New Method for Liquid-Chromatographic Measurement of Erythrocyte Protoporphyrin and Coproporphyrin

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We describe a “high-pressure” liquid-chromatographic method for separating all the porphyrins in the heme biosynthetic pathway. The preliminary extraction and purification method is such that only erythrocyte porphyrins with fewer than five carboxyl groups, which includes protoporphyrin and the much smaller amount of coproporphyrin present, are measured. The porphyrins extracted from erythrocytes are chromatographically separated by use of a simple eluent system and fluorometrically detected.

Additional Keyphrases: reference intervals • anemia • lead poisoning

Protoporphyrin and coproporphyrin are present in normal human erythrocytes. Protoporphyrin IX and the reduced form of coproporphyrin, coproporphyrinogen, are both intermediates in heme biosynthesis. Normally they are present in very small amounts: the molar ratio of protoporphyrin to heme in the circulating erythrocytes is about 1:30,000, and coproporphyrin comprises only 5% or less of the total porphyrin in erythrocytes. In some circumstances the amounts of the erythrocyte porphyrins may be increased or decreased, reflecting disturbances in heme biosynthesis.

The best-known disturbances that increase the amount of erythrocyte protoporphyrin are lead intoxication (1–5), iron-deficiency anemia (1–5), and erythropoietic protoporphyria (6–8). In some cases of sideroblastic anemia, erythrocyte protoporphyrin may be decreased and coproporphyrin increased (9, 10). It has been claimed that the concentration of coproporphyrin in the erythrocyte is more closely connected to the rate of heme biosynthesis than is the protoporphyrin concentration and, accordingly, a better correlation should exist between the percentage of reticulocytes and the quantity of coproporphyrin (11). However, it is evident that there are only a few disease states in which increased erythrocyte coproporphyrin values are seen. These include, e.g., congenital erythropoietic porphyria (6), hereditary coproporphyria (6), and sideroblastic anemia (10).

In the solvent-partition methods that have been used to separate erythrocyte protoporphyrin and coproporphyrin, there is always the likelihood that protoporphyrin contaminates the coproporphyrin fraction, because of the great excess of protoporphyrin as compared with coproporphyrin. In fact, Christensen and Romslo (12) have shown that up to 30% of 2-carboxyl porphyrins (including protoporphyrin) dissolved in ether is extracted into 0.1 mol/L HCl. Furthermore, the formation of the hematoporphyrin having almost the same HCl-number (0.1) as coproporphyrin (0.09) (13) is likely to lead to a falsely high result for coproporphyrin and a falsely low one for protoporphyrin. The thin-layer chromatographic methods, even if more nearly quantitative in separating protoporphyrin and coproporphyrin, are too laborious for routine use (14, 15).

Here we describe a “high-pressure” liquid-chromatographic method with which erythrocyte porphyrins can be quantitatively separated from one another. This method is more specific than the solvent-partition methods used before and is sensitive enough to be used in the study of erythrocyte porphyrin metabolism in general.

Materials and Methods

Apparatus

The chromatographic system we used consisted of two solvent-metering pumps (Model 110 A; Altex Scientific Inc., Berkeley, CA 94710), a syringe-loading injector (Model 7125; Rheodyne Inc., Berkeley, CA 94710), a solvent programmer (Model 1601 A, Altex), a fluorescence detector for liquid chromatography (Model FS-970; Schoeffel Instrument Corp., Westwood, NJ 07675), and a strip-chart recorder (Omni Scribe Series B-500; Houston Instrument, Austin, TX 78753). A 30 cm × 3.9 mm μBondapak C18 column (Waters Associates, Milford, MA 01757) was used throughout the study. The excitation filter used in the fluorescence detector was a broad-band filter 7-59, with half-transmission from 320 nm to 432 nm and maximum transmission at 396 nm, and the emission filter was a cut-off filter KV 470, with transmission of 10−22 at 430 nm and 0.99 at 505 nm. A Varian Cary spectrophotometer (Model 118; Varian Instrument Division, Cary Products, Palo Alto, CA 94303) was used to measure the porphyrin spectra.

Reagents

Porphyrins: We used a Porphyrin Acid Chromatographic Marker Kit (lot no. D5; Porphyrin Products, Logan, UT 84321) containing measured amounts of the following six porphyrins of the type I series: uroporphyrin, 7-carboxyl porphyrin, 6-carboxyl porphyrin, 5-carboxyl porphyrin, coproporphyrin, and mesoporphyrin IX.

Protoporphyrin IX dimethyl ester (lot. no P-5889) and coproporphyrin III tetramethyl ester (lot no. C-1880) were from Sigma Chemical Co., St. Louis, MO 63167.

Internal standard: Fluorescein (a “pro analysis” product from Merck, Darmstadt, F.R.G.) was used as an internal standard in the samples.

Solvents: Methanol and glacial acetic acid were both purchased from Merck and were “p.a.” quality.

Other reagents: Ethyl acetate (p.a., Merck), sodium acetate, solid (p.a., Merck), HCl, concd. (p.a., Merck), and diethyl ether, peroxide-free (Orion, Helsinki, Finland).

Preparation of standards: For routine work, protoporphyrin IX dimethyl ester was hydrolyzed to the free acid according to Bonkowski et al. (16) and coproporphyrin III tetramethyl ester was hydrolyzed to coproporphyrin III according to Mauzeral et al. (17). These porphyrins were >95% pure, as confirmed by thin-layer chromatography. The standards were dissolved in solution A (see below). The concentration of the protoporphyrin IX solution was 13.1 μmol/L,
that of the coproporphyrin III solution 7.3 μmol/L. In the standard porphyrin mixture the concentration of each porphyrin was 10 μmol/L. The concentration of the fluorescein solution was 59.7 μmol/L.

Procedures

The chromatographic procedure. For the chromatographic analyses we used a solvent system with two solutions, A and B, in a gradient system. Solution A was methanol/water/glacial acetic acid (6/4/1, by vol) and B was methanol/glacial acetic acid (10/1, by vol) (18). In the course of a gradient run the percentage of solvent B increases from 0 to 100% and the methanol concentration thus increases from 55 to 91%. In this “reversed-phase” liquid-chromatographic gradient system the most polar porphyrin will be eluted first and the least polar porphyrin last. The solvents were degassed in the ultrasonic bath under reduced pressure (water pump) before use. In the gradient system the flow rate was 1 mL/min. The final conditions were reached in 10 min. The excitation wavelength of a tungsten lamp for the porphyrins eluting from the column was 404 nm, and the resulting fluorescence was measured at 595 nm. The absorption maxima for coproporphyrin and protoporphyrin were 390 and 400 nm in solvents with compositions corresponding to the concentrations of the gradient where the compounds were eluted. The absorption maxima were measured with a Varian Cary 118 spectrophotometer. As a spectrophotometric fluorometer is not technically corrected to give an absolute excitation or emission spectrum it is to be expected that the excitation wavelength found for the sensitivity maximum in liquid-chromatography may deviate from the real absorption maximum. All the chromatographic separations were performed at room temperature.

Procedure for extracting erythrocyte porphyrins. The following method, modified after Rimington (19), was used: Place about 10 mL of erythrocytes from heparinized blood samples (centrifuged and measured in a graduated centrifuge tube) in a beaker. (In our experience, it is not necessary to wash the cells with isotonic saline.) Slowly add 150 mL of a mixture of ethyl acetate/glacial acetic acid (3/1), with stirring, and leave the beaker in the dark at -20°C for at least an hour. Then filter the mixture by suction through a sintered-glass funnel and wash the residue twice with the ethyl acetate/acetic acid mixture. Combine filtrate and washings in a separatory funnel and wash once with half a volume of saturated sodium acetate solution. Re-extract the washings with a small amount of ethyl acetate and add it to the main organic phase. Then extract this phase once with 20 mL and twice with 10 mL of HCl/water (15/85 by vol) to remove all the porphyrins. Add solid sodium acetate to the HCl solution until Congo Red test paper no longer turns blue and extract all porphyrins by shaking twice with 20-mL portions of ether. Evaporate the ether under nitrogen and, just before measurement, dissolve the porphyrins in the residue in 2 mL of solvent A. In principle, all the volumes used in the extraction procedure can be reduced by a factor of five, if necessary—e.g., in pediatric practice.

Chromatographic analysis. The volume of each standard solution injected onto the column was 5 μL. The samples, which sometimes might contain traces of heme, were injected onto the column in volumes of 10 to 90 μL, depending on the porphyrin concentration. The range of the fluorescence detector is usually kept at 0.05 μA with only exceptional changes for samples with very high protoporphyrin concentration. The porphyrin contents were calculated from their peak heights, according to the following equation:

$$C_1 = \left(\frac{C_2}{PH_2}\right) \cdot PH_1 \cdot \left(\frac{N_A}{N_B}\right) \cdot \left(\frac{L_A}{L_B}\right)$$

where

- $C_1$ = the protoporphyrin or coproporphyrin concentration of the sample, in nmol/L of erythrocytes
- $C_2$ = the amount of the standard injected onto the column, in nanomoles
- $PH_1$ = the peak height of the sample protoporphyrin or coproporphyrin in millimeters
- $PH_2$ = the peak height of the standard protoporphyrin or coproporphyrin, in millimeters
- $N_A$ = the initial volume of the sample, in milliliters (in this system, always 2 mL)

Fig. 1. Gradient elution separation of the standard porphyrin mixture

Uroporphyrin I (7), 7-carboxyl porphyrin (2), 6-carboxyl porphyrin (3), 5-carboxyl porphyrin (4), coproporphyrin I (5), mesoporphyrin IX (6). Column: Waters μBondapak C18; initial mobile phase: methanol/water/acetic acid (8/4/1); final mobile phase: methanol/acetic acid (10/1); gradient shape: linear; gradient duration: 10 min; flow rate: 1 mL/min. The line over the diagram represents the change in methanol concentration (i.e., the gradient) in the eluent.

Fig. 2. Gradient elution separation of fluorescein (7), coproporphyrin III (2), and protoporphyrin IX (3)

Chromatographic conditions as in Fig. 1.

Fig. 3. Gradient elution separation of the porphyrins from an erythrocyte sample

Fluorescein (7), coproporphyrin (2), protoporphyrin (3). The sample contained protoporphyrin (453 nmol/L cells) and coproporphyrin (65 nmol/L cells). Volume applied to column, 80 μL. Chromatographic conditions as in Fig. 1.
Results and Discussion

Separation of the six component porphyrins of the standard mixture is good, almost complete (Figure 1). The porphyrins are eluted from the column in order of decreasing polarity. The most polar porphyrin, uroporphyrin (with eight carboxyl groups), is thus eluted first and mesoporphyrin (with only two carboxyl groups) last. The amount of each porphyrin injected onto the column was 50 pg. The elution times for uroporphyrin, coproporphyrin I, and mesoporphyrin IX were 4.5, 9.0, and 12.5 min (elution volumes 4.5, 9.0, and 12.5 mL), respectively. The solvent-extraction system used in this study, however, only makes possible the measurement of porphyrins with fewer than five carboxyl groups. Measurement of erythrocyte uroporphyrin and other porphyrins with at least five carboxyl groups requires that the protein residue and the saturated sodium acetate washing should be processed further according to Rimington (19). To prove this, we did an experiment in which to a known erythrocyte sample a known amount of the standard porphyrin mixture was added. Only mesoporphyrin, coproporphyrin, and about 25% of 5-carboxyl porphyrin were detected in the final sample.

Figure 2 shows an elution diagram of the standard solutions of protoporphyrin and coproporphyrin. The amounts injected into the column were 65 and 37 pg, respectively. The retention time for coproporphyrin III was 9 min (elution volume 9 mL) and for protoporphyrin IX 14 min (elution volume 14 mL). The protoporphyrin solution was freshly prepared every day, but the coproporphyrin solution could be preserved for about two weeks. The coproporphyrin IX in this diagram elutes from the column later than mesoporphyrin IX, as shown in Figure 1.

Coproprophyrin isomers I and III are not separated. The coproporphyrin shown in Figure 1 is isomer I and that in Figure 2 is isomer III; they have the same retention time, as confirmed by co-chromatography. The first compound eluted in all chromatograms, except in Figure 1, is the internal standard, fluorescein, for which the retention time is 5 min.

Figure 3 shows an example of an elution chromatogram of erythrocyte porphyrins. Heme sometimes causes mild quenching in the fluorescence just before the coproporphyrin elutes, but on the basis of our recovery studies it does not influence the observed coproporphyrin concentration. A known amount of coproporphyrin was added to the sample, which was still slightly colored by heme, and it was found that the heme "quenching" did not lower the peak in the chromatogram. The significance of the two small peaks seen in Figure 3 just before and after the protoporphyrin peak is not yet known.

Figure 4 shows standard curves for protoporphyrin and coproporphyrin. A tendency to level off is obvious in the highest concentration area. Therefore, we routinely inject at least two different volumes of the samples to ensure that at least one will fall within the concentration range over which the standard curves are linear. The porphyrin contents were assayed from peak heights, but nonlinearity of the standard curve is also seen when the porphyrin contents are measured from enclosed areas.

Analytical recovery is about 98% for the protoporphyrin and about 80% for the coproporphyrin (about 20% being lost during the extraction procedure) in this extraction and measurement system. The recovery is the same if coproporphyrin is added to blood instead of to buffer before the extraction procedure.

As was expected from the difference of the absorbance maxima for coproporphyrin and protoporphyrin, the wavelength of maximum sensitivity for coproporphyrin was also somewhat shorter than for protoporphyrin in the liquid chromatography detector. Also, the height ratios of coproporphyrin and protoporphyrin peaks changed with wavelength, being at 396 nm 0.77, at 400 nm 0.75, and at 404 nm 0.71. The differences in the fluorescence intensities at these different wavelengths were not remarkable. (No difference was seen in fluorescence intensities in routine erythrocyte coproporphyrin measurements at 400 nm versus 404 nm.) This is well

### Table 1. Erythrocyte Protoporphyrin and Coproporphyrin Concentrations in 19 Ostensibly Healthy Adults

<table>
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<tr>
<th></th>
<th>Protoporphyrin</th>
<th>Coproporphyrin</th>
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<tbody>
<tr>
<td>Mean</td>
<td>519</td>
<td>14.5</td>
</tr>
<tr>
<td>SD</td>
<td>200</td>
<td>7.8</td>
</tr>
<tr>
<td>Range</td>
<td>220–934</td>
<td>2.2–30.1</td>
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### Table 2. Reported Erythrocyte Porphyrin Concentration in Healthy Normal Persons

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<tr>
<th>Sex</th>
<th>Protoporphyrin</th>
<th>Coproporphyrin</th>
<th>No. subjects</th>
<th>Ref. no.</th>
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<tr>
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<td>nmol/L cells, mean (SD)</td>
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<td>Solvent-partition system</td>
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<tr>
<td>M</td>
<td>507 (80)</td>
<td>4.6 (6.0)</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>F</td>
<td>742 (105)</td>
<td>10.7 (9.2)</td>
<td>30</td>
<td></td>
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<tr>
<td>M</td>
<td>302</td>
<td>—</td>
<td>23</td>
<td>20</td>
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<tr>
<td>F</td>
<td>320</td>
<td>—</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>534 (133)</td>
<td>19.9 (6.1)</td>
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<td>13</td>
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<tr>
<td>F</td>
<td>658 (178)</td>
<td>18.3 (10.7)</td>
<td>20</td>
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<tr>
<td></td>
<td>335</td>
<td>5.4</td>
<td>8</td>
<td>21</td>
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<tr>
<td></td>
<td>617 (299)*</td>
<td>24.5 (36.7)*</td>
<td>22</td>
<td>22</td>
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<tr>
<td></td>
<td>835 (265)</td>
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<td>TLC method</td>
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<td>M</td>
<td>502</td>
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<td>31</td>
<td>14</td>
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<tr>
<td>F</td>
<td>378</td>
<td>&lt;15</td>
<td>28</td>
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<td>HPLC method</td>
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<td></td>
<td>519 (200)</td>
<td>14.5 (7.8)</td>
<td>19</td>
<td>This paper</td>
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* 2 SD.
understood when we take account of the bandwidths of the coproporphyrin and protoporphyrin absorbance spectra in gradient system solutions in which they elute. The bandwidth at half height in the absorbance scale was 30 nm, both for coproporphyrin and protoporphyrin. The effective bandwidth of the excitation monochromator was 5 nm.

Table 1 lists our results for ostensibly healthy normal persons (mostly hospital personnel). To test our method we also have measured erythrocyte porphyrins in several patient groups. Rather interesting porphyrin profiles were obtained, e.g., in hereditary sideroblastic anemia, porphyria cutanea tarda, and in certain preleukemic states. These results will be published when the study is completed.

Concentrations used as reference values by other authors compare well with our reference material (Table 2), except that we saw no sex-related difference.

Our method offers several advantages for the analysis of erythrocyte porphyrins. It provides quantitative porphyrin profiles with high specificity and good recoveries. Accordingly, it obviates hematoporphyrin contamination in the coproporphyrin fraction as well as coproporphyrin contamination in the protoporphyrin fraction, which are likely to be pitfalls in other systems. In addition, an analysis can be done reasonably easily and quickly. Furthermore, in some cases, disorders of heme synthesis lead to an accumulation of porphyrins with more than four carboxyl groups in erythrocytes, for which our method is equally useful if the washings with saturated sodium acetate obtained during the extraction procedure are processed further according to Rimington (19).

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References