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Note

Rapid and simultaneous determination of pyrazinamide and its major metabolites in human plasma by high-performance liquid chromatography

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Pyrazinamide is the amide of pyrazinoic acid and is widely used in combination with other drugs for the treatment of tuberculosis. After rapid absorption of pyrazinamide via the gastrointestinal tract, it has been shown to be metabolized to 5-hydroxypyrazinamide, which presumably undergoes microsomal deamination to give 5-hydroxypyrazinoic acid, and to pyrazinoic acid, which is hydroxylated to 5-hydroxypyrazinoic acid [1,2]. When pyrazinamide is used in the treatment of tuberculosis, its measurement and that of pyrazinoic acid in plasma is required in order to prevent their associated side-effects, especially that of hyperuricaemia. 5-Hydroxypyrazinoic acid is of interest because it is not detected in the urine of xanthinuric patients who are administered pyrazinamide. This suggests that only xanthine oxidase converts pyrazinoic acid into 5-hydroxypyrazinoic acid and, therefore, the measurement of 5-hydroxypyrazinoic acid is considered useful in studying patients with xanthinuria.

Pyrazinamide and its three major metabolites have been determined by column chromatography [3] and gas chromatography [1]. However, these methods are time consuming and tedious. Ratti et al. [4] reported a high-performance liquid chromatographic (HPLC) technique for the determination of pyrazinamide and pyrazinoic acid, but this method required a very long and difficult extraction. Brouard et al. [5] reported the determination of pyrazinamide in plasma by HPLC, but its metabolites were not determined. In contrast, the method described in this paper features the rapid and simultaneous determination of pyrazinamide and its metabolites.

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EXPERIMENTAL

Reagents

Pyrazinamide and pyrazinoic acid were kindly provided by Sankyo (Tokyo, Japan). 5-Hydroxypyrazinamide and 5-hydroxypyrazinoic acid were separated from pyrazinamide and pyrazinoic acid, respectively, by column chromatography as described previously [3] after reacting pyrazinamide (0.8 mmol) or pyrazinoic acid (0.72 mmol) with xanthine oxidase (50 U) in a 10-ml or 30-ml reaction mixture at pH 7.0 for 15 h.

2,3-Pyrazinedicarboxamide (97% purified) was purchased from Wako (Osaka, Japan) and was repurified by column chromatography. In brief, 50 mg of 2,3-pyrazinedicarboxamide were dissolved in 50 ml of distilled water and applied on a 16×2.0 cm I.D. column of Bio-Rad Cl-X8 resin (200–400 mesh). 2,3-Pyrazinecarboxamide was eluted first at a rate of 80 ml/h with distilled water.

Xanthine oxidase was purchased from Sigma (St. Louis, MO, U.S.A.) and allopurinol was kindly provided by Tanabe (Osaka, Japan). Other chemicals were purchased from Wako.

Apparatus and technique

The chromatographic system consisted of an LC-6A high-performance liquid chromatograph (Shimadzu, Kyoto, Japan), an RF 530 fluorescence HPLC monitor (Shimadzu) and a C-R3A Chromatopac recorder (Shimadzu). A 10-μm μBondapak C_{18} column (30 cm×3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) was used at a column temperature of 25°C. The mobile phase was 0.02 *M* KH_2PO_4 (pH 2.56) and the flow-rate was 2.0 ml/min. A fluorescence detector was used at 410/365 nm. The detection limits of 5-hydroxypyrazinoic acid, 5-hydroxypyrazinamide, pyrazinoic acid and pyrazinamide were 3, 3, 30 and 30 ng, respectively, with an apparatus detection limit (signal-to-noise ratio of 1) of 0.45 pg per 12 μl cell.

Sample preparation

A 100-μl aliquot of 2 *M* perchloric acid was placed in a 5-ml glass tube containing 0.5 ml of plasma, 0.3 ml of distilled water and 100 μl of 10 m*M* 2,3-pyrazinedicarboxamide as the internal standard, with thorough mixing for 10 s. After centrifugation at 1500 *g* for 10 min, a 200-μl aliquot of supernatant was neutralized with 48 μl of 1 *M* sodium hydroxide solution and 60 μl of the supernatant were loaded on to the column.

Calibration graph

To 0.5 ml of drug-free plasma were added 0.1 ml of a standard solution containing pyrazinamide, pyrazinoic acid, 5-hydroxypyrazinamide and 5-hydroxypyrazinoic acid, 0.1 ml of 10 m*M* 2,3-pyrazinedicarboxamide and 0.3 ml of distilled water to obtain a calibration between 5 and 80 μg/ml for pyrazinamide, 2.5 and 50 μg/ml for pyrazinoic acid, 1 and 10 μg/ml for 5-hydroxypyrazinamide and 2.5 and 0.25 μg/ml for 5-hydroxypyrazinoic acid. Standard samples were then treated in the same way as the plasma samples.

TABLE I

ACCURACY AND REPRODUCIBILITY OF THE DETERMINATION OF PYRAZINAMIDE AND ITS METABOLITES IN HUMAN PLASMA ($n=10$)

Compound	Amount added (μg/ml)	Amount found (mean ± S.D.) (μg/ml)	Accuracy (%)	Coefficient of variation (%)
Pyrazinamide	60	60.8 ±0.5	101	0.8
	40	39.7 ±0.9	99	2.3
	20	19.3 ±0.5	97	2.6
Pyrazinoic acid	40	41.2 ±1.1	103	2.7
	20	20.3 ±0.7	101	3.4
	10	9.5 ±0.4	95	4.2
5-Hydroxypyrazinamide	10	10.2 ±0.4	102	3.9
	5	5.2 ±0.2	104	3.8
	2	2.0 ±0.1	100	5.0
5-Hydroxypyrazinoic acid	2	2.09 ± 0.08	105	3.8
	1	1.02 ± 0.04	102	3.9
	0.5	0.49 ± 0.008	98	1.6

RESULTS

Accuracy and reproducibility of the determination of pyrazinamide, pyrazinoic acid, 5-hydroxypyrazinamide and 5-hydroxypyrazinoic acid in human plasma

Linearity of peak area versus concentration was first tested on standards in aqueous solution at various concentrations, and the linearity was excellent up to 100, 50, 10, 2.5 and 150 μg/ml for pyrazinamide, pyrazinoic acid, 5-hydroxypyrazinamide, 5-hydroxypyrazinoic acid and 2,3-pyrazinedicarboxamide, respectively. Next, accuracy and reproducibility studies were performed at three concentrations of pyrazinamide, pyrazinoic acid, 5-hydroxypyrazinamide and 5-hydroxypyrazinoic acid in plasma, using 2,3-pyrazinedicarboxamide as the internal standard. Table I gives the results.

Pyrazinamide and its metabolite levels in plasma

Fig. 1 shows the chromatograms of drug-free plasma and plasma extracted from a healthy volunteer who had been given 3 g of pyrazinamide. Fig. 2 shows the plasma profile of pyrazinamide and its metabolites in a healthy volunteer who had been given 3 g of pyrazinamide. Fig. 3 shows the chromatogram of plasma extracted 2 h after a healthy volunteer had been given 3 g of pyrazinamide and 200 mg of allopurinol. The peak of 5-hydroxypyrazinoic acid disappeared completely.

DISCUSSION

A small peak of an endogenous compound in plasma overlapped the peak of 5-hydroxypyrazinamide under the chromatographic conditions used (Fig. 1). We therefore established a calibration graph by adding standard solution to drug-free

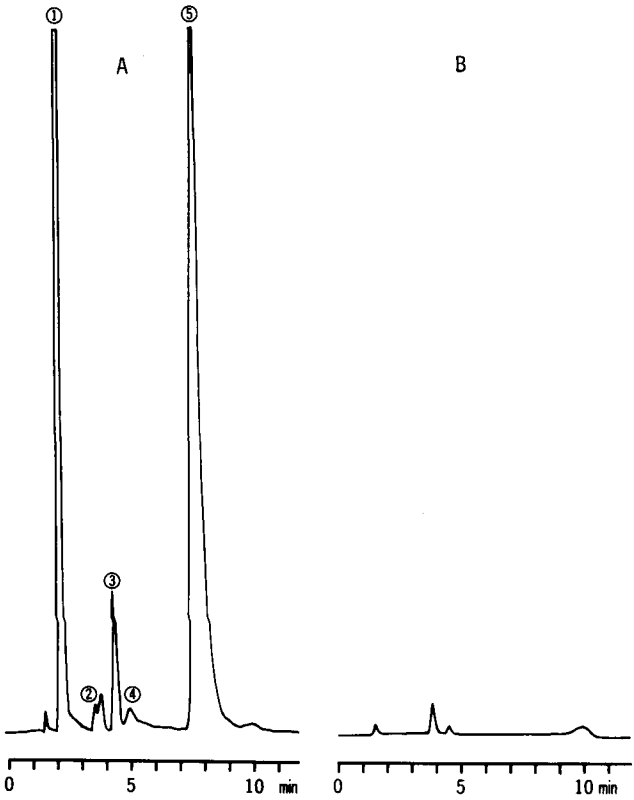

Fig. 1. (A) Chromatogram of plasma extract containing pyrazinamide and its metabolites 4 h after administration of pyrazinamide; (B) chromatogram of blank plasma. Peaks: 1 = 2,3-pyrazinedicarboxamide; 2 = 5-hydroxypyrazinoic acid (0.36 μg/ml); 3 = 5-hydroxypyrazinamide (5.74 μg/ml); 4 = pyrazinoic acid (6.14 μg/ml); 5 = pyrazinamide (73.91 μg/ml).

plasma. Using this method, we investigated the accuracy and reproducibility of the determination of pyrazinamide and its metabolites in plasma, and the results were satisfactory in comparison with those described by Ratti et al. [4] and Brouard et al. [5]. Next, we measured concentrations of pyrazinamide and its metabolites in plasma after a healthy volunteer had been given 3 g of pyrazinamide. The concentrations of pyrazinamide and pyrazinoic acid were comparable to those reported previously [6]. No peak of 5-hydroxypyrazinoic acid was found in the plasma 2 h after administration of pyrazinamide and allopurinol, the latter of which is a xanthine oxidase inhibitor. This result strongly suggests the possibility that only xanthine oxidase converts pyrazinoic acid into 5-hydroxypyrazinoic acid. The concentration of 5-hydroxypyrazinamide (2.30 μg/ml) was lower 2 h after administration of pyrazinamide and allopurinol than 2 h after administration of only pyrazinamide (4.69 μg/ml), suggesting that xanthine oxidase in vivo converts pyrazinamide into 5-hydroxypyrazinamide.

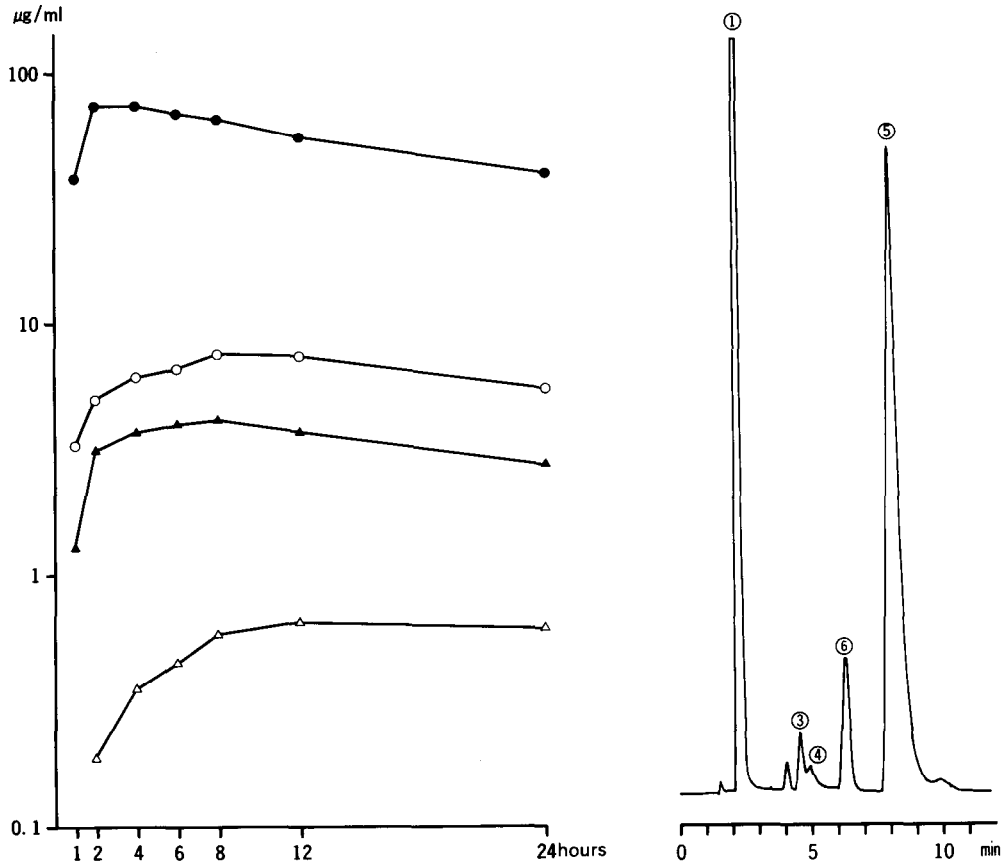

Fig. 2. Concentrations of pyrazinamide (●) and its metabolites pyrazinoic acid (○), 5-hydroxypyrazinamide (▲) and 5-hydroxypyrazinoic acid (△).

Fig. 3. Chromatogram of plasma extract containing pyrazinamide, its metabolites and oxypurinol. Peaks: 1 = 2,3-pyrazinedicarboxamide; 3 = 5-hydroxypyrazinamide (2.30 μg/ml); 4 = pyrazinoic acid (4.37 μg/ml); 6 = oxypurinol; 5 = pyrazinamide (67.60 μg/ml).

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