Simultaneous Liquid-Chromatographic Determination of Zinc Protoporphyrin IX, Protoporphyrin IX, and Coproporphyrin in Whole Blood

George R. Gotelli, Jeffrey H. Wall, Pokar M. Kabra, and Laurence J. Marton

We describe a method for simultaneously measuring concentrations of coproporphyrin, zinc protoporphyrin IX, and protoporphyrin IX in whole blood by liquid chromatography, with use of reversed-phase ion-pair system, fluorometric detection, and internal standardization. Each analysis requires 10 μL of whole blood and 15 min total analysis time. Analytical recovery ranged from 84 to 92%, day-to-day precision (CV) from 5 to 12%. Uroporphyrin, though not studied in detail, can also be detected by this method.

Additional Keyphrases: uroporphyrin • lead poisoning • pediatric chemistry • chromatography, reversed-phase ion-pair • fluorometry

Solvent-extraction methods are commonly used to isolate porphyrins from biological materials. These methods, in addition to being laborious, only partly separate the numerous porphyrins in materials such as blood, urine, and feces. These porphyrins are better separated by use of thin-layer chromatographic methods (1, 2), but such methods are semi-quantitative and require conversion of the isolated porphyrin free acid to the corresponding methyl ester, a procedure that may present problems (4). Recently, “high-performance” liquid chromatography has simplified analysis for porphyrins (5–8). However, similar to thin-layer chromatographic methods, in most liquid-chromatographic methods methyl esters are separated on silica. Silica columns retain polar biological compounds, and these retained compounds may interfere with the compounds of interest; thus, reversed-phase columns are more suitable for the separation of many polar biological compounds. In 1976, Adams et al. (3) separated urinary porphyrins by liquid chromatography on a reversed-phase column. Similarly, Bonnet et al. (4), using ion-pair chromatography on a reversed-phase column, successfully chromatographed the porphyrin free acids in urine. Until recently porphyrin measurement in erythrocytes has received relatively little attention. The current effort to detect lead poisoning in children by measuring erythrocyte protoporphyrin IX has prompted numerous extraction methods (9–14). In 1974, Lamola and Yamane (15) reported that zinc protoporphyrin IX is the erythrocyte porphyrin predominantly increased in lead poisoning. Accordingly, methods were developed to measure zinc protoporphyrin IX (16–17). In 1979, Culbreth et al. (18) developed a liquid-chromatographic method for protoporphyrin IX and related porphyrins, and they demonstrated that this method was sufficiently sensitive to measure erythrocyte protoporphyrin IX in acid extracts of whole blood.

We describe here a method for erythrocyte porphyrins that combines reversed-phase ion-pair chromatography with fluorometric detection. This method is capable of simultaneously determining coproporphyrin, zinc protoporphyrin IX, and protoporphyrin IX in whole blood and involves internal standardization. It is suited to use with pediatric samples, because only 10 μL of whole blood is required, and total analysis time is 15 min.

Materials and Methods

Instrumentation

We used a Series 1 liquid chromatograph (Perkin-Elmer Corp., Norwalk, CT 06856) equipped with a Model 7105 sample injection valve (Rhodyne, Berkeley, CA 94710) and a Model 204-A fluorescence spectrophotometer (Perkin-Elmer) with a Model 150 Xenon power supply (Perkin-Elmer). Excitation wavelength was set at 400 nm. Emitted fluorescent light of all wavelengths above 560 nm was allowed to reach the detector by using a 560-nm cutoff filter. Both excitation and emission slit widths were set at 20 nm. A 30-cm × 4-mm (i.d.) prepacked reversed-phase column (μ-Bondapak C18; Waters Associates, Inc., Milford, MA 01757) was mounted in a Model LC-100 oven (Perkin-Elmer), which was maintained at 50 °C. A Sigma 10 (Perkin-Elmer) data system was used for all data analysis.

The column was eluted with a mixture of 50 mmol/L tetrabutylammonium hydroxide (pH 7.5) and acetonitrile (34/66, by vol), at a flow rate of 2 mL/min. An in-line valve was placed just prior to the column inlet, which when closed would stop flow, holding any eluted material within the fluorometer flowcell. We used a 3-cm precolumn packed with LiChromprep RP-18, to protect the analytical column.

Reagents

Acetonitrile (“ultraviolet” grade; Burdick and Jackson Laboratories, Inc., Muskegon, MI 49442). Tetrabutylammonium hydroxide (Eastman Kodak Co., Rochester, NY 14650). A 50 mmol/L solution in water was adjusted to pH 7.5 with concentrated phosphoric acid. Triton X-1002 surfactant (J.T. Baker, Phillipsburg, NJ 08865).

n-Propanol (Burdick and Jackson).

Mobile phase. The mobile phase was prepared by mixing 34 parts of the aqueous tetrabutylammonium hydroxide solution with 66 parts of acetonitrile.

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2 Registered trademark of Rohm and Haas Co.
Protoporphyrin IX, zinc protoporphyrin IX, and uroporphyrin III octamethyl ester (internal standard) were purchased from Porphyrin Products, Logan, UT 84321.

Coproporphyrin I and uroporphyrin III were purchased from Sigma Chemical Co., St. Louis, MO 63178.

"Proto-solv." This porphyrin solvent was purchased from Porphyrin Products.


Porphyrin reference standard. A mixed porphyrin reference standard was prepared by placing 1 to 2 mg of each of coproporphyrin I, zinc protoporphyrin IX, protoporphyrin IX, and uroporphyrin III octamethyl ester into separate containers. About 1 mL of Proto-solv was added to each and mixed. A measured aliquot of each porphyrin solution was diluted 100-fold with 1 mol/L hydrochloric acid, except for the uroporphyrin III octamethyl ester solution, which was diluted 100-fold with chloroform. We determined the concentration of each porphyrin solution from its absorptivity as described in Specifications and Criteria for Biochemical Products (National Academy of Sciences, Washington, DC, 1972). Each known porphyrin solution was then diluted to equal concentration with Proto-solv, combined in equal volumes, and stored at −10 °C. This porphyrin reference standard is stable at −10 °C for at least 60 days. For use, it was diluted with mobile phase to give a concentration of 1 mg/L. This working reference standard is stable at 4 °C for 24 h.

Blood-lysing reagent. This reagent lyses the erythrocytes and contains the internal standard. It is prepared by adding to 10 mL of n-propanol an appropriate amount of a uroporphyrin III octamethyl ester in chloroform solution to give a final concentration of 10 mg/L. This solution is then diluted to 0.25 mg/L with 50 mmol/L tetrabutylammonium hydroxide (pH 7.5) containing 50 mL of Triton X-100 per liter.

Blood samples. Blood samples were collected in an ethylenediaminetetraacetate (EDTA) preservative. These samples are stable for at least 20 days at 4 °C. Blood samples processed as described in the procedure are stable for at least 6 h at 4 °C.

Procedure

Add 300 μL of blood-lysing reagent to 100 μL of whole blood, vortex-mix for 15 s, centrifuge to sediment the erythrocyte ghosts, and inject 25 μL of the supernatant fluid onto the column.

Results

Figure 1A shows a typical chromatogram of the working porphyrin reference standard, each peak representing 0.25 ng. Figure 1B illustrates that for a normal blood containing 390 μg of zinc protoporphyrin IX and 110 μg of protoporphyrin IX per liter. Figure 1C shows a chromatogram of blood from a patient with lead poisoning; the zinc protoporphyrin IX concentration was 10 mg/L and the protoporphyrin IX concentration was 690 μg/L. The coproporphyrin concentration was too low for accurate calculation in Figure 1B or 1C.

Sensitivity. We could readily detect 0.1 ng of zinc protoporphyrin IX, protoporphyrin IX, and coproporphyrin I per sample.

Linearity. Coproporphyrin I, zinc protoporphyrin IX, and protoporphyrin IX were added to normal whole blood in amounts equivalent to 500, 1000, and 1500 μg/L. At least three samples at each concentration were processed as described. The concentration of each porphyrin and its peak area relative to that of the internal standard were linearly related over the stated range.

Precision. We evaluated within-run precision by processing 20 aliquots of a whole-blood sample at low and high porphyrin concentrations. Day-to-day precision was evaluated by processing aliquots of a normal blood sample on 20 consecutive days.

Table 1. Precision of Assay for Zinc Protoporphyrin IX and Protoporphyrin IX in Whole Blood

<table>
<thead>
<tr>
<th></th>
<th>Within-run (n = 20)</th>
<th>Day-to-day (n = 20)</th>
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<tbody>
<tr>
<td></td>
<td>Conc (low) µg/L (SD)</td>
<td>CV, %</td>
</tr>
<tr>
<td>Zinc protoporphyrin IX</td>
<td>250 (6.7)</td>
<td>2.65</td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td>150 (6.0)</td>
<td>4.74</td>
</tr>
<tr>
<td>Coproporphyrin I</td>
<td>a</td>
<td>a</td>
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</tbody>
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Table 2. Analytical Recovery of Porphyrins Added to Normal Whole Blood

<table>
<thead>
<tr>
<th>Added Porphyrin, µg/L</th>
<th>Recovered</th>
<th>Recovered, %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coproporphyrin I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>425–440</td>
<td>85–88</td>
</tr>
<tr>
<td>1000</td>
<td>920–1060</td>
<td>92–106</td>
</tr>
<tr>
<td>1500</td>
<td>1350–1410</td>
<td>90–94</td>
</tr>
<tr>
<td>Zinc protoporphyrin IX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>430–480</td>
<td>86–96</td>
</tr>
<tr>
<td>1000</td>
<td>880–910</td>
<td>88–91</td>
</tr>
<tr>
<td>1500</td>
<td>1365–1395</td>
<td>91–93</td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>385–405</td>
<td>77–81</td>
</tr>
<tr>
<td>1000</td>
<td>810–850</td>
<td>81–85</td>
</tr>
<tr>
<td>1500</td>
<td>1335–1380</td>
<td>89–92</td>
</tr>
</tbody>
</table>
working days. Results of these studies are tabulated in Table 1.

Analytical recovery. Known amounts of coproporphyrin I, zinc protoporphyrin IX, and protoporphyrin IX were added to normal whole blood and processed as described. At least three recoveries were measured at each indicated concentration (Table 2).

Comparison with an established method. To assess the suitability of this method, we compared it with the method of Piomelli (9). Because of the acid extraction reagent used in the Piomelli method, zinc protoporphyrin IX is converted to protoporphyrin IX; thus both porphyrins are measured as protoporphyrin IX. Piomelli referred to this total as “free erythrocyte protoporphyrin.” Therefore, for the purposes of the regression analysis, we combined the zinc protoporphyrin IX and protoporphyrin IX values from our method and compared that total with the free erythrocyte protoporphyrin value obtained by the method of Piomelli. The results of the regression analysis of the two methods were: \( r = 0.951 \), slope = 1.04, \( y \)-intercept = 0.29, and \( n = 20 \).

Although we elected to utilize all emitted fluorescent light above 560 nm to detect all porphyrin species in blood, one can increase specificity and sensitivity by using the specific emission wavelength of the porphyrin of interest (see Table 3). This technique also eliminates from consideration any non-porphyrin fluorescence that may be present in plasma.

Table 4 tabulates the individual zinc protoporphyrin IX and erythrocyte protoporphyrin IX concentrations obtained in 22 unselected blood samples. Coproporphyrin concentrations were too low to calculate in any of these samples.

Because it rarely is present in blood, uroporphyrin was not included in our reference standard, but it can also be detected by this method. It is eluted just after the coproporphyrin peak. Uroporphyrin was not detected in any of the blood samples we tested; trace amounts of coproporphyrin were consistently detected.

Discussion

Commonly used methods for determination of erythrocyte protoporphyrin IX involve solvent extraction at a low pH. The low pH needed to partition the porphyrins into the extractant also converts zinc protoporphyrin IX to protoporphyrin IX. Thus, these methods measure total blood porphyrins, commonly called “free erythrocyte protoporphyrin.” In addition, these methods require back-extraction of the porphyrins from the organic phase into mineral acid before fluorometry, to eliminate the fluorescence-quenching effect of hematin.

Liquid chromatography offers significant advantages over these methods, the most notable being that the individual erythrocyte porphyrins—zinc protoporphyrin IX, protoporphyrin IX, and coproporphyrin—can be separately quantitated on a single run. In addition, hematin does not interfere in this liquid-chromatographic method.

The stop-flow technique allows additional qualitative confirmation of each peak by allowing one to scan its excitation and emission maxima. The resulting spectra permit unequivocal identification of the porphyrin.

We saw a decrease in useful life of the column to about 150 samples per column, presumably ascribable to non-extraction of the sample.

References


