarda

Porphyropathies are diseases arising from inherited or acquired defects in the enzymes of the heme biosynthetic pathway. Quantitative and qualitative measurement of the urinary excretion of porphyrins and porphyrin precursors is an essential tool for the investigation and differential diagnosis of porphyria (1–3). Early methods involved solvent-extraction techniques, and only uroporphyrin and coproporphyrin could be separated and quantified (4). These methods were laborious and imprecise, and yielded inconsistent results for samples containing mixtures of highly carboxylated porphyrins. In the 1970s, “high-performance” liquid-chromatographic (HPLC) techniques were developed for biological samples; in these, porphyrins were esterified, separated on normal-phase columns, and quantified by their absorption at the Soret band (5–7). These approaches were an improvement over earlier techniques but the problems of poor resolution, low sensitivity, and an undetermined variable effect of esterification on the porphyrin pattern remained (8). The development of reversed-phase columns in the early 1980s eliminated the esterification step and the associated problems of chromatographic interpretation. Several authors (9–14) have used these reversed-phase systems, together with ultraviolet or fluorescence detection, to establish quantitative urine porphyrin analyses that involve minimal sample preparation. With all of these techniques, however, the retention times are long and the analytical columns are expensive, both of which limit their use in routine clinical laboratories.

Here we present a rapid method for quantitative and qualitative analysis for urinary porphyrins by HPLC. Sample preparation involves only acidification, filtration, and possibly dilution before the sample is injected into the chromatograph. Uroporphyrin, hepta-, hexa-, and pentacarboxylic acid porphyrins, and coproporphyrins are completely resolved in less than 5 min.

Materials and Methods

Standards and chemicals. Porphyrin standards were from Porphyrin Products, Logan, UT. Solutions of individual and mixed porphyrins were prepared by dissolving the hydrochloride salts in 3 mol/L HCl diluent. Subsequent dilution gave stock solutions of 1000 nmol/L in 0.1 mol/L HCl solution, which were stable for several weeks when stored in the dark at 4 °C. Working standards were prepared weekly and stored at 4 °C in the dark. Standard curves were prepared daily. HPLC-grade methanol was from Anachemia, Montreal, Quebec, Canada. Hydrochloric acid and sodium dihydrogen phosphate (NaH₂PO₄), both AR-grade, were from Fisher Scientific Co., Fair Lawn, NJ.

Chromatographic system. We used a Series 4 liquid chromatograph (Perkin-Elmer Canada Ltd., Montreal, Quebec, Canada) equipped with a Model 7125 20-µL loop injection valve (Rheodyne Inc., Cotati, CA). The analytical column was a Perkin-Elmer 3 cm × 0.43 cm (i.d.) cartridge column containing 3-µm-diameter octadeysilylsil particles. For quantification we used a Perkin-Elmer LS-4 flowthrough fluorescence spectrophotometer with excitation wavelength at 365 nm and emission wavelength at 624 nm (slit widths 15 and 20 nm, respectively).

Chromatographic conditions. All solutions were filtered through 0.20-µm (pore-size) filters (Millipore, Mississauga, Canada) before use. Porphyrins were eluted with a methanol/sodium phosphate gradient mobile phase. The column was equilibrated with an equivolume solution of methanol/sodium phosphate buffer (0.1 mol/L NaH₂PO₄, pH 3.5) at a flow rate of 2.5 mL/min. A linear gradient was automatically initiated on injection to bring the mobile phase to 95% methanol in 2 min, held there for 2 min, then returned to initial conditions by a 1-min linear gradient. A 3-min re-equilibration period brought the per-sample chromatographic run time to 8 min. All porphyrins were eluted from the column 5 min after sample injection, and were quantified by peak height. The direct method of sample preparation obviated use of an internal standard.

Specimens. Twenty-four-hour urine specimens collected with sodium carbonate (5 g/L) present were stored in the dark at 4 °C until analysis. (Specimens to be stored for longer than a week before analysis were maintained at −20 °C.) For analysis, we acidified a 2-ml aliquot of urine to pH 2.5–3.0 with 6 mol/L HCl, then filtered it through a 0.45-µm (pore-size) Millipore filter attached to a 1-mL disposable syringe (Becton Dickinson Canada Inc., Mississauga, Canada). The filtration process and the small injection volume obviated the need for sample degassing procedures, allowing direct injection into the HPLC.

Results and Discussion

Figure 1 illustrates a chromatogram of the commercially available mixed standard. These standards included mesoporphyrin, a dicarboxylic acid analog of protoporphyrin (however, dicarboxylic acid porphyrins are not excreted in the urine). Uroporphyrin, hepta-, hexa-, and pentacarboxylic acid porphyrins, and coproporphyrin are completely

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resolved within 5 min of sample injection. The detection limit is 0.5 pmol (25 nmol/L). As previously reported (15), sensitivity is greater with the detector excitation wavelength set at 400 nm, but high background fluorescence, owing to contaminants in the methanol of the mobile phase, necessitated that the excitation wavelength be 365 nm. The fluorometer response was linear over the range 0.5 to 6 pmol (25–300 nmol/L). Table 1 gives the precision of the assay at 25 and 300 nmol/L.

Figure 2 shows a chromatogram of urine from a normal individual; it contains baseline concentrations of uroporphyrin and 80 nmol of coproporphyrin per liter. The urinary porphyrin excretion pattern for a patient with porphyria cutanea tarda is shown in Figure 3. This pattern is consistent with a defect in uroporphyrinogen decarboxylase (EC 4.1.1.37), the enzyme responsible for the stepwise decarboxylation of uroporphyrinogen to hepta-, hexa-, and pentacarboxylic acid porphyrinogens, and ultimately to coproporphyrinogen (1). This defect subsequently results in an increased urinary excretion of the highly carboxylated porphyrins (relative excretion in terms of number of carboxylic acid groups per molecule: 8 > 7 > 6 > 5 > 4). In contrast, in a patient with porphyria variegata, deficiency of the enzyme protoporphyrinogen oxidase (EC 1.3.3.4) results in increased urinary excretion of the moderately carboxylated porphyrins (relative excretion: 4 > 5 > 6 > 7 > 8), as also illustrated in Figure 3. An unidentified peak is eluted between penta- and hexacarboxylic acid porphyrins in the sample from the patient with porphyria cutanea tarda. This peak did not correspond to any of the porphyrin standards and was seen only in urine from patients with this disease. An unidentified peak between pentacarboxylic acid porphyrin and coproporphyrin was observed in the patient with porphyria variegata, but low amplitudes of this peak were also observed in some urines from normal individuals. The identities of these peaks are currently being investigated. They may represent chromatographic resolution of alternative decarboxylation products. Clearly, the quantitative and qualitative patterns of these two porphyrinas are distinct and predictable from the specific site of each enzyme defect.

Table 1. Within-Run Coefficients of Variation for Two Porphyrin Standard Solutions

<table>
<thead>
<tr>
<th>Porphyrin</th>
<th>CV, % (n = 6 replicates)</th>
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<tr>
<td></td>
<td>25 nmol/L</td>
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<tr>
<td>Uroporphyrin</td>
<td>7</td>
</tr>
<tr>
<td>Heptacarboxylic</td>
<td>4</td>
</tr>
<tr>
<td>Acid</td>
<td>9</td>
</tr>
<tr>
<td>Hexacarboxylic</td>
<td>12</td>
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<td>Acid</td>
<td>12</td>
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<tr>
<td>Pentacarboxylic</td>
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<td>Acid</td>
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In porphyria cutanea tarda, the porphyrin concentrations were: uroporphyrin (u), 1540 nmol/L; heptacarboxylic acid porphyrin (7), 640 nmol/L; hexacarboxylic acid porphyrin (6), 50 nmol/L; pentacarboxylic acid porphyrin (5), 70 nmol/L; and coproporphyrin (c), 380 nmol/L. In porphyria variegata, uroporphyrin and hepta- and hexacarboxylic acid porphyrins were <25 nmol/L; pentacarboxylic acid porphyrin, 80 nmol/L; and coproporphyrin, 1850 nmol/L.

Fig. 1. Chromatogram of porphyrin standards, each at 6 pmol/injection: u: uroporphyrin; 7: heptacarboxylic acid porphyrin; 6: hexacarboxylic acid porphyrin; 5: pentacarboxylic acid porphyrin; c: coproporphyrin; and m: mesoporphyrin. Figs. 1–3: fluorescence detection; excitation 365 nm (15-nm slit), emission 624 nm (20-nm slit)

Fig. 2. HPLC separation of porphyrins in urine from a healthy volunteer. Coproporphyrin, c (80 nmol/L)

Fig. 3. HPLC separation of porphyrins in urine of a patient with porphyria cutanea tarda (left) and porphyria variegata (right)
excretion. The high-speed cartridge columns we used in this assay showed remarkable longevity (>700 samples or standards assayed per column), even in the absence of a guard column. This, coupled with minimal sample preparation, short chromatography time, and low relative cost of each cartridge (compared with traditional C18 columns), renders this system ideal for routine use in a clinical chemistry laboratory. Furthermore, because porphyric urines invariably require extensive dilution before assay, HPLC systems involving less-sensitive fluorometers, but suitably equipped with a red-sensitive photomultiplier tube, would probably be able to readily distinguish normal from porphyric urines.

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References