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QUANTITATIVE DETERMINATION OF PORPHYRINS, THEIR PRECURSORS AND ZINC PROTOPORPHYRIN IN WHOLE BLOOD AND DRIED BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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SUMMARY

Methods for the determination of porphyrins, δ-aminolevulinic acid (ALA), porphobilinogen (PBG) and zinc protoporphyrin of heme biosynthesis in whole blood and dried blood are described. Erythrocyte porphyrins and the precursors ALA and PBG were extracted from whole blood (50 μl) with 0.3 ml of methanol and 1.5 *M* hydrochloric acid (2:1, v/v). Zinc protoporphyrin was extracted with an acetone–pyridine–Sterox solution. Other major interfering metabolites were removed by centrifugation. An aliquot of the supernatant was injected onto the reversed-phase C_{18} column for detection of porphyrins with excitation wavelength at 405 nm and emission wavelength at 630 nm. The mobile phase was 0.1 *M* phosphate–methanol–tetrahydrofuran (18:30:16, v/v/v), pH 5.38. The ALA and PBG were derivatized with *o*-phthalaldehyde before injection. The detection excitation wavelength occurred at 330 nm and the emission wavelength at 418 nm. The mobile phase was 0.1 *M* phosphate–methanol (7.5:5), pH 3.38. For the dried blood specimen of filter paper, two 0.64-cm discs punched out from the blood-impregnated filter paper were placed in a test tube containing 200 μl of 0.9% saline for 60 min or longer at room temperature and then treated as whole blood.

INTRODUCTION

Disturbances in the heme biosynthetic pathway are characterized by the excess accumulation of porphyrins and the precursors in body fluids and tissues [1–4]. With the advances of high-performance liquid chromotography (HPLC) as a tool for the analysis of these metabolites in biological fluids and urine, a number of techniques using solvent gradient and isocratic analysis have been described [5–10]. HPLC analysis and other methods have been successfully used in determination of total erythrocyte porphyrin concentration [6–9]. Acid extraction, however, dissociates zinc from zinc protoporphyrin (ZnPP), and the quantifi-

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cation of ZnPP is, therefore, measured as free protoporphyrin (unchelated). The quantification of each porphyrin species and the precursors in blood would provide important information in diagnosis of diseases. As a convenient method of sample collection blood was spotted on filter paper (S&S No. 903™). This is the current method of blood collection used in screening tests for inherited metabolic diseases [11].

The present paper describes a simple and inexpensive acetone–pyridine–Sterox extraction of ZnPP, coupled with a sensitive methanol–1.5 *M* hydrochloric acid extraction technique to facilitate an accurate quantification of each detectable porphyrin and the precursors concentration by a reversed-phase HPLC procedure modified from the method previously described [5].

EXPERIMENTAL

Materials

The dihydrochlorides of uroporphyrin I, coproporphyrin I, mesoporphyrin, protoporphyrin IX and zinc protoporphyrin IX, and the methyl esters of penta-, hexa- and heptacarboxylporphyrin I were purchased from Porphyrins Products (Logan, UT, U.S.A.). Porphobilinogen (PBG), δ-aminolevulinic acid (ALA), *o*-phthalaldehyde (OPA) and 2-mercaptoethanol were obtained from Sigma (St. Louis, MO, U.S.A.). HPLC-grade methanol and tetrahydrofuran were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Sterox solution was purchased from Banco Anderson Labs., Fort Worth, TX, U.S.A.). All other chemicals are reagent grade. Filter papers were purchased from Schleicher & Schuell (Keene, NH, U.S.A.).

Instrumentation

Experiments were performed on a Model 5000 liquid chromatograph (Varian Assoc., Palo Alto, CA, U.S.A.) equipped with a Rheodyne 7126 injector fitted with a 500-μl loop. A μBondapak C_{18} column (30 cm$\times$0.38 cm I.D., average particle size 10 μm, Waters Assoc., Milford, MA, U.S.A.), protected by a 10-μm Ultrasphere octadecylsilane (ODS) guard column (Whatman) was used for all experiments. A variable-wavelength spectrofluorometer (Varian, Model SF-330) fitted with a 40-μl flow cell attachment was used. All chromatograms were recorded with a Hewlett-Packard 3388A integrator. All pH readings were taken on a Model 601 digital ionalyzer with a Ross combination pH electrode, both from Orion Research (Cambridge, MA, U.S.A.).

Methods

The procedures described here are essentially the same as those previously reported [5]. However, ZnPP and uroporphyrin standards were prepared differently to maintain the stability of the solutions for a considerably longer time. In addition, a μBondapak C_{18} column was used in place of the Whatman Partisil-10-ODS column (25 cm$\times$0.46 cm I.D.) in an attempt to improve the resolution and efficiency. Better resolution between peaks was observed with the modified mobile phase compositions at a new flow-rate of 1.3 ml/min.

Standard solutions of copro-, meso-, penta-, hexa-, hepta- and protoporphyrins, and ALA and PBG were prepared as before [5]. ZnPP and uroporphyrin solutions were dissolved in minimum amount of pyridine and diluted to the desired volume with methanol. The excitation and emission wavelengths were set at 405 nm (slit width, 10 nm) and 630 nm (slit width, 20 nm), respectively, for the detection of porphyrins. The wavelengths were monitored at 330 and 418 nm, respectively, to detect ALA and PBG. Reversed-phase HPLC separation of porphyrins was performed under isocratic elution with the ternary mobile phase, 0.1 *M* phosphate–methanol–tetrahydrofuran (18:30:16, v/v/v), pH 5.38. The separation of ALA and PBG was achieved with a binary mobile phase, 0.1 *M* phosphate–methanol (7.5:5), pH 3.38. The isocratic elution of porphyrins and the precursors was performed at 1.3 ml/min at ambient temperature.

Extraction of erythrocyte porphyrins, ALA and PBG

Whole blood. Whole blood (50 μl) was mixed with 0.3 ml acidified methanol (1.5 *M* hydrochloric acid–methanol, 1:2, v/v). The extraction was achieved with vortex of the mixture for 2 min and sonication in a water-bath for 3 min. The mixture was then spun down at 2500 *g* in a table-top centrifuge for 5 min. The yellowish supernatant was transferred to a clean test tube. The 10 μl of the supernatant were derivatized with OPA solution as described earlier [5]. The remaining supernatant was injected directly onto the column for the analysis of porphyrins. A 20-μl volume of the derivatized supernatant was separately injected onto the column for the determination of ALA and PBG.

Dried blood. Blood (70 μl) was spotted on filter paper (S&S No. 903) according to the instruction on the back of the paper and allowed to dry overnight at room temperature. Two 0.64-cm discs were punched out from the blood-impregnated filter paper and placed in a test tube containing 200 μl of 0.9% saline for 60 min or longer at room temperature. The extraction was achieved by adding 600 μl acidified methanol to the test tube and completed by vortex and centrifugation as before. A 10-μl volume of the supernatant was used for derivatization and the remaining supernatant was used for the analysis of porphyrins.

Extraction of ZnPP

Whole blood. The only acid-unstable porphyrin, ZnPP, of heme biosynthesis required a different procedure. Another 50 μl of whole blood were mixed with 300 μl of acetone, 15 μl of pyridine and 15 μl of Sterox solution. The mixture was vigorously agitated with vortex for 2 min, then centrifuged at 2500 *g* for 5 min. The supernatant was injected onto th column for the analysis of ZnPP.

Dried blood. Extraction of ZnPP from dried blood specimen required soaking of another two 0.64-cm discs in saline solution for about 1 h or longer prior to extraction with the procedure described in the previous paragraph.

For the recovery experiments, standard solutions (2 μl) of each compound were added to 120 μl whole blood. Then, 50 μl of the blood specimen were processed as before. The remaining blood sample (70 μl) was spotted on filter paper and treated similarly. The concentrations of these compounds in each blood specimen were determined as described above.

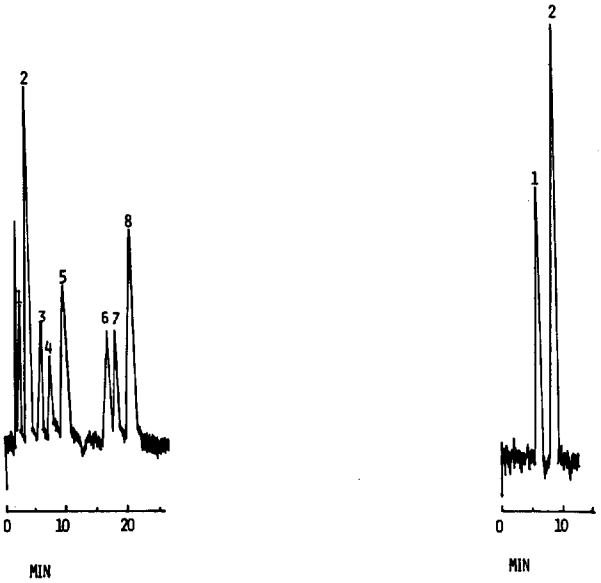

Fig. 1. Chromatogram of porphyrins. Eluent: 0.1 *M* phosphate–methanol–tetrahydrofuran (18:30:16), pH 5.38. Peaks: 1 = uroporphyrin (7.97 pmol); 2 = coproporphyrin (11.9 pmol); 3 = heptaporphyrin (1.39 pmol); 4 = hexaporphyrin (3.29 pmol); 5 = pentaporphyrin (1.34 pmol); 6 = mesoporphyrin (8.50 pmol); 7 = protoporphyrin (8.60 pmol); 8 = zinc protoporphyrin (32.4 pmol).

Fig. 2. Chromatogram of 3.84 nmol of ALA (1) and 0.65 nmol of PBG (2). Eluent: 0.1 *M* phosphate–methanol (6:7), pH 3.38.

RESULTS

Chromatography

The chromatograms of eight porphyrins and two precursor standards are shown in Figs. 1 and 2. The elution profile of erythrocyte porphyrins extracted from whole blood is shown in Fig. 3A. The two derivatized precursors, ALA and PBG, in blood were chromatographed separately with the binary mobile phase as shown in Fig. 3B. The ZnPP concentration was determined separately by the acetone–pyridine–Sterox extraction technique and the HPLC method previously reported [5] (Fig. 3C).

Calibration and quantification

The quantification of each porphyrin in the blood required the addition of mesoporphyrin as an internal standard to compensate for various analytical errors due to variations in separation parameters on peak size, including sample-size fluctuations. Internal standard calibrations were constructed by chromatographing appropriate volumes of calibration mixtures containing various porphyrins with a constant concentration of mesoporphyrin. The ratios of peak areas or peak heights of porphyrins to that of internal standards are plotted versus the amounts in pmol of porphyrins. Each calibration line was calculated using the least-squares

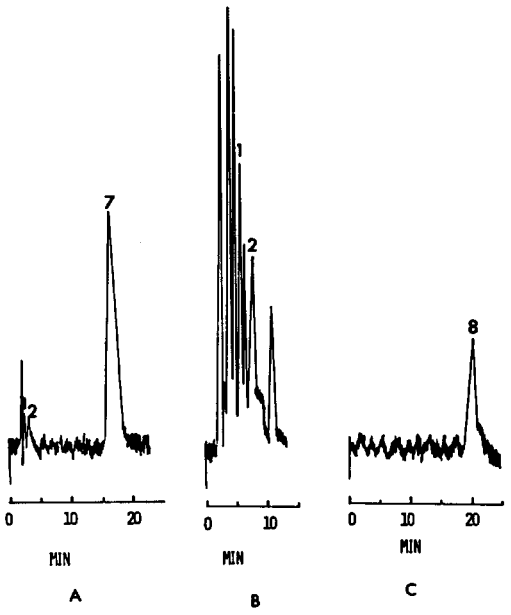

Fig. 3. Chromatograms of (A) erythrocyte porphyrins, (B) ALA (1) and added PBG (2) and (C) ZnPP (8), from extraction of blood specimen. Peak 7 = protoporphyrin.

method. The correlation parameters of linearity of the line graph are summarized in Table I.

Concentrations were computed from either the peak-area ratio or peak-height ratio directly from the calibration line. Quantification of ALA and PBG was computed from the calibration graph (peak height versus mmol). The efficiency of extraction and determination of each intermediate is summarized in Table II. Absolute recoveries of intermediate metabolites and the internal standard were calculated using the internal method as was discussed above.

TABLE I

COEFFICIENTS OF CORRELATION OF PEAK-AREA RATIO, PEAK-HEIGHT RATIO TO THE AMOUNT IN PICOMOLES OF EACH PRECURSOR

Heme precursor	Peak-area ratio	Peak-height ratio
Uroporphyrin	0.89	0.99
Coproporphyrin	0.99	0.96
Heptaporphyrin	0.70	0.87
Hexaporphyrin	0.98	0.96
Pentaporphyrin	0.75	0.93
Protoporphyrin	0.89	0.98
Zinc protoporphyrin	0.85	0.85

TABLE II

EFFICIENCY AND RECOVERY FROM EXTRACTION OF PORPHYRINS AND THEIR PRECURSORS IN BLOOD

N.D. = not detected in blood.

Compound	Recovery* (%)	Absolute quantity** (μg/dl)
δ-Aminolevulinic acid	98 ± 2	$5.16 \cdot 10^3$
Porphobilinogen	96 ± 2	N.D.
Uroporphyrin	94 ± 5	5.03
Coproporphyrin	96 ± 3	1.39
Heptaporphyrin	96 ± 2	N.D.
Hexaporphyrin	96 ± 2	N.D.
Pentaporphyrin	95 ± 2	N.D.
Protoporphyrin	95 ± 4	24.94
Zinc protoporphyrin	92 ± 3	23.05

*Average ± standard deviation from triplicates of control blood samples with internal standards.
**The actual amount in blood.

DISCUSSION

Selectivity and sensitivity

Chromatograms of blood specimens indicate that there are no detectable interfering peaks of other metabolites throughout the elution when detection was monitored at excitation wavelength of 405 nm (slit width 10 nm) and the emission fluorescence was measured at 630 nm (slit width 20 nm). In contrast, the solvent selectivity of the binary phase does not eliminate other metabolites being detected in the same chromatogram when the excitation and the emission wavelengths were monitored at 330 and 418 nm, respectively. Separate experiments showed that those peaks appearing before derivatized ALA were mainly composed of amino acids including glycine, the precursor of the heme biosynthetic pathway. However, there were no detectable PBG in blood samples. Sensitivity tests were performed using standard samples of known porphyrin concentration. The detection limit, defined as the minimum of each compound determined simultaneously in a single chromatogram, is in the subpicomole range for most porphyrins and ca. 1.6 pmol for ZnPP. The system noise level increases considerably at lower excitation and emission wavelengths. Thus, the simultaneous determination of ALA, PBG and porphyrins in picomole amounts is not possible as before [5]. However, ALA and PBG can be separated and detected in nanomole quantities with different detector parameters to minimize the noise level. Though calibration graphs obtained for blood sample were linear for a wide range of concentrations tested as shown in Table I, peak-height ratios were used for more accurate quantification.

Recovery

The overall recovery was detemined using internal standard method (Table II). The results show that the extraction of porphyrins and the precursors is

quantitative. Better extraction efficiency of ZnPP, porphyrins and other metabolites was achieved by extracting the blood sample with more pyridine but resulted in precipitation of other metabolites in the column. Methanol–hydrochloric acid extraction technique permits accurate quantificaton of total erythrocyte porphyrins concentration by HPLC with fluorimetric detection. Acid extraction, however, displaces zinc from ZnPP, and protoporphyrin concentration is therefore measured as free protoporphyrin fluorescence [9]. In contrast, the other porphyrin intermediates of heme biosynthesis maintain their integrity throughout the extraction and separation process. Nevertheless, there were no detectable penta-, hepta- or hexaporphyrins in blood specimens tested which is in agreement with the observations of others [3]. Under the conditions described here for the extraction of porphyrins with hydrochloric acid–methanol (1.6%) for about 10 min, methyl esters were not found in detectable amounts. Near quantitative conversion of porphyrins to methyl esters required longer time (overnight) and excess of stronger acid–methanol (5%) solution [12]. Nevertheless a mixture of porphyrin free acids and porphyrin methyl esters was used as the standards to test for any possible conversion of porphyrins to esters in picomole amounts. The results indicated that the described conditions here did not incur any conversions. The use of methyl esters as standards proved unnecessary for analysis of blood samples which contain mainly coproporphyrin, protoporphyrin free acid, ZnPP and a very trace amount of uroporphyrin free acid [3]. Thus the isocratic conditions make it an efficient and improved method for the determination of all the detectable porphyrin free acids in blood. The extraction efficiency of erythrocyte porphyrins and the precursors with acetone–pyridine–Sterox solution was further examined by treating the protein pellet remaining after acetone extraction with methanol–hydrochloric acid. The result indicates that the acetone extraction of ZnPP is quantitative but is not efficient enough to extract other porphyrins and the precursors. The acid extraction of free erythrocyte porphyrins and the precursors is essentially complete with acidified methanol. However, the absence of PBG in blood samples prompted a recovery study which showed that the stability and reactivity of PBG were not affected by experimental conditions.

Stability of dried blood specimens

Dried blood specimens are as stable as whole blood stored in heparinized Vacutainer tubes at 10°C in the dark for about four weeks [11]. Hence, dried blood specimens have been extensively used in screening tests for different inborn errors since its introduction for detecting phenylketonuria (PKU) in large populations [13]. It proves to be a more economical and safer method for blood collection for screening tests. Also, dried blood specimens can be stored and disposed of much more easily than liquids. Dried blood specimen are more stable and are readily available from patients. A good estimate of stability of porphyrins in dried blood speciment is six to nine monthes at −10°C in the dark. The concentration of ZnPP decreased considerably as indicated by the simultaneous elevation of protoporphyrin concentration in blood specimen. Thus the stability of ZnPP is the limiting consideration for blood specimen used in these tests. However, the retention time of erythrocyte protoporphyrin slightly decreased when the dried blood

specimen was not kept at low temperature and protected from the light. A standard protoporphyrin solution showed the same shift in retention if not refrigerated at $-10\,^{\circ}C$ in the dark.

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