Copper Coproporphyrin Excretion in Familial Coproporphyria

Robert E. Carlson,¹ D. Dolphin,¹,³ and Melvyn Bernstein²,³

Analysis of stool specimens from a patient with familial coproporphyria by high-performance liquid chromatography revealed that 112 μg per gram of dry feces (14% of the total porphyrin present) was copper coproporphyrin. Examination of stool specimens from other patients with this disease confirmed the presence of significant amounts of both copper coproporphyrin and coproporphyrin. Further investigation showed that the copper coproporphyrin was probably formed by a nonenzymic incorporation of copper by the coproporphyrin in either the bile or feces.

Additional Keyphrases: inherited disorders  •  porphyrin metabolism  •  trace elements  •  fecal porphyrins

In 1949, Watson et al. (1) described two men who excreted large amounts of coproporphyrin in their feces. Six years later, Berger and Goldberg (2) described a family with the same symptoms and subsequently named the condition hereditary coproporphyria. Recently Elder et al. (3) used cultured skin fibroblasts from three patients with hereditary coproporphyria to show that this condition is associated with decreased activity of the enzyme coproporphyrinogen oxidase. While developing a high-performance liquid-chromatographic method to measure porphyrins in biological material, we observed an unusual compound in the stool of a patient suffering from an acute attack of hereditary coproporphyria.

This paper describes the characterization of this compound as copper coproporphyrin and gives a probable account of its origin.

Methods and Materials

Ultraviolet–visible spectra were recorded with a Cary 17 spectrophotometer. Mass spectra were recorded with a Varian/ mat CHAB using direct probe insertion at 70 eV and 230–330 °C. The chromatograph was a Waters Associates' ALC 202. The water used was distilled and de-ionized.

Specimens

Twenty-four hour fecal samples, collected from all patients and controls, were stored frozen at −40 °C until analyzed. No preservatives were used.

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Patients

A.B., a 38-year-old woman, presented with acute porphyria after ingesting diet pills of dubious origin. We could never ascertain exactly what these were. At the time she was first seen, her urine was positive for porphobilinogen and coproporphyrin and she was excreting large amounts of coproporphyrin in her feces. Two fecal samples were collected from her, one at this time and another four months later, at which time her urine was completely free of porphyrins, with no porphobilinogen present. The fecal coproporphyrin had also decreased considerably.

G.H. is an 18-year-old boy who suffered brain damage, with the removal of an intracranial hemangioma, in 1962. Since then, he has suffered from mild mental retardation, left hemiparesis, and epilepsy. His epilepsy is controlled with drugs, including phenobarbital, hydantoins, and primadone. His urine has been frequently positive for porphobilinogen and coproporphyrin and his feces contain large amounts of coproporphyrin. It has proved impossible to take him off his antiepileptic medication and so he continues to excrete large amounts of coproporphyrin in his feces.

F.H. is the father of G.H., age 45 years. Routine screening after G.H.'s diagnosis showed increased coproporphyrin in his feces. He is asymptomatic.

L.H. is a sister of G.H., age 16 years. She is asymptomatic. However, increased coproporphyrin was found in her feces.

C.D. was admitted as an emergency with ascending polyneuropathy. Her urine was positive for porphobilinogen and porphyrins. Her feces were strongly positive, with increased protoporphyrin and coproporphyrin. She required treatment with intermittent partial pressure respiration and has gradually recovered neurological function. During the latent phase all porphyrins and porphobilinogen disappeared from the urine. The feces showed increased prophylin and porphyrin X complexes. Variegate porphyria was diagnosed.

S.A. This sample, from Dr. L. Eales, was from a patient diagnosed as having erythropoietic protoporphyrinia.

Control specimens were collected from hospital patients who did not have liver disease and who gave negative tests on screening for porphyrins. None had any condition that would be expected to affect porphyrin metabolism.

Sample Preparation

We added 20 ml of methanol and 2 ml of boron trifluoride etherate (BF₃·Et₂O) or 1 ml of concd. H₂SO₄ to the fecal sample (0.5 g wet or 0.2 g dry) and stirred the mixture overnight. Esterified material was extracted with two 100-ml portions of methylene dichloride after adding 80 ml of water.
Table 1. Results of Chromatography of Copper Coproporphyrin Test Samples as Compared with Coproporphyrin Feces

<table>
<thead>
<tr>
<th>Sample</th>
<th>Estification</th>
<th>TLC analysis</th>
<th>Result $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feces</td>
<td>MeOH/BF$_3$</td>
<td>A</td>
<td>Cu-copro (+)</td>
</tr>
<tr>
<td>Feces</td>
<td>MeOH/H$_2$SO$_4$</td>
<td>A</td>
<td>Cu-copro (+)</td>
</tr>
<tr>
<td>Copro</td>
<td>MeOH/BF$_3$</td>
<td>A</td>
<td>Cu-copro (+)</td>
</tr>
<tr>
<td>Proto</td>
<td>MeOH/BF$_3$</td>
<td>B</td>
<td>Cu-proto (-)</td>
</tr>
<tr>
<td>Feces + copro</td>
<td>MeOH/BF$_3$</td>
<td>A</td>
<td>Cu-copro (+)</td>
</tr>
<tr>
<td>Feces + proto</td>
<td>MeOH/BF$_3$</td>
<td>A</td>
<td>Cu-copro (-)</td>
</tr>
</tbody>
</table>

$^a$ See experimental section for solvent system used.

$^b$ (+) = present, i.e., copper coproporphyrin Soret ca. 25% of coproporphyrin Soret; (-) = absent, i.e., copper coproporphyrin Soret <1% of coproporphyrin Soret; (±) = greatly reduced relative to total coproporphyrin.

40/1/1 by vol; copper coproporphyrin $R_F$ 0.71) or solvent B (methylene dichloride/methanol/formic acid 400/3/3 by vol); copper protoporphyrin and copper coproporphyrin had $R_F$ values of 0.35 and 0.17, respectively, in this system.

Identification of Copper Coproporphyrin

The stop-flow visible scan of the compound in the tricarboxylic acid–porphyrin region of the liquid-chromatographic analysis of fecal samples from A.B. gave a metalloporphyrin spectrum:

<table>
<thead>
<tr>
<th>Peak (nm)</th>
<th>Relative absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>399</td>
<td>27.8</td>
</tr>
<tr>
<td>525</td>
<td>1.00</td>
</tr>
<tr>
<td>561.5</td>
<td>1.72</td>
</tr>
</tbody>
</table>

By thin-layer chromatography (solvent A), a sample of this compound was obtained by collection of the pink, non-fluorescent band at $R_F = 0.71$. This compound was demetallated with H$_2$SO$_4$ but not with HCl. On thin-layer chromatography in solvent-system A, the metal-free porphyrin had the same $R_F$ (0.55) as an authentic sample of coproporphyrin. A synthetic sample of copper coproporphyrin had the same $R_F$ in solvent A and the same visible spectrum in methylene dichloride as did the unknown. The unknown was confirmed to be copper coproporphyrin by its mass spectrum ($m/e = 774$).

Metallation Studies

Stock solutions of copro- and protoporphyrin were prepared immediately before use by dissolving the porphyrin in conc. HCl, followed by dilution to give a 0.01 mol/liter HCl and 0.16 mmol/liter porphyrin solution. The copper(II) acetate (0.80 mmol/liter) was prepared in water; 5 and 0.5 mol/liter NaOH solutions were used to adjust the pH.

Rate of porphyrin metallation was studied by monitoring the change of absorbance for the change of free metal to metalloporphyrin (coproporphyrin, 398 nm; protoporphyrin, 407 nm) in solutions prepared from 0.50 ml of porphyrin stock adjusted to the appropriate pH and diluted to 0.88 ml with water. At time zero, 0.12 ml of the copper acetate stock was added.

Competition studies were performed on solutions prepared from 0.50 ml of the copro- and protoporphyrin stock, adjusted to the appropriate pH. We added 0.17 ml of copper acetate stock and stirred the solution for 5 min. The porphyrins were esterified in the usual way by adding 25 ml of H$_2$SO$_4$/methanol (5/95 by vol). Separation/quantitation was by liquid chromatography.

Fig. 1. Chromatogram of a coproporphyrionic fecal sample containing copper coproporphyrin

$^n$Heptane/methyl acetate (75/25 by vol) at 1.5 ml/min

The methylene dichloride solution was dried over anhydrous sodium sulfate, filtered, and the solvent removed with a rotary evaporator.

The residue was dissolved in methylene dichloride (1.0 ml/100 mg of dry feces) for analysis.

Chromatography

For the chromatographic analysis we used a $\mu$-Porsil (Water Associates) column and the Cary 17 detection system described previously (4). The solvent system of Gray et al. (5) was used with the indicated ratios by volume.

Thin-layer chromatographic analysis was performed with solvent A (methylene dichloride/ethyl acetate/methanol, 12/8/1 by vol) at 1.5 ml/min.
Table 2. Analysis of Porphyric Fecal Samples for Copper Coproporphyrin

<table>
<thead>
<tr>
<th>Patient</th>
<th>Porphyrin (number of carboxyl groups)</th>
<th>µg/g dry weight</th>
<th>Cu coproporphyrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.B. (acute)</td>
<td>58 — 790</td>
<td>— — — —</td>
<td>112</td>
</tr>
<tr>
<td>A.B. (remission)</td>
<td>16 36 564</td>
<td>— — — —</td>
<td>74</td>
</tr>
<tr>
<td>G.H.</td>
<td>52 62 1220</td>
<td>T* — —</td>
<td>42</td>
</tr>
<tr>
<td>F.H.</td>
<td>43 43 96</td>
<td>— — — —</td>
<td>8</td>
</tr>
<tr>
<td>L.H.</td>
<td>69 18 199</td>
<td>— — —</td>
<td>5</td>
</tr>
<tr>
<td>C.D.</td>
<td>686 161 985</td>
<td>57 73 42 57</td>
<td>20</td>
</tr>
<tr>
<td>S.A.</td>
<td>490 T 73</td>
<td>13 T — —</td>
<td>1</td>
</tr>
</tbody>
</table>

* Trace.

Table 3. Competition Studies: Metallation of Copro and Protoporphyrin by Copper

<table>
<thead>
<tr>
<th>pH</th>
<th>% Metallation</th>
<th>% Metallation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>5.5</td>
<td>45</td>
</tr>
<tr>
<td>8.0</td>
<td>17</td>
<td>91</td>
</tr>
</tbody>
</table>

Results

Liquid chromatographic analysis of the fecal porphyrins from a patient with familial coproporphyria led to the isolation and identification of copper coproporphyrin (Figure 1). Analysis of suitable test samples by liquid- (Figure 2) and thin-layer (Table 1) chromatography proved that the copper coproporphyrin was not formed during the esterification/extraction procedure, either from extraneously introduced copper (8) or from copper present in the feces but not already combined with coproporphyrin. Reagent blank tests with coproporphyrin showed <0.1% copper coproporphyrin.

Analysis and quantitation of additional porphyric fecal samples gave the results summarized in Table 2. Additionally, four of five nonporphyric control fecal samples contained small amounts of copper coproporphyrin (0.1–1.0 µg/g) as well as the expected protoporphyrins (15–53 µg/g) and coproporphyrins (1.1–2.0 µg/g).

No other metallated porphyrins, in particular, no copper protoporphyrin, were observed in samples from any of the patients or controls.

The apparent preference of coproporphyrin for the available copper was found to be the result of the difference in the rate of metallation. In both competition (Table 3) and rate (Figure 3) studies, the formation of copper coproporphyrin was clearly favored by the affinity of coproporphyrin for copper and the rate of reaction at physiological pH. Formation of copper protoporphyrin is much less favored under comparable conditions.

Discussion

Although copper and zinc porphyrins have occasionally been observed in porphyric samples (6, 7), the presence of metallated porphyrins has usually been regarded as resulting from extraneously introduced copper (8).

The chromatographic analyses of coproporphyrin, protoporphyrin, and copper-supplemented fecal samples from a hereditary coproporphyria show that the coproporphyrin fecal samples contain amounts of copper coproporphyrin that are well above those that could be accounted for by contamination. Analysis of samples from additional patients with hereditary coproporphyria and other forms of porphyria, as well as samples from nonporphyric controls, showed the presence of copper coproporphyrin in all but one of the samples. No other copper porphyrins were observed in any of the samples.

Although the apparent specificity of copper coproporphyrin formation may be the result of a specific in vivo process (e.g., enzyme-mediated metallation) the results of in vitro coproporphyrin and protoporphyrin metallation studies (see also Doss (9)) indicate that the absence of copper protoporphyrin could result from the extremely high affinity of coproporphyrin for copper. However, the absence of any copper protoporphyrin in the non-coproporphyrin porphyric samples where protoporphyrin concentrations are significantly increased is particularly difficult to explain on the basis of pure competition. However, pH may be a significant additional factor, because acidic conditions can result in the preferential precipitation of the protoporphyrin.

The observed variability in the amounts of copper coproporphyrin is also probably due to the different rates of incorporation with changes of pH. Thus, the pH of the bile and feces, which are the likely sites of copper coproporphyrin formation, may affect the percentage of copper coproporphyrin formed at given concentrations of the porphyrin and copper, as well as preventing the formation of copper protoporphyrin.

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References


