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Note

Reversed-phase high-performance liquid chromatography of urinary porphyrin free acids

MISAKO TAKAYAMA

Department of Medical Technology, Tottori University College of Medical Care Technology, 133-2 Nishimachi Yonago, 683 Tottori (Japan)

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Uroporphyrin and coproporphyrin are present in normal human urine, but the other porphyrins (hepta-, hexa- and pentacarboxylic porphyrin) are either absent or present in very small concentrations. In some conditions the levels of urinary porphyrins may be increased or decreased, reflecting disturbances in heme biosynthesis.

The use of high-performance liquid chromatography (HPLC) for the separation and quantification of porphyrins in biological specimens has been reviewed [1–6]. Some authors used acidified samples and chromatographed them with different buffers [1, 2], or used ion-pair reagents dissolved in organic solvent and phosphate buffer as eluents [3] and coating techniques employing tetraethylenepentamine [4]. As for porphyrin methyl ester, the methods of determination required complicated steps in which urine samples were absorbed on talcum powder and esterified by boron trifluoride in methanol [5] or esterified by direct treatment of the methanol–sulphuric acid [6]. Since esterification is time-consuming, an HPLC analysis of urinary free porphyrins was developed using solvent partition methods. Furthermore, the effects of direct sunlight on recovery and washing the organic solvent with water were examined.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile was HPLC grade; potassium dihydrogenphosphate and acetic acid were reagent grade; porphyrin standards were purchased from Sigma (St. Louis, MO, U.S.A.).

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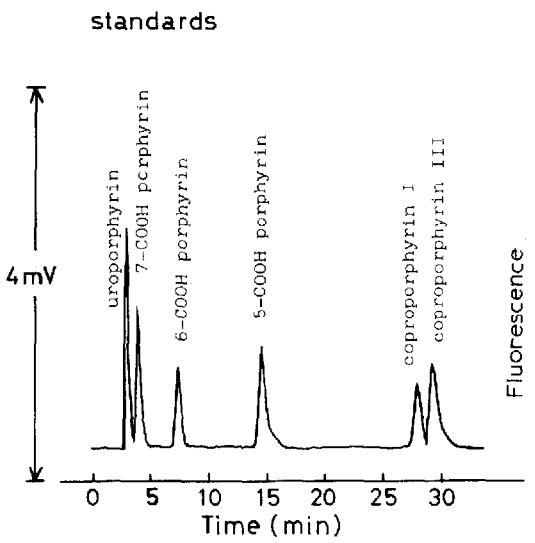

Fig. 1. Chromatogram of porphyrin standards. Conditions as in Experimental. Fluorescence detector set at 393 nm excitation and 610 nm emission wavelength. Amounts of standards: UP, 11.5 ng; 7-COOH-P, 10.0 ng; 6-COOH-P, 9.2 ng; 5-COOH-P, 8.2 ng; CP-I, 6.6 ng; CP-III, 8.0 ng. Injection volume, 10 μl.

To make the standards stock solution, ca. 1 mg of each of the respective methyl ester porphyrins were hydrolysed individually to the free acid forms by dissolving them in 1 ml of 6 *M* hydrochloric acid, and they were left in the dark overnight at room temperature.

For the working solutions, aliquots of each stock solution were diluted with 0.1 *M* hydrochloric acid, and the final concentrations were calculated on the basis of molar extinction coefficients. The UV absorbance was measured with a Hitachi 200-10 spectrophotometer (Tokyo, Japan).

Apparatus

A Hitachi 635 liquid chromatograph and a Hitachi F1100 fluorescence detector, set at excitation and emission wavelengths of 393 and 610 nm, respectively, were used. The injector was a Rheodyne 655-0890 valve fitted with a 20-μl loop. The separation was performed on a 5 μm, 150×4 mm I.D., ODS column (Hitachi, Tokyo, Japan). The guard column was a 30×4 mm I.D. Hitachi ODS column (5 μm). Solvent A for gradient elution was 10% acetonitrile containing 0.5% acetic acid and 0.05% potassium dihydrogenphosphate, and solvent B was 90% acetonitrile in water.

The column was equilibrated with a 6:4 (v/v) mixture of solvent A and solvent B before the sample was injected. Porphyrins were eluted with a 30-min linear gradient from 60% A to 40% A. The flow-rate was 1.5 ml/min, and the analyses were carried out at room temperature.

TABLE I

ANALYTICAL RECOVERY OF PORPHYRINS ADDED TO URINE

Compound	Added (ng)	Found (ng)	Recovery (%)
UP	9.6	8.6	90
7-COOH-P	7.4	5.7	78
6-COOH-P	11.8	10.0	85
5-COOH-P	12.2	9.5	78
CP-I	9.9	9.1	92
CP-III	14.8	13.6	92
UP	19.2	15.6	82
7-COOH-P	14.8	10.7	72
6-COOH-P	23.6	18.4	78
5-COOH-P	24.4	17.6	72
CP-I	16.6	13.8	83
CP-III	24.8	18.1	73
UP	28.8	23.0	80
7-COOH-P	22.2	17.1	77
6-COOH-P	35.4	28.4	80
5-COOH-P	36.6	27.3	75
CP-I	23.2	18.6	80
CP-III	34.8	27.6	80

Sample preparation

Urine (10 ml) was placed in a separatory funnel and extracted with 20 ml of ethyl acetate–acetic acid (4:1, v/v), and then with an additional portion of the same solvent until the aqueous phase showed no further fluorescence under UV light for the urine containing a large amount of porphyrin. The extracts were combined and washed with water (10 ml). The organic phase was evaporated in a rotary evaporator at ca. 50°C under reduced pressure and then to dryness in the dry block (80°C). The residue was dissolved with 0.5 ml of 0.1 *M* hydrochloric acid, and the solution was filtered through a 0.45-μm membrane filter before use.

Creatinine determination

Creatinine was analysed by the method described in ref. 7.

RESULTS AND DISCUSSION

The chromatogram of porphyrin carboxylic acids is shown in Fig. 1. The standard mixture containing uroporphyrin, hepta-, hexa-, penta- and tetracarboxylic porphyrins and coproporphyrin I and III was completely separated within 30 min. The linearity of the response–concentration curve was established by adding various amounts of porphyrins to urine. The correlation was linear up to ca. 180 ng for each porphyrin ($r=0.9075$). The detection limit was ca. 0.3 ng at a signal-to-noise ratio of 2.

In order to determine the extraction efficiencies for the porphyrins, known

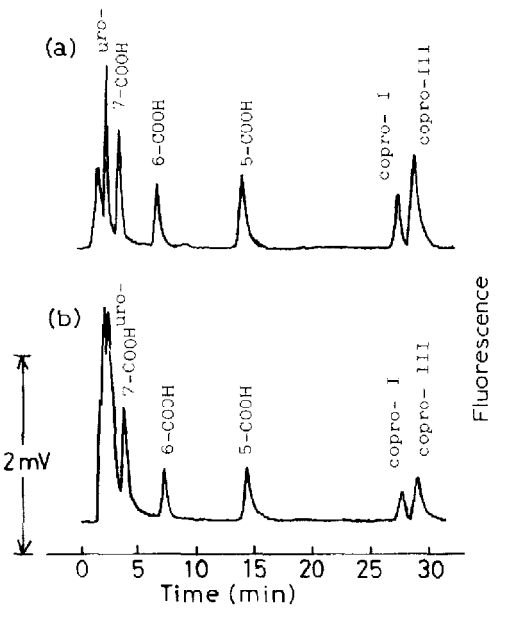

Fig. 2. Effect of washing by water of the organic solvent after extraction: (a) washing, (b) no washing. UP (11.5 ng), 7-COOH-P (10.0 ng), 6-COOH-P (9.2 ng), 5-COOH-P (8.2 ng), CP-I (6.6 ng) and CP-III (8.0 ng) were added to urine. The analyses were carried out as described in the text. Chromatographic conditions as in Experimental and Fig. 1.

amounts of the porphyrin free acid standards were added to urine. These samples were extracted as outlined above and analysed by HPLC. The results of the recovery studies are summarized in Table I.

The within-analysis precision of the assay was determined using a urine sample

TABLE II

EFFECT OF DIRECT SUNLIGHT ON RECOVERY

	Compound	Added (ng)	Found (ng)	Recovery (%)
Under direct sunlight	UP	11.6	5.09	44
	7-COOH-P	10.0	6.29	63
	6-COOH-P	9.2	6.46	70
	5-COOH-P	8.2	5.84	71
	CP-I	7.6	6.28	83
	CP-III	10.4	6.29	61
In dim light	UP	11.6	9.09	83
	7-COOH-P	10.0	8.53	85
	6-COOH-P	9.2	7.64	83
	5-COOH-P	8.2	6.51	79
	CP-I	7.6	5.94	78
	CP-III	10.4	8.03	77

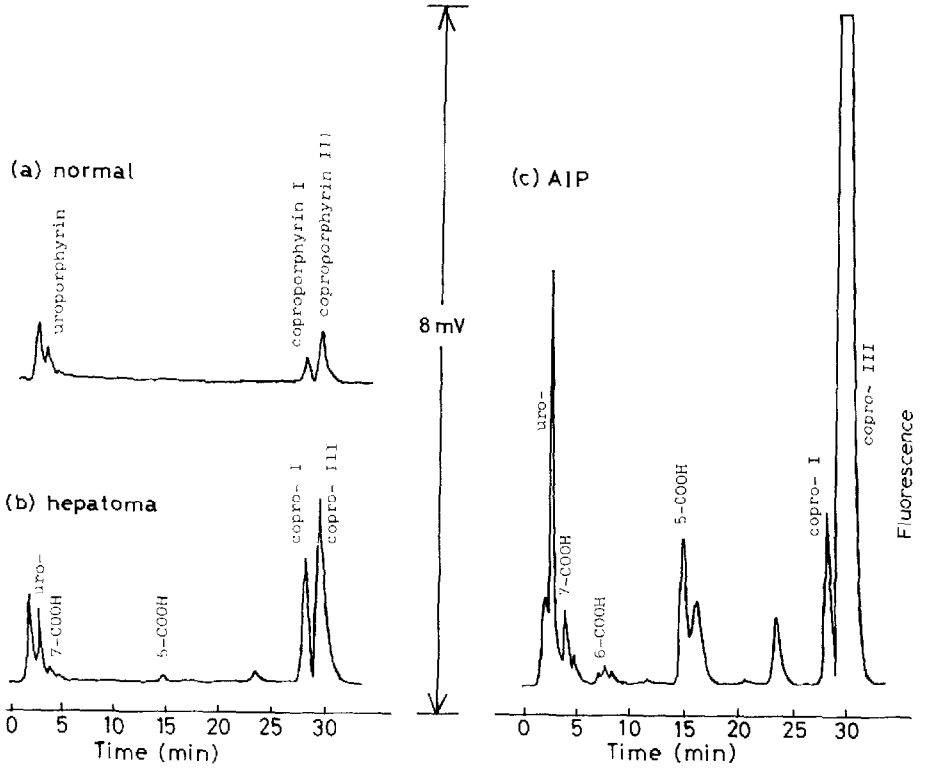

Fig. 3. Chromatograms of urine from (a) a healthy adult, (b) a patient with hepatoma and (c) a patient with acute intermittent porphyria. The analyses were carried out as described in the text. Chromatographic conditions as in Experimental and Fig. 1.

mixed with porphyrin free acid standards. The coefficient of variation (C.V.) for each porphyrin ranged from 6.1 to 9.1% ($n=10$): uroporphyrin (UP), 6.1% (mean 4.93 ng); heptacarboxylic porphyrin (7-COOH-P), 9.1% (4.01 ng); hexacarboxylic porphyrin (6-COOH-P), 7.1% (3.52 ng); pentacarboxylic porphyrin (5-COOH-P), 8.2% (3.22 ng); coproporphyrin I (CP-I), 7.4% (3.80 ng); coproporphyrin III (CP-III), 7.6% (5.27 ng).

The between-assay precision of the retention times was examined. The C.V. for each porphyrin ranged from 2.39 to 3.53% ($n=10$): uroporphyrin, 3.13% (mean retention time 2.81 min); heptacarboxylic porphyrin, 2.53% (3.83 min); hexacarboxylic porphyrin, 3.53% (7.35 min); pentacarboxylic porphyrin, 3.21% (14.60 min); coproporphyrin I, 2.40% (27.93 min); coproporphyrin III, 2.39% (29.30 min).

When the organic solvent was not washed with water, the peaks of uroporphyrin and heptacarboxylic porphyrin were not fully separated from the early-eluting substances in the samples (Fig. 2).

The effect of sunlight on the extraction was investigated, and the results are shown in Table II. Because of the photosensitivity of porphyrin, the recovery was not high under direct sunshine, so such exposure must be avoided.

To establish the normal values, random urine samples from five volunteers were analysed. The mean levels and standard deviations of uroporphyrin, hep-

tacarboxylic porphyrin and coproporphyrin I and III were 16.1 ± 4.2, 0.97 ± 1.3, 13.7 ± 3.7 and 28.8 ± 5.5 $\mu g/g$ of creatinine, respectively.

Fig. 3a shows a urinary porphyrin profile of a healthy subject. Uroporphyrin, heptacarboxylic porphyrin and coproporphyrins I and III were detected. Hexa- and pentacarboxylic porphyrin were not detectable. Fig. 3b and c show urinary profiles of a patient with hepatoma and a patient suffering from acute intermittent porphyria, respectively.

The HPLC method described here, combined with the simple extraction procedure, appears suitable for the routine analysis of urinary porphyrins.

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