Biochemical Diagnosis and Monitoring Therapeutic Modulation of Disease Activity in an Unusual Case of Congenital Erythropoietic Porphyria

Sudhir K. Mukerji, Neville R. Pimstone, Shoba N. Gandhi, and Kwan T. Tan

We describe the methodology used for quantifying and characterizing porphyrins in various tissues and in excreta, in the diagnosis and monitoring of the therapeutic modulation of biochemical disease activity in a 53-year-old white man who has a rare form of familial porphyria cutanea tarda with bone marrow rather than hepatic expression of the disease. Liquid-chromatographic and thin-layer chromatographic analyses of the patient's urine and skin showed predominantly hepta-carboxylic porphyrin and uroporphyrin, whereas his stool and bile contained isocoproporphyrin and coproporphyrin as the major products. The data reflect defective uroporphyrinogen decarboxylation. Both analytical methods gave quantitatively similar results for urinary and fecal porphyrins. A triple-lumen perfusion study of samples procured both at the ampulla of Vater and 15 cm downstream provided data for porphyrins excreted in the bile and their reabsorption in the small intestine. We evaluated: (a) suppression by hypertransfusion of bone marrow overproduction of porphyrins and (b) reduction of enteral absorption of porphyrins by orally administered charcoal (Acta Char) and cholestyramine.


Porphyria cutanea tarda (PCT), the most common human porphyria, is characterized by diminished activity of uroporphyrinogen decarboxylase (EC 4.1.1.37), a cytochrome involved in heme biosynthesis (I). In familial PCT, an autosomal dominantly inherited disorder, the enzyme activity is reportedly about half of normal in the liver and erythrocytes (2-4), with a concomitant decrease in immuno-reactive activity of the enzyme in erythrocytes (5-7). In the sporadic subtype of the disease, however, the catalytic (3, 8) and immunoreactive enzyme activities (5, 6) in the erythrocytes are normal, despite deficient enzyme activity in the liver (2, 3, 8, 9). There are rarer forms of spontaneous uroporphyrinogen decarboxylase deficiency (10), one of which is expressed in a patient (G.B.) in whom results of erythrocyte studies in kindred are consistent with familial PCT (11). In this patient, erythrocyte uroporphyrinogen decarboxylase activity is less than 50% of control values (12), and two of his seven children exhibit similarly decreased enzymic activity but without porphyria. The expression of the metabolic defect in the bone marrow has been ascribed to a congenital dyserythropoietic anemia type I (11), in which the skin porphyrins derived from those circulating in the plasma have resulted in disfiguring photomutilation.

Patient G.B., a 53-year-old white man, presented a therapeutic challenge to develop new modalities of therapy. This provided an ideal clinical situation to evaluate and modify existing "high-performance" liquid-chromatographic (HPLC) and thin-layer chromatographic (TLC) methods for measuring porphyrins in excreta and in various tissues. By these methods, the quantitative characterization of porphyrins in the urine, bile, feces, plasma, erythrocytes, and skin yielded a classic porphyrin profile of uroporphyrinogen decarboxylase deficiency. Moreover, we evaluated the existence of an enteral limb of an active enterohepatic circulation of porphyrins in a triple-lumen perfusion study, by which we obtained samples from the intestinal lumen at various locations in the proximal small bowel. Quantifying porphyrins in plasma, feces, and urine allowed for critical monitoring of the efficacy of hypertransfusion to suppress overproduction of porphyrin in the bone marrow and the ability of drugs such as charcoal and cholestyramine, which bind porphyrins in vitro, effectively to interrupt the enteral absorption of porphyrins. Finally, we directly tested the ability of an acute oral charcoal challenge to deplete the skin of porphyrins. Here we review in depth the methodologies used as well as their usefulness in monitoring the course of the disease and the impact of various therapies.

Materials and Methods

Procurement of Samples

Specimens of skin, colonic mucosa, plasma, bile, stool, and urine were procured from patient G.B., who was postulated to have bone-marrow expression of familial PCT. His photomutilation has included loss of fingers, earlobes, and lips (Figure 1), caused by massive accumulation of porphyrins in the skin. The enterohepatic circulation of porphyrins was evaluated by a triple-lumen perfusion study. The details of this will be published elsewhere, but bile-stained intestinal perfusate at the ampulla of Vater and 15 cm downstream was procured by aspiration from proximal and distal ports. Porphyrin quantification (allowing for water flux) at these two sites demonstrated highly efficient enteral absorption of endogenous porphyrins (13).

By monitoring plasma porphyrins in serum for a week, we evaluated the effects of treatment with porphyrin-binding drugs such as cholestyramine (4 g every 6 h) and charcoal (30 g every 3 h) as well as the effect of hypertransfusion with 14 pints of blood. We also quantified and characterized the concentrations of porphyrin in skin tissues, before and after therapy with charcoal.

Materials

A porphyrin ester marker kit containing eight-, seven-, six-, five-, and four-carboxylic porphyrins, and mesoporphyrin of the isomer I series was from Porphyrin Products, Logan, UT; TLC aluminum sheets pre-coated with silica gel 60 were from Wilshire Chemical Co., Gardena, CA; all other
HPLC. We redissolved the porphyrin methyl esters in 100 μL of dichloromethane, then injected 10–20 μL of this into a Waters Associates chromatograph (Model ALC/GPC 201) equipped with two Model 6000 A pumps and a normal-phase 30 x 0.39 cm μPorasil column. We equilibrated the column with ethyl acetate/cyclohexane (40/60 by vol), which also was the mobile phase. The absorbance of the column effluent at 405 nm was recorded with a Waters Model 440 detector and the porphyrin methyl esters were identified by comparing their retention times with those of a standard mixture of porphyrin methyl esters. The porphyrins were quantified from their peak areas, with use of a Hewlett Packard 3390A Reporting Integrator.

TLC. The porphyrin methyl esters, in dichloromethane, were spotted onto the TLC plate and chromatographed with known porphyrin esters as markers. The solvent, CCl₄/CH₂Cl₂/ethyl acetate/ethyl propionate (2/2/1/1, by vol), was allowed to move to within 1 cm from the top edge of the plate. After drying the plate, we enhanced and stabilized the fluorescence of the separated porphyrin ester bands by immersing the plate in a second solvent containing dodecane/hexadecane/CHCl₃ (3/3/14, by vol) (15). The fluorescent spots corresponding to the porphyrins were identified, then quanitified with a Schoeffel SD 300 densitometer set at an excitation wavelength of 399 nm and an emission wavelength of 620 nm. Isocoprotoporphyrin was characterized according to its mobility on TLC plates, as reported by Day et al. (15).

Results

Quantitative Analyses for Porphyrins in Urine, Stool, Plasma, Bile, and Colonic Mucosa

Analysis for patient GB's urinary porphyrins by HPLC and TLC (Figure 2 and Table 1) showed heptacarboxylic porphyrin to be the major component (49% of total), uroporphyrin (27%) the second most abundant. In stool, coproporphyrin (40%) and isocoprotoporphyrin (25%) were the major porphyrins (Figure 3). Isocoprotoporphyrin, a distinct peak by TLC, was barely separated from coproporphyrin by HPLC. Both methods gave about the same values for total urinary and fecal porphyrins, i.e., approximately 10 mg/24 h and 250 μg/g dry weight, respectively.

Hexacarboxylic (30% of total), pentacarboxylic (23%), and heptacarboxylic (17%) porphyrins were the major components in the patient's plasma (Figure 4). The porphyrin peaks were clearly separated in both methods. TLC analysis, however, revealed an additional single peak for isocoprotoporphyrin (10%) and a porphyrin peak co-migrating to about the same distance as the coproporphyrin marker. The latter probably represents a complex between coproporphyrin and an unknown molecule characterized as "Pu" by Day et al. (15). When we quantified plasma porphyrins, the values obtained by TLC were about 20% higher than those obtained by HPLC.

TLC analysis for porphyrin in bile (Figure 5) gave distinctly superior results, although both TLC and HPLC revealed coproporphyrin as the major compound (approximately 49% of total) in bile. Copro-, isocopro- and pentacarboxylic porphyrins were clearly separated by TLC.

HPLC analysis for porphyrins in biopsied colonic mucosa (13.4 mg fresh weight) showed clear separation of 2- to 8-carboxylic porphyrins (Figure 6 and Table 1).

Effect of Oral Administration of Charcoal or Cholestyramine, and of Suppression of Bone Marrow, on Porphyrin Concentrations in Plasma

The data shown in Figure 7 indicate a dramatic and rapid decline in plasma porphyrins after administration of char-
Fig. 2. HPLC (A) and TLC (B) separation of porphyrin methyl esters extracted from the urine of patient G.B.
(A): Mobile phase: ethyl acetate/cyclohexane (40:60 by vol); flow rate 1.5 mL/min. Vertical axis, absorbance at 405 nm. The arrow notes an anomalous peak. 3, uroporphyrin; 7, heptacarboxylic porphyrin; 6, hexacarboxylic porphyrin; 5, pentacarboxylic porphyrin; 4, coproporphyrin

Table 1. Proportions of Porphyrins with Two- to Eight-Carboxyls in Urine, Stool, Plasma, Bile, Colonic Mucosa, and Skin of the Patient*

<table>
<thead>
<tr>
<th>Samples</th>
<th>Uro</th>
<th>Hept</th>
<th>Hesx</th>
<th>Penta</th>
<th>Copro and isotocopher</th>
<th>Proto</th>
<th>Total porphyrin contentb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>26.7</td>
<td>49.4</td>
<td>14.2</td>
<td>4.1</td>
<td>5.8</td>
<td></td>
<td>10.5 mg/24 h</td>
</tr>
<tr>
<td>Stool</td>
<td>—</td>
<td>2.5</td>
<td>6.1</td>
<td>6.7</td>
<td>65.4</td>
<td>19.4</td>
<td>227.6 µg/g dry wt</td>
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<tr>
<td>Plasma</td>
<td>6.9</td>
<td>16.9</td>
<td>30.5</td>
<td>22.7</td>
<td>9.9</td>
<td>13.2</td>
<td>539 µg/L</td>
</tr>
<tr>
<td>Bile</td>
<td>—</td>
<td>2.4</td>
<td>6.2</td>
<td>35.6</td>
<td>48.0</td>
<td>6.9</td>
<td>19.7 mg/L</td>
</tr>
<tr>
<td>Colon</td>
<td>4.7</td>
<td>7.6</td>
<td>15.1</td>
<td>—</td>
<td>15.9</td>
<td>56.9</td>
<td>5.3 µg/g wet wt</td>
</tr>
<tr>
<td>Skin</td>
<td>27.0</td>
<td>46.3</td>
<td>10.6</td>
<td>6.8</td>
<td>5.6</td>
<td>3.9</td>
<td>1-5 µg/g wet wt</td>
</tr>
<tr>
<td>Before charcoal</td>
<td>7.6</td>
<td>9.9</td>
<td>21.2</td>
<td>24.5</td>
<td>36.8</td>
<td>—</td>
<td>0.01-0.04 µg/g wet wt</td>
</tr>
<tr>
<td>After charcoal</td>
<td>—</td>
<td>14.3</td>
<td>10.6</td>
<td>6.8</td>
<td>5.6</td>
<td>3.9</td>
<td>1-5 µg/g wet wt</td>
</tr>
</tbody>
</table>

*Porphyrins analyzed by HPLC. bNormal values: urine <250 µg/24 h; stool <140 µg/g dry wt; plasma <48 µg/L.

coal and cholestyramine, to values that became constant in 15 h, remaining at these concentrations throughout the seven days of the study. Cholestyramine caused a decrease of about 60%, whereas with charcoal the decrease was to near normal by 15 h.

Hypertransfusion with bone marrow suppression resulted in a 70 to 80% decrease in plasma porphyrin concentrations, more effective than cholestyramine, but the rate of decrease was in days rather than in hours.

Effect of Orally Ingested Charcoal on Skin Porphyrins

In Figure 8A and B, we show elution profiles of skin porphyrins measured by HPLC method before and after therapy with charcoal. A punch skin-biopsy sample of 15 mg fresh weight before therapy with charcoal sufficed for quantitative analysis for 2- to 8-carboxylic porphyrins, which were clearly resolved under our analytical conditions. Analysis of as little as 5 mg (wet weight) of skin showed clear separation of porphyrin peaks. Here, heptacarboxylic porphyrin (46%) and uroporphyrin (27%) were the major porphyrins. Thus, the pattern of porphyrins in the patient's skin and urine were very similar. Within two days after therapy with charcoal was begun, the patient's total skin porphyrin content had declined from about 1-5 µg/g wet
weight to about 0.01–0.04 μg/g wet weight (Table 1). Heptacarboxylic porphyrin and uroporphyrin in particular were decreased by the treatment.

**Discussion**

This is an atypical case of congenital erythropoietic porphyria (II). The presence of fluorescent normoblasts, the absence of measurable porphyrins in the patient's (nonfluorescent) liver, and the defective erythrocyte uroporphyrinogen decarboxylase enzyme present in the proband and in two of his seven children strongly suggest that his disease represents the expression of familial PCT by bone marrow instead of by the liver (II). His photomutilation, a classic feature of congenital erythropoietic porphyria, is accompa-
and these porphyrins were clearly separated by TLC. Isocoproporphyrin, a specific marker of uroporphyrinogen decarboxylase deficiency, is also a major fecal porphyrin in patients with PCT (17). Quantitatively, HPLC and TLC methods yielded the same results for urinary and fecal porphyrin.

TLC analyses of the patient's plasma and bile also revealed the presence of isocoproporphyrin. Patient's bile contained about 49% coprop- and isocoproporphyrins (Table 1). These data are relevant to current understanding of the disease: i.e., defective decarboxylation of pentacarboxylic porphyrinogen favors the formation of dehydroisocoproporphy- rinogen, instead of coproporphyrinogen, owing to the conversion of the 2-propionate group of pentacarboxylic porphyrinogen to a vinyl group before decarboxylation of the acetic acid residue at position 5 (17, 18). Excess dehydroisocoproporphyrin is excreted in the bile of PCT patients. This is modified by bacterial enzymes in the intestine, which act on the vinyl groups to produce isocoproporphyrin (19). Plasma porphyrins, presumably derived from the bone marrow and from equilibration with the enterohepatic cycling of endogenous porphyrins, are in a rapid turnover pool.

The striking decrease in plasma porphyrin (Figure 7) after hypertransfusion to suppress bone marrow (20) confirms bone marrow expression of the metabolic lesion, but monitoring of the plasma porphyrins over a year has indicated that this suppression was effective for only about seven to 10 days (data not shown).

The triple-lumen perfusion study revealed the passage of more than 200 mg of porphyrin per day at the level of the ampulla of Vater with a near 95% reabsorption of all porphyrin elements in the proximal 15 cm of the small bowel (data not shown).

The ability accurately to quantify porphyrins in bile-stained intestinal perfusate permitted evaluation of the effectiveness of the orally administered drugs, charcoal and cholestyramine, which bind porphyrins in vitro, as an intraluminal sump entrapping porphyrins in the gut. These drugs rapidly depleted the systemic plasma porphyrins (Figure 7), suggesting rapid equilibration with the depleted portal venous porphyrin pool. The methodology also has allowed long-term surveillance of the biochemical efficacy of charcoal therapy (21).

With regard to the skin, heptacarboxylic porphyrin (46%) followed by uroporphyrin (27%) were the major porphyrins—a profile similar to that of urinary porphyrins. The high initial skin porphyrins (1–5 μg/g wet weight) were decreased to insignificant concentration (0.01–0.04 μg/g wet weight) by two days of oral therapy with charcoal, as documented by HPLC of a 15-mg punch-biopsy sample.

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References
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