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Note

Determination of coproporphyrin I and III isomers by high-performance liquid chromatography

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The coproporphyrin (CP) III is led to heme in the biosynthetic pathway, but CP I is not involved in the metabolic pathway. Therefore, a deficiency in the metabolism may be observed from the analysis of CP I and CP III.

Previously published methods for the CP isomers were a paper chromatographic method [1] and thin-layer chromatographic methods [2, 3]. But these published methods present both problems of precision and are time consuming. Battersby et al. [4] reported the separation of CP isomers (I, II, III, IV) using a high-performance liquid chromatographic (HPLC) method, but the method required a recycling system for separation of all the isomers. Recently, Englert et al. [5] reported a direct method for the isolation and measurement of porphyrins in biological samples using HPLC. Unfortunately, the method was intricate; we also examined a direct method for the determination of CP isomers in urine, but were not able to obtain adequate results. Therefore, we investigated an HPLC combined solvent extraction method, and obtained good results.

In this paper, a simple HPLC method for the separation of CP I and CP III is described. The chromatographic analysis was realized in less than 20 min without the need for temperature programming.

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EXPERIMENTAL

Reagents

Free coproporphyrin I and coproporphyrin III tetramethyl ester were purchased from Sigma (St. Louis, MO, U.S.A.). Coproporphyrin I (5 μg per vial) was dissolved in 5 ml of 0.1 *N* hydrochloric acid. Coproporphyrin III tetramethyl ester was hydrolyzed in 7 *N* hydrochloric acid overnight, and diluted with water until a concentration of 0.1 *N* hydrochloric acid was obtained; the concentration of CP III was calculated from the molecular extinction [6].

Acetonitrile was purchased from Wako Chemical (Tokyo, Japan) and all other reagents were reagent grade.

Instrumentation

The liquid chromatographic system consisted of the following components: a Model 635 High-performance liquid chromatograph (Hitachi, Tokyo, Japan); a Hitachi 3053 reversed-phase column 5 μm (150 mm × 4 mm); a Model 204S fluorescence detector (Hitachi); and a Hitachi Model 056 recorder.

Procedure

The chromatographic procedure was as follows: for the mobile phase 5 ml of acetic acid and 0.25 g of potassium dihydrogen phosphate were dissolved in water and made up to 500 ml with water. Then, 500 ml of acetonitrile were added. The solvents were degassed in an ultrasonic bath under reduced pressure (water pump) before use. The flow-rate was 1.3 ml/min. The excitation wavelength of the xenon lamp was 392 nm and the emission wavelength was measured at 610 nm. The chart speed of the dual-channel recorder was 0.5 cm/min. All chromatographic separations were performed at room temperature. The volume of the samples injected into the column was 10 μl.

Urine analysis

The method was applied to the analysis of urine levels in ten healthy volunteers. Urine (10 ml) was placed in a separating funnel, acidified with 2 ml of acetic acid and extracted with 20 ml diethyl ether until the aqueous phase showed no fluorescence under UV light. The extracts were combined and washed once with a little water. The extract was evaporated to dryness using a rotary evaporater and the CP I and CP III isomers in the residue were dissolved in 0.5 ml of 0.1 *N* hydrochloric acid. Then the 0.1 *N* hydrochloric acid solution was filtered through a membrane filter (Sartorius 0.45 μm).

RESULTS AND DISCUSSION

Fig. 1 shows the effect of acetonitrile concentration in the mobile phase on the separation of CP I and CP III. On decreasing the acetonitrile concentration, the retention times increased, and other variables were kept constant. A concentration of 50% (v/v) acetonitrile was chosen for the determination of CP I and CP III. The effect of acetic acid concentration on the separation was studied at different concentrations of 0, 0.25, 0.5, 1.0 and 4.0% (v/v).

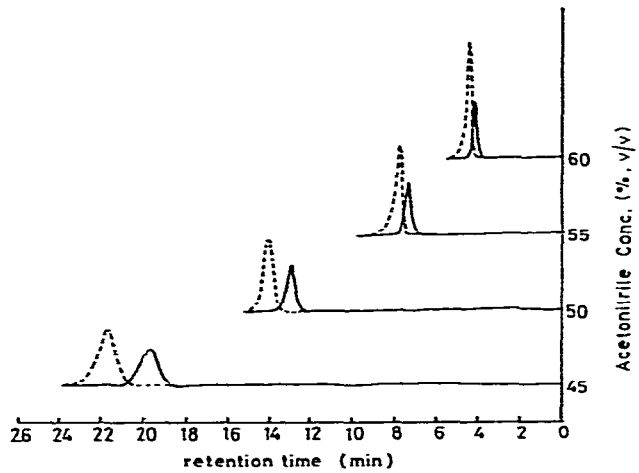

Fig. 1. Effect of acetonitrile concentration in the mobile phase on the separation and retention times of the CP isomers. Solid line: CP I; dotted line: CP III. Stationary phase: Hitachi Gel 3053 (150 × 4 mm), mobile phase: see Experimental. Flow-rate: 1.3 ml/min. Fluorophotometer: excitation wavelength 392 nm, emission wavelength 610 nm.

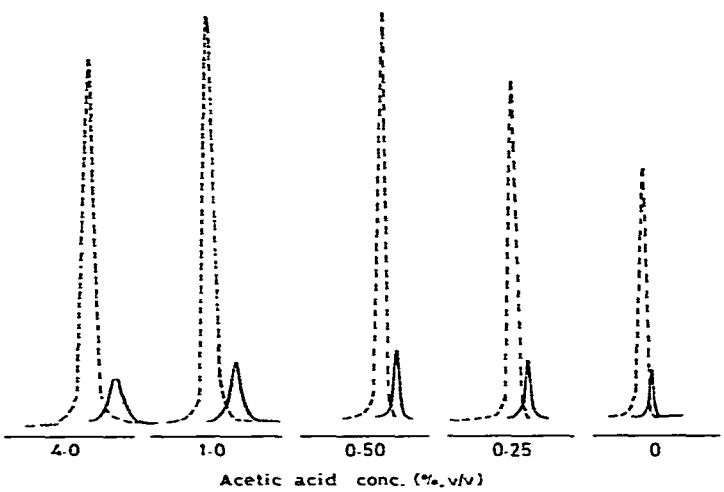

Fig. 2. Effect of acetic acid concentration in the mobile phase on the separation and retention times of the CP isomers. Solid line: CP I, dotted line: CP III. Potassium dihydrogen phosphate 0.05% (w/v) in acetonitrile—water (1:1) solution.

A good separation was obtained at a concentration of 0.5% (v/v) acetic acid (Fig. 2). Effect of the potassium dihydrogen phosphate concentration was studied at concentrations of 0, 0.025, 0.05, 0.1 and 0.2% (w/v) with both a 50% (v/v) solution of acetonitrile and 0.5% (v/v) solution of acetic acid. A good separation was obtained at a concentration of 0.025% (w/v) (Fig. 3). Therefore, to determine CP I and CP III, concentrations of 50% (v/v) acetonitrile, 0.5% (v/v) acetic acid and 0.025% (w/v) potassium dihydrogen phosphate were chosen for the mobile phase.

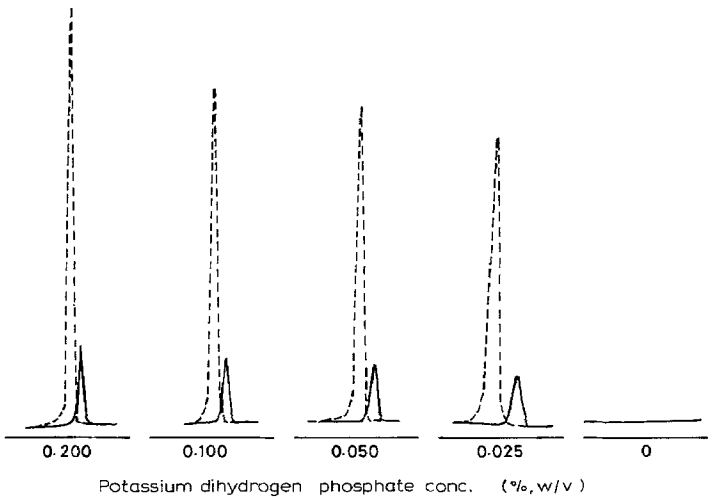

Fig. 3. Effect of potassium dihydrogen phosphate concentration in the mobile phase on the separation and retention times of the CP isomers. Solid line: CP I, dotted line: CP III. Acetic acid 0.05% in acetonitrile—water (1:1) solution.

Linearity

Under the optimal conditions, a good linear relationship between the peak heights and concentrations of CP I and CP III in the ranges 2—10 ng and 2.7—13.5 ng, respectively, is found. Sensitivity of both CP I and CP III in the described method was 0.5 ng.

Comparison of results using spectrophotometric and spectrofluorometric detection

The two detectors were connected in series directly after the column, first the photometer and secondly the fluorometer. Fluorometric detection depends on two parameters, the emission and the excitation wavelength, and yields a higher specificity than the VIS detection (Fig. 4).

Reproducibility

The reproducibilities of ten replicate analyses of CP I (200, 400, 1000 μg/l) and CP III (270, 540, 1350 μg/l) were examined. Coefficients of variation were 4.0, 2.7, 1.3% and 3.7, 2.4, 1.2%, respectively.

Application

The CP content in urine was calculated from peak heights, according to the following equation:

$$A = S \times \frac{PH_a}{PH_s} \times \frac{0.5}{0.01} \times \frac{V}{10} \times \frac{1}{1000}$$

where A = CP I or CP III concentration in the sample (μg/day); S = amount of the standard injected into column (ng); PH_a = peak height of CP I or CP III in the sample (mm); PH_s = peak height of the standard CP I or CP III (mm);

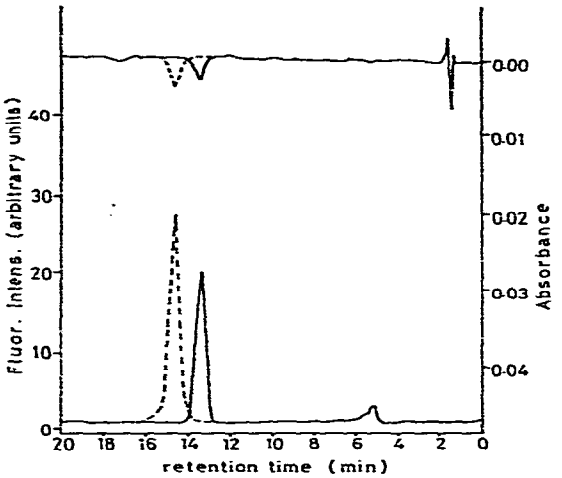

Fig. 4. Comparison of results using spectrophotometric and spectrofluorometric detection. Solid line: CP I, dotted line: CP III. Hitachi spectrophotometer 100-50 at 401 nm, 0.05 a.u.f.s.; Hitachi fluorometer 204-S, excitation wavelength 392 nm, emission wavelength 610 nm. Chromatographic conditions were as given in the text.

V = total volume of the sample (ml); 0.5 = final volume of the sample (ml); 10 = initial volume of urine (ml); 0.01 = volume of the sample injected into the column (ml); and $\frac{1}{1000}$ = conversion factor into micrograms.

Normal urine contains about 31 ± 15 μg per day CP I and about 72 ± 27 μg per day CP III. Total CP value obtained with the present method was in a good agreement with previous reports [7, 8]. CP III per total CP found in urine in ten normal individuals was 60—75%, which was in agreement with the value reported by Aziz et al. [9].

For recovery studies, distinct amounts (10 ng per 10 μl) of CP I were added to urine samples. The mean recovery of ten experiments was 85% for CP I. Thus this method might be expected to be useful in the routine analysis of CP I and CP III.

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