Simultaneous Separation and Quantification of Free and Metal-Chelated Protoporphyrins in Blood by Three-Dimensional HPLC

Haruo Sato, Ken-ichi Ido, and Ken Kimura

In this new method for simultaneous separation and quantification of free and metal-chelated porphyrins in blood, the porphyrins are extracted from blood samples with a mixture of diisopropylamine:water:methanol (25:100:900, by vol) and separated by HPLC elution. The data are collected in three-dimensional form with a microcomputer. This method permits a high recovery of protoporphyrin (PP) and Zn-chelated PP as well as heme from blood. By this method, the amounts of these analytes in erythrocytes from normal or abnormal subjects can be determined more accurately than by conventional methods.

Indexing Terms: erythrocytes/heme/zinc protoporphyrin/three-dimensional spectra: fluorescence (or absorbance) vs wavelength vs time

Porphyrins exist in human blood and tissues in both metal-free and metal-chelated forms. Separation and quantification of these porphyrins are of great biological and clinical significance because abnormal species and (or) excessive amounts of porphyrins are found in a variety of disorders, including lead poisoning and iron-deficiency anemia. Both protoporphyrins (PP) and zinc protoporphyrin (ZnPp) are found in normal erythrocytes (RCs), but their amounts increase in abnormal ones. Numerous methods have been reported for extracting porphyrins from RCs (1–8). One of the most general methods involves a mixture of acetic acid:ethanol acetate (1:3, by vol; AcOH/AcOEt) as the solvents; dilute HCl (1.5 mol/L) is then used to isolate the porphyrins [e.g., PP and coproporphyrin (CP)] after the extraction and to remove the heme (9).

HPLC methods have been developed for the assay of porphyrins (10–18), most of which use AcOH/AcOEt for extraction and the 1.5 mol/L HCl solution (referred to as simply HCl hereafter) to remove heme before analysis by HPLC. However, HCl is not really suitable for the analysis of porphyrins: It does not dissolve heme but does cause transformation of β-substituents on the PP macrocycles (6) and may remove metal ions from metal-chelated PP (M-PP). Therefore, a new method that can extract, separate, and quantify PP and M-PP on HPLC simultaneously, without any transformation of the side chains or removal of the metal ions from M-PP, is desirable.

We report here on the use of a mixture of diisopropylamine:water:methanol (25:100:900, by vol; DWM) as a solvent for this purpose. In this solvent, hemoglobin (Hb) dissociates to heme and globin, and the globin and other proteins are precipitated out, whereas PP and M-PP (heme and ZnPp) remain completely soluble. An aliquot of the extract can be injected directly for HPLC and analyzed by using DWM as an eluting solvent.

Materials and Methods

Standards

Protoporphyrin dimethyl ester (PPME) was prepared from hemin (19). ZnPME was prepared from PPME as follows: Zinc acetate (10 mg) in dimethylformamide (10 mL) was added to a solution of PPME (0.1 g) in dichloromethane (100 mL), and the porphyrin-containing precipitates were collected. The purity of these porphyrins was confirmed by thin-layer chromatography (Wakogel B-5F; Wako Pure Chemicals, Tokyo, Japan) developed with dichloromethane (100%). Disodium PP and ZnPp were prepared by the following procedures: PPME (1 g) was dissolved in 200 mL of boiling toluene. After 30 min, 0.135 g of NaOH (2 mol of NaOH per mole of PPME) dissolved in 10 mL of methanol was added, and the mixture was boiled for 1 h more. Precipitates of PPNa2 were collected, rinsed with toluene, and dried under reduced pressure. Similarly, ZnPpNa2 was prepared quantitatively from ZnPME. The purity of the salts was confirmed by elemental analysis.

Standard solutions of the porphyrins were prepared by dissolving PPNa2 or ZnPpNa2 in deoxygenated and distilled water (10 mg in 100 mL) and then diluted 1000-fold with DWM (0.1 mL/100 mL). Hemin was dissolved (10 mg/100 mL) in a mixture of diisopropylamine: methanol (25:975, by vol; DM), and diluted in the same solvent. Use of ultrasound for 30 min completely dissolved the porphyrins in the solvent.

Human Blood Samples

Samples were collected from four apparently healthy men (staff members at Jichi Medical School, ages 23–45 years) and one lead-poisoning patient (a 43-year-old man). Human subjects were treated according to the Helsinki Declaration of 1975, as revised in 1983. These samples were collected into tubes coated with 0.1 mL of heparin sodium salt solution and then centrifuged
(1600g, 20 min) to separate the plasma from the RCs. Both fractions were stored frozen at −20°C.

Procedures

RC samples were thawed at room temperature (20°C) before analysis. Porphyrins and heme were extracted by mixing 200 μL of the RC fraction with 9800 μL of DM and then centrifuging at 1600g, for 20 min. The clear supernate [200 μL (or 50 μL, for the heme assay)] was injected into the HPLC column and analyzed. For the exact estimation of porphyrins in the RCs, we measured the weight of each 200 μL of RC sample and corrected by calculating from specific gravity. To standardize the analysis for heme, we dissolved 100 μL of the standard solution of Hb (guaranteed content by Wako Pure Chemicals, 150 g/L) in DM to a final volume of 10 mL. This mixture was centrifuged, and 100 μL of the supernate was injected into the HPLC column.

To compare our new method with a conventional HPLC method, we assayed the same RC samples, using HCl and the HPLC method of Smith et al. (13) to separate the porphyrins. In the conventional method, we mixed 50 μL of RCs with 5 mL of AcOH/AcOEt, centrifuged, and mixed the supernate with 5 mL of HCl solution. The relative emission intensity (REI) of the separated HCl layer was measured at 605 nm after excitation at 400 nm. The quantity of porphyrin was calculated by Piomelli’s equation (9), based on the REI of the standard solution of CP (methyl ester; from Wako Pure Chemicals) in HCl.

We also determined the recovery of PP from RCs to which various amounts of PPNa₂ has been added, using DWM as an extraction and elution solvent. We compared the results with those obtained by the method of Smith et al. (13). Each sample in the recovery study was prepared by mixing 0.5 mL of each concentration of PPNa₂ solution (0.4, 1.2, 2, or 4 mg/mL) with 0.5 mL of RCs (all from the same subject, in which the concentration of PP had been determined by the DWM method to be 56.6 μg/L). From each 1-mL sample (one at each concentration of added PPNa₂) 100 μL was extracted with 1.9 mL of DM solution or AcOH/ACOEt. After the extracts were centrifuged, 100 μL of each supernate was injected into an HPLC column for analysis by each method.

HPLC Assays

The HPLC system (Waters, Milford, MA) consisted of a pump (Model 501) and an automated sampler (Model 710B). Porous polymer gel (Type HP-125; Showa Denko, Tokyo, Japan) was packed into a 4 mm (i.d.) × 250 mm stainless-steel column with a mixture of water and methanol (1:9, by vol). HP-125 gel is made from poly-styrene and is usually used to separate nucleic acids or drugs. The mobile phase (DWM) was used at a flow rate of 0.4 mL/min, and the column was kept at 30°C. For the method of Smith et al. (13) we used a 5-μm particle-size μBondaspher C₁₈ column (3.9 mm (i.d.) × 150 mm; Waters) and eluted the sample components with acetic acid:water:methanol (4:7:39, by vol, pH 3.4). Elutions were performed isocratically in both methods.

Reagents were all analytical grade (Wako Pure Chemicals), and water was redistilled before use. Before use, all mixtures of solvents were filtered through a Millipore membrane (Type FH, 0.5-μm pores; Waters).

The REI and absorbance of the eluant from the HPLC columns were collected three-dimensionally: the retention time along the x-axis, the absorbance or the REI along the y-axis, and the wavelength along the z-axis. That is, the photometric data were collected in optional time intervals between optional wavelengths on a microcomputer (Model FM-11AD₂; Fujitsu, Tokyo, Japan), which was connected to the spectrophotometers (see below) with a communication interface (Model 150-20; Hitachi, Tokyo, Japan). After the analysis, the filed data could be presented in two- or three-dimensional (3D) form by a computer program. To accomplish this, the flow cell for detecting absorbance was connected with a 0.02 mm (i.d.) × 300 mm stainless-steel tube to follow the flow cell used to detect the REI. The concentrations of porphyrins were determined by measuring the REI with a fluorescence spectrophotometer (Model 650-40; Hitachi) equipped with a xenon source lamp and an R928 photomultiplier. Concentrations of heme were determined by measuring absorbance at 398 nm with a spectrophotometer (Model 510; Hitachi).

Results

Standards

The results of an analysis of a mixture of standard samples (PPNa₂, ZnPPNa₂, and hemin) with the DWM/HPLC method are shown in Figs. 1 and 2. Fig. 1A shows in 3D form the changes in the REI from 573 to 660 nm over time. The REI data were collected by repeated scanning from 573 to 660 nm. Each REI was obtained in 1 s, so that the 30 values for each 3-nm interval from 573 to 660 nm were collected in 30 s. The scanning was repeated 50 times, and all the data were assembled in one 3D display. The net analysis time was 30 min per sample because resetting the detector to 573 nm after completing the scan at 660 nm took 6 s for each scanning. Similarly, absorbance was measured with 50 scans from 370 to 426 nm at 2-nm intervals. Fig. 2A shows the absorbance data in a 3D format.

Figure 1B shows the time-coursed spectrum (TCS; the usual HPLC pattern), which was obtained by plotting over time the REI values at 630 nm from the filed data. The quantity of PP was estimated from the REI at 630 nm on the wavelength-scaning spectrum (WSS) at 22.2 min, and corrected (Fig. 1C) by subtracting the baseline REI attributable to fluorescence components other than porphyrins. The baseline was estimated by use of a WSS obtained at a wavelength where the peak of porphyrin was not observed; for the PP analysis, we used the final WSS (the 50th) as the baseline. The peak for ZnPP was observed at 13.8 min (the 23rd scan) on the TCS at 588 nm (Fig. 1D). The corrected emission intensity of ZnPP was obtained from the 23rd WSS and the 27th WSS, the latter being used as the baseline (Fig. 1E).

The TCS of absorbance at 398 nm, extracted from the data of Fig. 2A, shows a peak ascribed to heme at 12.6
min (Fig. 2B). The quantity of heme can be estimated (Fig. 2C) from the corrected absorbance obtained from the WSS at 12.6 min (the 21st) and the baseline (the last WSS).

Calibration Curves and Linearity

Each concentration of PPNa<sub>2</sub> and ZnPPNa<sub>2</sub> was linearly related to the corrected REI value at 630 and 585 nm, respectively, extracted from the 3D spectra obtained by the DWM/HPLC method (Fig. 3). As can be seen, the corrected REI gives analytical results accurate enough to estimate the amounts of the porphyrins. The equation expressing the calibration of PP from 10 pg to 250 ng (x) in one 100-μL injection by HPLC is \( y = 1.80x \), where \( y \) is the corrected REI. For ZnPP, the equation is \( y = 0.60x \) (from 30 pg to 1 μg per 100 μL). The average SD for three injections of PPNa<sub>2</sub> of each quantity was 3.3%; that for ZnPPNa<sub>2</sub> was 3.4%. The lower limits for quantifying PP and ZnPP by DWM/HPLC were 10 and 30 pg per 100 μL, respectively.

To quantify >1 μg of ZnPP or >3 μg of PP per 100 μL, we can use their absorbances at 400 nm, there being a linear relation between the amount of hemin standard \( x \) injected into HPLC (250 ng to 10 μg per 100 μL) and absorbance at 398 nm (y): \( y = 0.075x \). The average SD for three injections of hemin at each quantity was 8.6%.

The results obtained with the comparison method (13) were linearly related between 10 and 50 ng of PP or ZnPP per 100 μL, and the respective equations of calibration were \( y = 0.150x \) and \( y = 0.074x \). The average SD for three injections of PP was 4.0% and that of ZnPP was 4.2%. Outside this range of concentrations, however, especially for PP <0.1 ng and >50 ng per 100 μL, the calibration line for the comparison method was no longer straight. In particular, the REI for 100 ng/100 μL was nearly the same as that for 50 ng/100 μL. The SD
Retention time, mm

Concentration, ng/100 µL

Retention time, min

Absorption wavelength, nm

Absorption

Concentration, ng/100 µL

Fig. 3. DWM/HPLC calibration curve for PP (○) and ZnPP (■) at 630 and 585 nm.

n = 3 samples for each point.

Fig. 4. Calibration curve for PP at 630 nm by Smith's method (■) and by DWM/HPLC method (○).

Note difference in y-axis scales.

Table 1. DWM/HPLC analyses of PP, ZnPP, and heme in RC from five men.

<table>
<thead>
<tr>
<th></th>
<th>PP</th>
<th>ZnPP</th>
<th>Heme</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/L</td>
<td>µg/L</td>
<td>µg/L</td>
<td>g/L</td>
</tr>
<tr>
<td>SD, %</td>
<td>SD, %</td>
<td>SD, %</td>
<td>SD, %</td>
</tr>
<tr>
<td>58.4</td>
<td>166.5</td>
<td>10.61</td>
<td>4.0</td>
</tr>
<tr>
<td>53.5</td>
<td>143.5</td>
<td>10.25</td>
<td>1.2</td>
</tr>
<tr>
<td>25.2</td>
<td>114.9</td>
<td>10.1</td>
<td>0.6</td>
</tr>
<tr>
<td>49.1</td>
<td>132.9</td>
<td>8.81</td>
<td>2.2</td>
</tr>
<tr>
<td>357.9</td>
<td>1845</td>
<td>8.07</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* The first four listed were apparently healthy; the fifth man was a patient with chronic lead poisoning.

n = 3 for each determination.

The average value of PP in the four apparently healthy men we studied was 46.5 µg/L; ZnPP was 139.5 µg/L and heme was 9.95 g/L. For the lead-poisoning patient, the respective values were 357.9 µg/L, 1845 µg/L, and for three injections was 65.7% (Fig. 4). In contrast, the calibration curve for the DWM/HPLC method shows a straight line between the same concentration, and the REI signal was 20 times stronger than that by the method of Smith et al.

Concentrations of PP, ZnPP, and Heme in RC

Because we obtained a linear calibration line for each standard porphyrin by the DWM method, as shown in Fig. 3, we applied the method to the analyses for porphyrins in RCs. The results for the normal men's RCs by the DWM/HPLC method are summarized in Table 1.
8.07 g/L. This patient's RCs contained >7.68 times the PP and >13.2 times the ZnPP of the healthy men.

Table 2 compares the concentrations of Hb calculated from the concentration of heme obtained by our DWM method with those obtained from the subjects' clinical reports. For example, for the concentration of heme in RCs of 10.61 g/L we estimated the quantity of Hb in RCs to be 277.4 g/L, using the relative molecular masses of heme (MW = 616.4) and Hb (MW = 64,456). Given that the hematocrit of this subject (determined from his clinical report) was 49.2%, we could calculate his concentration of Hb in whole blood to be 136.5 g/L. The difference between this value and the 185 g/L obtained by the cyanmethemoglobin method (20) will be discussed later.

Comparisons with Other Methods
To assay the samples by the conventional fluorometric assay, we successively extracted the RCs with AcOH/AcOEt and HCI solution (14). The RCs were the same as those we analyzed by DWM/HPLC. This conventional fluorometric method gave 294.7 µg/L (average SD = 12% for triplicate analyses) for PP, as estimated by Piomelli's equation (9) with use of a standard solution of CP. This method did not separate porphyrins, and the amount of PP was only estimated by an empirical equation. HPLC analysis by the conventional method (13) gave 168.5 µg/L (average SD = 46% for triplicate analyses) for PP measured in the AcOH/AcOEt extracts of the same RCs as for the DWM method.

Analytical Recovery
Figure 5 shows the percentage of recovery of PP by DWM/HPLC and by the method of Smith et al. (13) for analyses of RCs to which given concentrations of PPNa2 had been added. The average recovery ratio of PP was 71.0% by DWM/HPLC; in contrast, the method of Smith et al. gave not only a much larger value (468.5%) for PP in the concentration range from 1 to 5 ng/100 µL added but also a much smaller value (26.4%) for 10 ng/100 µL added.

Discussion
The advantages of our method over the conventional methods are as follows. First, for our method we use a basic solvent (DWM) for extraction and elution from the HPLC column. This solvent dissolves much more porphyrin than conventional acidic or neutral ones, and metal-chelated porphyrins are more stable in a basic medium and do not undergo chemical transformations. Second, porphyrins show much stronger REI in the basic medium than in an acidic or a neutral medium, resulting in much smaller standard deviations with the DWM method than with a conventional method.

DWM dissolves not only porphyrins but other emitting materials without a porphyrin ring in blood samples. The influence of these emitting components can be removed by a 3D detection procedure, the third merit of our method. In the conventional analysis of porphyrins, we measured the REI at 630 nm, at which free porphyrins show the maximum REI, although ZnPP has a maximum at 588 nm. In the 3D method REIs are collected from 573 to 660 nm continuously, so each porphyrin can be analyzed at its REI in the one injection. This procedure reduced the error from a fixed-wavelength analysis and made it possible to identify each porphyrin by WSS. Another problem with conventional HPLC (13) is that the quenching by heme obstructs correct measurement of PP and ZnPP. Therefore, heme has to be removed before analysis (18). Our 3D method can distinguish between the emission peak of porphyrin and the quenching by heme from each optional time-fixed WSS, so it is not necessary to remove heme in the DWM/HPLC method.

PP in normal RCs was reported as 170–770 µg/L (21), although a few methods of analysis showed a much smaller value (22). The DWM/HPLC method gave a much smaller value (25.2–55.4 µg/L) of PP than the conventional method of Smith et al. (120–279 µg/L) for the same samples. The latter method may remove Zn from ZnPP, and the reported amount of PP may include PP from ZnPP in the original RCs.

Similar excessive amounts of PP were reported by the other methods involving a nonacidic medium. One of them was the ethanol extraction method (2) and the other examined the fluorescence of blood samples directly (4). Those analyses were based on the quantity of PP that was obtained by the conventional AcOH/AcOEt

---

**Table 2. Comparison of Hb concentrations estimated by DWM/HPLC method with those obtained clinically.**

<table>
<thead>
<tr>
<th>Measured heme/RC, g/L</th>
<th>In RC</th>
<th>In WB</th>
<th>HM, %</th>
<th>Hb/WS, µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.61</td>
<td>277.4</td>
<td>136.5</td>
<td>49.2</td>
<td>165</td>
</tr>
<tr>
<td>10.25</td>
<td>288.0</td>
<td>105.7</td>
<td>42.6</td>
<td>145</td>
</tr>
<tr>
<td>10.10</td>
<td>264.2</td>
<td>122.1</td>
<td>43.2</td>
<td>153</td>
</tr>
<tr>
<td>8.81</td>
<td>230.4</td>
<td>104.9</td>
<td>45.5</td>
<td>149</td>
</tr>
<tr>
<td>8.08</td>
<td>211.1</td>
<td>58.1</td>
<td>27.5</td>
<td>87</td>
</tr>
<tr>
<td>4.48*</td>
<td></td>
<td>171.3*</td>
<td></td>
<td>150*</td>
</tr>
</tbody>
</table>

* Standard sample of Hb; other results are from the same five subjects as in Table 1.

WB, whole blood; HM, hematocrit.

---

**Fig. 5. Percentage of PP recovered from PP added to RC by DWM/HPLC (○) and by Smith's method (□).**

n = 3 for each point.
method, or without comparison with an accurate pure PP, respectively.

The quantities of Hb in whole blood could be calculated on the basis of the heme determined by the DWM method, but those values were smaller than in clinical reports. This difference is clearly explained by Fig. 6. The TCS at 398 nm indicated another small peak at 4.2 min, besides the peak in heme. That is ~8% of heme from the peak area. An analysis of the standard Hb by the DWM method showed 4.48 g/L heme; the calculated Hb from heme was 117.3 g/L, 78.2% of the guaranteed value of the standard (150 g/L).

In a currently used method, heme is estimated by the cyanmethemoglobin method (20), and porphyrins are analyzed by the conventional AcOH/AcOEt method independently. Heme is a product in porphyrin biosynthesis, so it is of great biological and diagnostic significance to estimate concentrations of porphyrins and heme in the same analysis. Use of the same solvent and HPLC conditions can remove errors caused by comparison of data from different methods as is done conventionally.

Finally, an abnormal metabolism of porphyrin in the lead-poisoning patient was investigated with the DWM method. The RCS of this patient had much more PP and ZnPp and slightly less heme than those of the normal men. In the future, this DWM method may contribute to clinical practice by use in examination of the relation between porphyrin metabolism and disease.

We are grateful to S. Sassa, The Rockefeller University, New York, NY, for his critical reading of this manuscript, and to I. Kumadaki, The Setsunan University, Osaka, Japan, for his helpful advice.

References
3. Blumberg WE, Eisinger J, Lemola AA, Zuckerman DM. Zinc protoporphyrin level in blood determined by a portable hemato-
14. Salmi M, Tenhunen R. New method for liquid-chromato-
graphic measurement of erythrocyte protoporphyrin and copropor-
17. Sakai T, Takeuchi Y, Araki T, Ushio K. Determination of erythrocyte porphyrins by reversed phase HPLC using capu-
20. Fairbanks VF, Klee GG. Measurement of hemoglobin concentra-
tion in whole blood. In: Tietsz NW, ed. Textbook of clinical chem-
22. Chisolm JJ, Hastings CW, Cheung DKK. Microphotofluoro-
metric assay for protoporphyrin in acidified acetone extracts of