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Short Communication

Simultaneous determination of caffeine and its primary demethylated metabolites in human plasma by high-performance liquid chromatography

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ABSTRACT

An improved high-performance liquid chromatographic method for the simultaneous determination of caffeine and its three primary metabolites (theophylline, theobromine and paraxanthine) in human plasma is described. The four substances were separated on a reversed-phase column (5 μm TSK gel ODS-80TM, 150 mm × 4.6 mm I.D.) by use of the mobile phase methanol–0.1 *M* NaH_2PO_4 (30:70, v/v) with a flow-rate of 0.8 ml/min. Absorbance was monitored at 274 nm. The detection limit was 5 ng/ml for theobromine and caffeine and 10 ng/ml for paraxanthine and theophylline. The linearity and reproducibility were sufficient for drug monitoring of caffeine and its primary methylxanthines.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine; CA) has been used as a model probe to estimate cytochrome P-448-dependent liver function [1–3]. Theophylline (1,3-dimethylxanthine; TP), paraxanthine (1,7-dimethylxanthine; PX), and theobromine (3,7-dimethylxanthine; TB) are produced as primary CA metabolites by N-demethylation [4].

Recently, Wang *et al.* [1] have reported the possibility of predicting the extent of hepatic disorder by studying changes in the blood concentrations of CA and its three primary metabolites after CA administration in patients with liver cirrhosis. Production of PX, TP and TB was suppressed in patients with liver disease. Therefore, it is necessary to measure plasma concentrations of low levels of CA metabolites. Previous analytical methods for CA and its N-demethylated metabolites are not sensitive enough for CA pharmacokinetic studies of a lower dose [5–18]. We have developed a simple, rapid, sensitive and reproducible method and carried out a pharmacokinetic study in humans by using the improved high-performance liquid chromatographic (HPLC) method.

EXPERIMENTAL

Reagents and standards

CA, TB, TP and 8-chlorotheophylline (internal standard, I.S.) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and PX was purchased from Sigma (St. Louis, MO, USA). Dichloromethane, methanol, hydrochloric acid and sodi-

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um phosphate (NaH_2PO_4) were all analytical grade from Wako (Osaka, Japan). Standard solutions (1 mg/ml) of the xanthines were prepared in the mobile phase, which remained stable for at least three months at 4°C. These solutions were then diluted as necessary to prepare the appropriate plasma standards for each drug and each assay. The I.S. solution (0.5 mg/ml) was also prepared in mobile phase and stored at 4°C.

Analytical procedure

To 0.5 ml of plasma (or standard) in a 15-ml culture glass tube were added 50 μl of I.S. (3 μg/ml), 100 μl of 1 *M* hydrochloric acid and 3 ml of dichloromethane. After vortex-mixing for 2 min, the tubes were centrifuged at 1200 *g* for 5 min and the aqueous (upper) phase was aspirated. The organic phase was transferred to a clean conical glass tube and evaporated in a water-bath at *ca.* 40°C under a gentle stream of nitrogen. The residue was dissolved in 100 μl of the mobile phase, and a 20 μl of the solution were injected into the HPLC apparatus.

Accuracy and reproducibility

The reproducibility and the accuracy from spiking drug-free plasma were calculated at concentrations of 0.02, 0.05, 0.1, 0.5, 1.0 and 5.0 μg/ml in plasma by comparing the peak-height ratio against I.S. with those obtained for aqueous solutions containing known concentrations of methylxanthine.

Chromatography

The HPLC apparatus (Model 114M, Beckman, CA, USA) was equipped with a variable-wavelength UV detector (Model 870-UV, Jasco, Tokyo, Japan), and separation was achieved using a C_{18} reversed-phase column (150 mm × 4.6 mm I.D., particle size 5 μm, TSK gel ODS-80TM, Tosoh, Tokyo, Japan). The mobile phase was methanol–0.1 *M* NaH_2PO_4 (30:70, v/v) with a flow-rate of 0.8 ml/min. The effluent was monitored at 274 nm. All instruments and the column were operated at ambient laboratory temperature (*ca.* 25°C).

Human study

Five healthy male subjects (aged 24–45 years; body weight 62–67 kg), who had given their informed consent, participated in the study. All subjects ingested 2 mg/kg CA in aqueous solu-

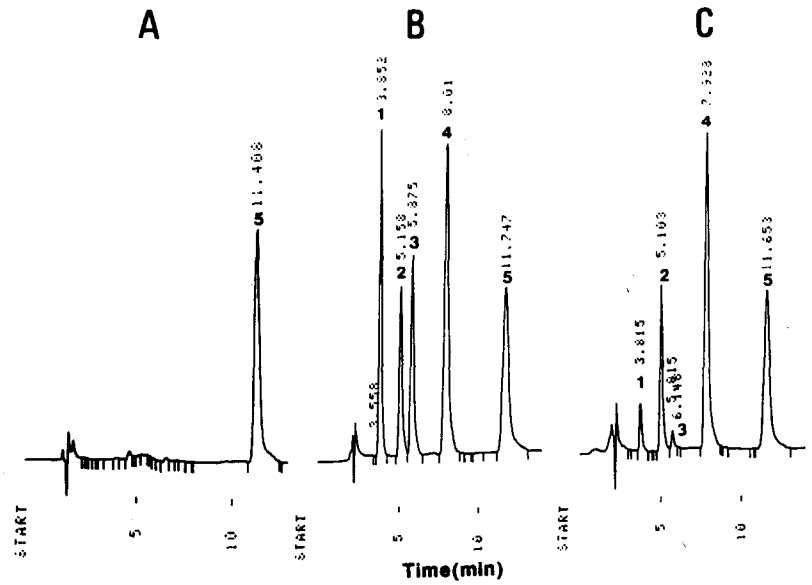

Fig. 1. Chromatograms of blank plasma (A), human plasma spiked with caffeine (CA) and its primary metabolites (0.6 μg/ml) (B), and a human plasma sample obtained 6 h after oral administration of CA (2 mg/kg) (C). Column, TSK gel ODS-80TM; mobile phase, methanol–0.1 *M* NaH_2PO_4 (30:70, v/v); flow-rate, 0.8 ml/min; detection wavelength, 274 nm. Peaks: 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = caffeine; 5 = 8-chlorotheophylline (I.S.).

tion, at 09:00 h after an overnight fast. Blood was sampled before drug administration and after 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h.

The half-life ($t_{1/2}$) of CA was estimated by linear regression analysis. The apparent volume of distribution (V_d) was calculated by dividing the dose of CA by the extrapolated value of the CA concentration at zero time. The total body clearance (Cl) of CA was calculated from the equation Cl = dose/AUC_0^∞, where AUC is the area under the concentration–time curve.

RESULTS AND DISCUSSION

Fig. 1 shows representative chromatograms obtained from human plasma spiked with CA and its primary metabolites (0.6 μg/ml) (B) and a human plasma sample obtained 6 h after oral administration of CA (C). There were no interfering peaks in blank plasma samples. The results indicate that CA, its three primary metabolites and the I.S. were well separated. The time needed was 20 min for sample preparation and 15 min for chromatographic analysis.

Table I demonstrates the accuracy and reproducibility of the analysis of the method for CA and its three metabolites. The standard curve produced by using pooled human drug-free plasma showed good linearity (r = 0.999–1.000) at concentrations of 0.02–5 μg/ml. The coefficient of variation (C.V.) was less than 10% (3–10%).

Previously reported detection limits were as follows: 0.5 μg/ml [9], 0.1 μg/ml [7], 0.08 μg/ml [8], 0.25 μg/ml [15] and 0.06 μg/ml [18]. With our method, the limits of quantitation, after extraction, were 5 ng/ml for TB and CA and 10 ng/ml for PX and TP. No interfering peaks appeared when the following drugs, which are usually coadministered with CA to patients, were added to plasma: acetaminophen, phenylbutazone, phenacetin, vitamin B_1, salicylic acid, phenobarbital, chlorpheniramine maleate, trimethadione, cortisol, prednisolone, prednisone, phenytoin, acetylsalicylic acid and ethenzamide.

We carried out a pharmacokinetic study of CA followig the administration of a lower dose of the drug. The results were as follows: $t_{1/2}$ = 4.6 + 0.88 h, V_d = 541 ± 51 ml/kg, Cl = 1.43 + 0.35 ml/min/kg. This method is sensitive enough for therapeutic monitoring and most pharmacokinetic studies after low-dose administration of CA. In addition, the method can also be applied to serum samples.

The data obtained suggest that this improved method is reliable in terms of simplicity, sensitivity, rapidity and reproducibility for the simultaneous determination of CA and its primary demethylated metabolites in human plasma.

TABLE I

ANALYTICAL ACCURACY AND REPRODUCIBILITY OF THE ANALYSIS OF CAFFEINE AND ITS PRIMARY METABOLITES IN HUMAN PLASMA

Each value represents the mean of five experiments assayed corresponding to each compound concentration. Concentration was calculated on the basis of the peak-height ratios against the I.S.

Concentration added (μg/ml)	Theobromine		Paraxanthine		Theophylline		Caffeine	
	Mean (μg/ml)	C.V. (%)	Mean (μg/ml)	C.V. (%)	Mean (μg/ml)	C.V. (%)	Mean (μg/ml)	C.V. (%)
0.02	0.019	10.0	0.021	6.7	0.020	6.7	0.021	10.0
0.05	0.051	7.2	0.050	4.8	0.049	5.9	0.051	8.8
0.1	0.09	5.6	0.11	5.5	0.10	9.0	0.11	8.2
0.5	0.48	8.3	0.48	6.3	0.51	9.8	0.49	8.2
1.0	0.98	8.2	0.99	6.1	1.06	6.6	0.96	9.4
5.0	4.92	5.3	5.10	4.7	4.92	4.3	4.95	6.5

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