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Note

Separation of hypoxanthine and xanthine from pyrazinamide and its metabolites in plasma and urine by high-performance liquid chromatography

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Pyrazinamide is an anti-tuberculous drug that completely blocks the secretion of uric acid in renal tubules. Therefore pyrazinamide has been used to investigate disturbance of the uric acid transport system in renal tubular sites in patients with renal hypouricaemia [1, 2]. Pyrazinamide is also known to influence the renal transport of oxypurines, which share the transport system with uric acid [3]. However, the transport mechanism of oxypurines has not been extensively investigated except in patients with xanthinuria [4]. In the past, separation of a pyrazinamide metabolite from hypoxanthine by direct use of reversed-phase high-performance liquid chromatography (HPLC) was often difficult. A column chromatographic method using Dowex 50 has been used to separate hypoxanthine and xanthine from pyrazinamide and its metabolites [4], but this method is tedious. Therefore, we developed a reversed-phase method that enables rapid and selective analysis of hypoxanthine and xanthine in plasma and urine without the interference of pyrazinamide metabolites, especially 5-hydroxypyrazinamide.

EXPERIMENTAL

Chemicals

Hypoxanthine, xanthine and xanthine oxidase in suspension (activity 25 U per 0.89 ml) were purchased from Sigma (St. Louis, MO, U.S.A.). Allopurinol was kindly provided by Tanabe (Osaka, Japan). Pyrazinamide and pyrazinoic acid were kindly provided by Sankyo (Tokyo, Japan). 5-Hydroxypyrazin-

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amide or 5-hydroxypyrazinoic acid was prepared as follows. The compounds were respectively separated from pyrazinamide and pyrazinoic acid by HPLC after treating pyrazinamide (0.8 m*M*) or pyrazinoic acid (0.24 m*M*) with xanthine oxidase (1 U) in a 10-ml reaction mixture at pH 7.0 for 24 h. The purified 5-hydroxypyrazinamide as identified by mass spectrometry (MS) and purified 5-hydroxypyrazinoic acid was identified by UV spectrophotometry. Other chemicals were obtained from Wako (Osaka, Japan).

Apparatus and chromatographic conditions

The mass spectrum was obtained using an M-60 mass spectrophotometer (Hitachi, Tokyo, Japan), by direct injection method at a probe temperature of 100°C. The operating conditions were the following: ionization beam, 20 eV; ion source temperature, 110°C; accelerating voltage, 3.2 kV. Wavelength scanning was performed using a U-3200 spectrophotometer (Hitachi).

The HPLC system consisted of an LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan), SPD-6AV UV—VIS spectrophotometric detector (Shimadzu) and a C-R3A Chromatopac recorder (Shimadzu). The column was a μBondapak C_{18} (30 cm × 3.9 mm I.D., 10 μm) (Waters Assoc., Milford, MA, U.S.A.). The mobile phase was 0.02 *M* potassium dihydrogen phosphate at pH 2.2 and a flow-rate of 2.0 ml/min. The detection wavelength was 254 nm. The hypoxanthine and xanthine peaks were identified in the samples by their retention times. This identification was confirmed by comparing the chromatographic profiles before and after treatment of 50 μl of plasma or 50 μl of urine (diluted 20-fold) with xanthine oxidase (0.33 U) for 3 h, using a modified Boulieu method [5].

Sample preparation

Standard solutions were prepared by dissolving hypoxanthine (42 μmol/l) and xanthine (39 μmol/l) in water. Blood (5 ml) was drawn into a heparinized tube from normal healthy humans 4 h after the intake of 3 g of pyrazinamide and it was immediately centrifuged at 1200 *g* for 10 min. The plasma samples were then stored at −20°C. Urine samples were collected for 24 h after the intake of 3 g of pyrazinamide and stored at −20°C. Next, 200 μl of 1.98 *M* perchloric acid was added to a 10-ml glass tube containing 1 ml of plasma, 600 μl of distilled water and 200 μl of 1.47 m*M* allopurinol and vigorously shaken with a Vortex agitator for 10 s. After centrifugation at 1200 *g* for 10 min, a 10-μl aliquot of the supernatant was loaded onto the column. The urine samples were centrifuged at 1200 *g* for 10 min. Then 1 ml of the supernatant was diluted ten-fold and a 10-μl sample was applied to the column.

RESULTS

The effect of a pH 2.2 mobile phase on the separation of oxypurines, nucleosides, allopurinol, pyrazinamide and metabolites of pyrazinamide

With a pH 2.2 mobile phase, the retention times of hypoxanthine, xanthine, guanine, adenine, 5-hydroxypyrazinoic acid, 5-hydroxypyrazinamide, pyrazinoic acid, allopurinol and pyrazinamide were 3.90, 5.90, 3.18, 3.21, 4.75, 5.31, 7.35, 8.53 and 9.57 min, respectively (Fig. 1).

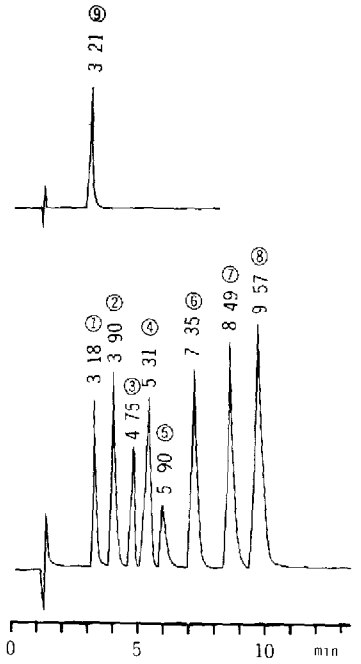

Fig. 1. HPLC and retention times of guanine (1), hypoxanthine (2), 5-hydroxypyrazinoic acid (3), 5-hydroxypyrazinamide (4), xanthine (5), pyrazinoic acid (6), allopurine (7), pyrazinamide (8) and adenine (9). Chromatographic conditions are described in Experimental.

Reproducibility and accuracy of the measurment for hypoxanthine and xanthine in plasma

Linearity (area versus concentration) was first tested against standards in an aqueous solution at various concentrations and the linearity was excellent up to 42, 39 and 140 μmol/l for hypoxanthine, xanthine and allopurinol, respectively. Next, reproducibility and accuracy studies were performed at three different concentrations of hypoxanthine and xanthine in plasma using allopurinol as an internal standard. Table I shows the results. 9-Methylxanthine could not be used as an internal standard because it overlapped with hypoxanthine under the chromatographic conditions described in this paper.

TABLE I

REPRODUCIBILITY AND ACCURACY OF HYPOXANTHINE AND XANTHINE ANALYSIS IN PLASMA

Compound	Concentration added (μmol l^{-1})	Concentration found (mean ± S.D., n = 10) (μmol l^{-1})	Coefficient of variation (%)
Hypoxanthine	4.46	4.51 ± 0.03	0.7
	13.36	13.25 ± 0.05	0.4
	26.64	26.50 ± 0.25	0.9
Xanthine	3.48	3.46 ± 0.04	1.2
	10.45	10.46 ± 0.05	0 8
	20.92	20.86 ± 0.20	1.0

Recoveries of oxypurines, and day-to-day and within-day coefficients of variation

The recoveries of hypoxanthine and xanthine were 99.8 and 99.7%, respectively. Day-to-day and within-day coefficients of variation were 1.0 and 0.9%, respectively, for hypoxanthine, and 1.1 and 1.1%, respectively for xanthine.

Effect of xanthine oxidase on the peaks of hypoxanthine and xanthine

We compared the chromatographic patterns before and after xanthine oxidase treatment to identify hypoxanthine and xanthine in plasma and urine

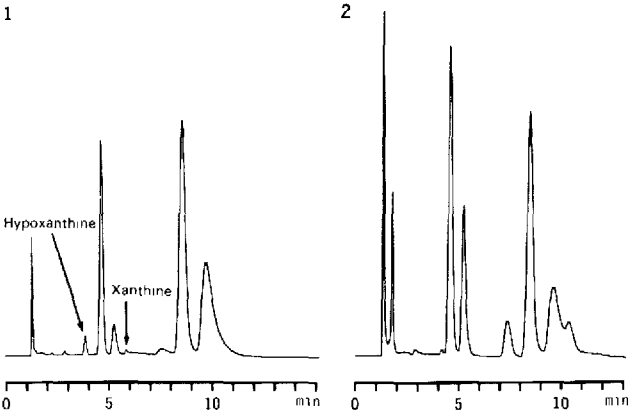

Fig. 2. Chromatograms of plasma before (1) and after (2) xanthine oxidase treatment.

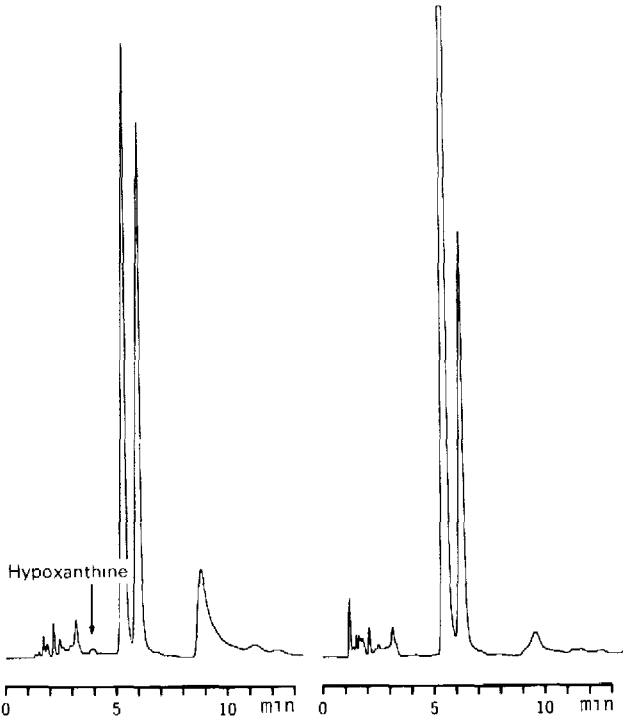

Fig. 3. Chromatograms of urine before (left) and after (right) xanthine oxidase treatment.

containing pyrazinamide and its metabolites. As shown in Fig. 2, the peaks of hypoxanthine and xanthine in the plasma disappeared completely after the treatment. The peak corresponding to xanthine could not be detected in the urine prior to the treatment, but hypoxanthine was detected. The peak of hypoxanthine in the urine specimen also disappeared completely after treatment (Fig. 3).

DISCUSSION

For the determination of oxypurines (hypoxanthine and xanthine), enzymatic methods [6], column chromatography [7], thin-layer chromatography [8], gas chromatography coupled to MS [9] and ion-exchange chromatography [10] have been used. The most popular method is reversed-phase HPLC, but hypoxanthine was not separated from guanine [11–13] except in the method reported by Boulieu et al. [5]. We therefore investigated whether the mobile phase at pH 2.2 could separate hypoxanthine from guanine, and obtained excellent results. We were unable to measure hypoxanthine in plasma and urine containing pyrazinamide and its metabolites by reversed-phase HPLC because of interfering by 5-hydroxypyrazinamide in the pH range 3.6–7.0 (data not shown). However, at pH 2.2, hypoxanthine could be separated clearly from 5-hydroxypyrazinamide. Next, we investigated the reproducibility, accuracy and recovery of hypoxanthine and xanthine in plasma containing pyrazinamide and its metabolites. These results were sufficiently good compared with those obtained by Boulieu et al. [5]. We used xanthine oxidase treatment to rule out the possibility of insufficient separation of hypoxanthine or xanthine from other endogenous compounds. This was confirmed by their complete disappearance after xanthine oxidase treatment.

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