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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DIMETHYLXANTHINE METABOLITES OF CAFFEINE IN HUMAN PLASMA

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SUMMARY

A normal-phase high-performance liquid chromatographic assay of caffeine and its metabolites, theophylline, theobromine and paraxanthine, in human plasma is described. The two internal standards ethyltheophylline and 1,3,7-trimethyluric acid are used simultaneously and cover the range of different polarities from caffeine to the three dimethylxanthines. Plasma (0.5 ml) in the presence of ammonium sulphate is extracted with chloroform—isopropanol (1:1, v/v). The extract is chromatographed with a LiChrosorb Si 60 5-μm column and a mobile phase of dichloromethane containing 2.5% of a formate buffer in methanol. Calibration is performed with six different calibration mixtures which take into account the large plasma concentration differences between caffeine and its metabolites in man. The method is suitable for the simultaneous determination of caffeine and its dimethylxanthine metabolites in plasma of healthy and diseased persons.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine), an exogenous substance extensively consumed in beverages and foodstuffs, undergoes practically complete absorption [1], is metabolized almost exclusively in the liver [2] and single doses (equivalent to two to three cups of coffee) may be considered innocuous. Its pharmacokinetics in healthy man are now well elucidated [1,3]. The recent surge of interest in caffeine is based on its potential therapeutic and diagnostic application. Thus, caffeine has been shown to be effective in the treatment of apnoea in the newborn [4,5]; further, its bronchodilating activity has been confirmed in adult asthmatics [6]. On the other hand, caffeine has been investigated as a probe to assess liver function [7,8].

Since caffeine is metabolized to the pharmacologically active dimethyl-

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xanthines (DMX) theobromine (3,7-DMX), theophylline (1,3-DMX) and paraxanthine (1,7-DMX), a more comprehensive knowledge of its effects in man requires the simultaneous measurement of these main metabolites. Therefore, a high-performance liquid chromatographic (HPLC) method was developed for the determination of caffeine and dimethylxanthines in human plasma. Because of the difference of polarity between caffeine and dimethylxanthines, the method described in this paper uses two internal standards with different polarities.

MATERIALS AND METHODS

Apparatus

Analysis was performed with a high-performance liquid chromatograph (Waters Model M6000A) equipped with an automatic sample injection system (Waters Model 710B WISP), a variable-wavelength absorbance detector (Kratos Spectro-Flow 773) and a recorder—integrator (Hewlett-Packard 3390 A). A Gerhardt shaking apparatus (Model LS 20), a Hettich Mikro Rapid centrifuge and a Sorvall RC-5 refrigerated centrifuge with HS-4 swinging bucket were used.

Reagents

All reagents were of analytical grade. Dichloromethane (HPLC grade), methanol (HPLC grade) and 1,3,7-trimethyluric acid were purchased from Fluka (Buchs, Switzerland). Hibar® RT 250-4 columns filled with LiChrosorb Si 60, 5 μm particle size, were obtained from E. Merck (Darmstadt, F.R.G.). 7-Ethyltheophylline and [3-methyl-^{14}C]caffeine were a kind gift from Dr. M.J. Arnaud (Nestlé Research Department, La-Tour-de-Peilz, Switzerland).

Mobile phase

The mobile phase of Midha et al. [9] and Sved and Wilson [10] was used. Ammonium formate (0.2 g) and 15 μl of formic acid were added to 100 ml of methanol; 25 ml of this solution were mixed with 975 ml of dichloromethane. The composition of the mobile phase formate buffer—dichloromethane varied slightly according to the age of the HPLC column between 35:965 (new column) and 18:982 (old column). The mobile phase was degassed with a gentle stream of helium during analysis.

Chromatographic conditions

The flow-rate decreased with the age of the column (from 2 to 1.2 ml/min). The back-pressure was approximately 7 MPa. UV detection was performed at 280 nm (0.06 a.u.f.s.). The injected volume varied between 25 and 50 μl and was kept constant throughout one series of samples. Peak heights were measured.

Extraction procedure

Ammonium sulphate (0.2 g) was weighed into a screw-capped glass centrifuge tube (20 ml) with a PTFE liner. Plasma (0.5 ml) and 0.10 ml of an aqueous solution of the internal standards containing 5 nmol of 7-ethyltheo-

phylline and 5 nmol of 1,3,7-trimethyluric acid were added, followed by 10 ml of a mixture of chloroform—isopropanol (1:1, v/v). The mixture was shaken for 30 min and thereafter centrifuged at 0°C and 3000 *g* (4000 rpm) for 10 min. The organic layer was decanted into a glass centrifuge tube and evaporated to dryness under a stream of nitrogen at 70°C. The residue was dissolved in 0.5 ml of mobile phase, shaken for 30 sec, transferred to a conical plastic tube (1-ml eppendorf) and centrifuged at 4°C (5 min, 11 000 *g*) in order to remove solid particles which could damage the HPLC system. The supernatant was transferred to a glass tube of the autosampler, and an aliquot was injected.

The recovery of caffeine was assessed by extraction of 0.5 ml of bovine plasma spiked with 150 000 dpm of [3-methyl-^{14}C]caffeine (in 50 μl of water) and 100 μl of calibration mixture 3 (Table I). The radioactivity in the sample was determined before and after the extraction procedure. The chloroform—isopropanol mixture in the counter vials was evaporated with a stream of nitrogen before counting in order to avoid quenching. Scintillation cocktail (10 ml) (Lumagel®, Lumac, The Netherlands) was added. A Packard Tri-Carb® 2660 liquid scintillation system (Packard Instrument International, Zurich, Switzerland) with the external channels ratio was used.

Calibration

Different stock solutions were prepared by dissolving each methylxanthine in 100 ml of water, as indicated in Table I. Aliquots of these stock solutions were mixed together (upper figures in Table I) yielding six calibration mixtures with four methylxanthines and two internal standards. For the calibration procedures either xanthine-free human or bovine plasma was used, which yielded no difference in the slopes or intercepts of the resulting calibration curves. Portions of 0.5 ml of this plasma were spiked with 0.1 ml of the calibration mixtures and analysed as described above. The ratio peak height of caffeine to peak height of 7-ethyltheophylline (internal standard 1) was plotted against the amount of caffeine in the spiked plasma samples (lower figures in Table I). Accordingly, the ratio of peak height of dimethylxanthine to peak height of 1,3,7-trimethyluric acid (internal standard 2) was plotted against the amount of dimethylxanthine. Peak height ratios were measured in the patient samples, and concentrations of methylxanthines were calculated from the calibration curves by linear regression.

RESULTS AND DISCUSSION

Fig. 1 shows a blank chromatogram and a chromatogram of methylxanthine standards from a plasma sample which was spiked with a calibration mixture and extracted. Baseline separation was achieved within 12 min. 7-Ethyltheophylline (internal standard 1) and caffeine elute after each other, which indicates similar polarities. The most polar xanthine in the sample is 1,3,7-trimethyluric acid (internal standard 2) which elutes after the three dimethylxanthines. The plasma used for calibration curves was free of interferences at the retention time of the methylxanthines (Fig. 1a). Retention times remained stable within one day. Calibration was performed every day. The calibration curves plotting peak height ratios were linear within the range given in Table I,

TABLE I

PREPARATION OF CALIBRATION MIXTURES

Compound	Molecular weight	Stock solution (mg per 100 ml)	Volume of stock solution in calibrator mixture (ml) Amount of methylxanthine per 100 µl of calibration mixture (nmol)						Internal standards for patient samples
			Calibrator 1	Calibrator 2	Calibrator 3	Calibrator 4	Calibrator 5	Calibrator 6	
Caffeine	194.2	77.6	0.625	1.25	2.5	5.0	10.0	20.0	
			2.5	5.0	10.0	20.0	40.0	80.0	
Theobromine	180.2	18.0	0.25	0.5	2.5	5.0	10.0	20.0	
			0.25	0.5	2.5	5.0	10.0	20.0	
Theophylline hydrate	198.2	19.8	0.25	0.5	2.5	5.0	10.0	20.0	
			0.25	0.5	2.5	5.0	10.0	20.0	
Paraxanthine	180.2	36.0	0.5	1.25	2.5	5.0	10.0	20.0	
			1.0	2.5	5.0	10.0	20.0	40.0	
7-Ethyltheophylline (internal standard 1)	208.2	10.4	10.0	10.0	10.0	10.0	10.0	10.0	10.0
			5.0	5.0	5.0	5.0	5.0	5.0	5.0
1,3,7-Trimethyluric acid (internal standard 2)	210.2	10.5	10.0	10.0	10.0	10.0	10.0	10.0	10.0
			5.0	5.0	5.0	5.0	5.0	5.0	5.0
Water (ml)			78.38	76.5	70.0	60.0	40.0	—	80.0
Total volume (ml)			100	100	100	100	100	100	100

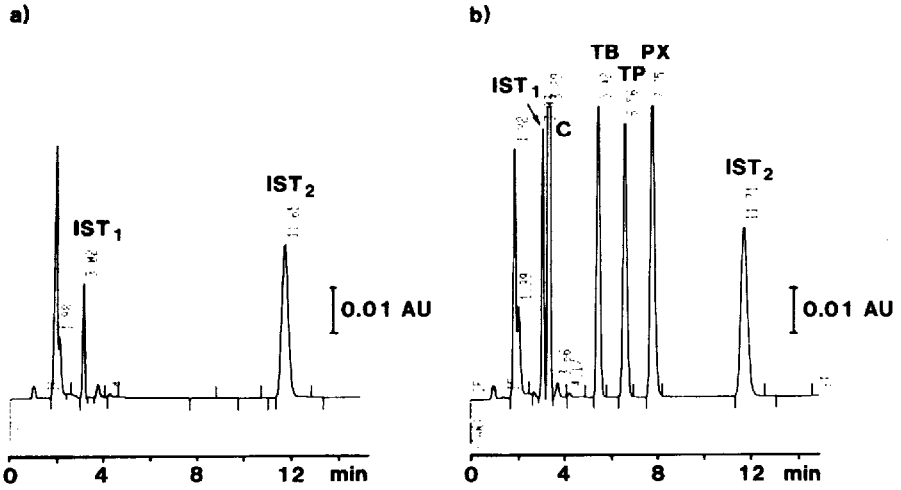

Fig. 1. (a) Chromatogram of a blank bovine plasma sample spiked with 7-ethyltheophylline (IST_1) and 1,3,7-trimethyluric acid (IST_2). (b) Chromatogram of calibration mixture 4 extracted from 0.5 ml of plasma spiked with 7-ethyltheophylline (IST_1), caffeine (C), theobromine (TB), theophylline (TP), paraxanthine (PX) and 1,3,7-trimethyluric acid (IST_2).

with linear correlation coefficients of >0.997. Inter-day reproducibility of the assay (Table II) measured with plasma of a healthy volunteer after coffee intake was below 4% coefficient of variation for caffeine, and below 8% for the metabolites, with the exception of theophylline. The higher coefficient of variation of the theophylline measurements presumably reflects the low theophylline concentration in this plasma sample.

There is a considerable difference of polarity between the trimethylxanthine caffeine and the dimethylxanthines theobromine, theophylline and paraxanthine. Therefore, the less polar internal standard ethyltheophylline was used for analysis of caffeine. Mean recovery of caffeine after the extraction procedure was 85% (n = 5), as determined with ^{14}C-labelled caffeine.

The more polar internal standard 1,3,7-trimethyluric acid was employed for analysis of all three dimethylxanthines (Fig. 2). This compound is a well known degradation product of caffeine, but no measurable concentration in human plasma has been reported, so far. Only less than 1% of an administered dose of caffeine can be found as 1,3,7-trimethyluric acid in urine [3,11]. In our laboratory no measurable peak could be detected in human samples (newborns, patients with liver disease, controls) at the retention time of this internal standard; therefore interference with the endogenous compound can be excluded.

Large variations in methylxanthine levels in humans had to be taken into account when the assay was developed. In liver disease the impaired metabolism of methylxanthines is reflected in increased plasma levels. Table III depicts the fasting plasma levels in ten patients with liver cirrhosis and ten healthy controls. Not only caffeine, but also its main metabolites may accumulate up to 40-fold compared to mean fasting concentrations in normal man. Total methylxanthines were increased on the average five-fold in the cirrhotic group. Representative chromatograms of each group are shown in Fig. 2.

TABLE II

REPRODUCIBILITY OF METHYLXANTHINE DETERMINATION

Compound	Number of determinations	Concentration* in plasma (μmol/l)	Coefficient of variation (%)
Intra-day			
Caffeine	15	27.3 ± 0.6	2.3
Theobromine	15	7.9 ± 0.3	4.3
Theophylline	15	1.4 ± 0.1	7.1
Paraxanthine	15	6.5 ± 0.2	3.5
Inter-day			
Caffeine	11	31.2 ± 1.1	3.7
Theobromine	11	3.7 ± 0.3	7.7
Theophylline	11	2.5 ± 0.3	11.2
Paraxanthine	11	14.0 ± 1.0	7.1

*Mean ± standard deviation. Different plasma samples of two healthy volunteers after coffee consumption.

TABLE III

FASTING PLASMA METHYLXANTHINE LEVELS IN PATIENTS WITH LIVER DISEASE AND CONTROLS

Values are expressed as μmol/l (mean ± S.D.), range in parentheses.

	Caffeine	Theobromine	Theophylline	Paraxanthine	Total methylxanthines
Cirrhosis (n = 10)	26.0 ± 23.1 (9.6—80.9)	13.7 ± 12.0 (2.0—38.2)	2.0 ± 1.6 (0.7—6.1)	9.1 ± 9.1 (0—30.6)	50.8 ± 24.4 (15.1—93.6)
Controls (n = 10)	2.0 ± 1.3 (0.1—4.1)	3.1 ± 2.0 (1.0—8.1)	0.9 ± 0.5 (0.2—1.8)	2.2 ± 1.8 (0—5.9)	9.3 ± 2.1 (2.1—14.4)

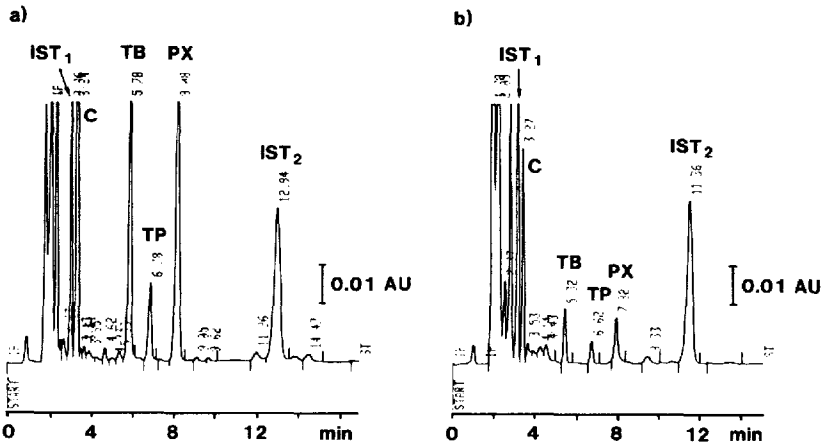

Fig. 2. Chromatogram of methylxanthines extracted from 0.5 ml of plasma of a patient with liver cirrhosis (a) and a healthy control (b). Peak symbols are the same as in Fig. 1.

These chromatograms demonstrate also the marked difference in concentration, especially between caffeine and theophylline.

Several HPLC methods for the analysis of methylxanthines in plasma have been published, most of them using reversed-phase HPLC columns [12—15]. However, baseline separation of paraxanthine and theophylline in the reversed-phase mode is difficult. Muir et al. [16] used an ion-pairing reversed-phase HPLC assay which allowed the separation of these two dimethylxanthines. The normal-phase solvent system presented here has the major advantage that the dimethylxanthines are well separated from the front peaks and also from each other, therefore allowing determination of caffeine and its main metabolites in one single isocratic run. This HPLC method facilitates the assessment of the metabolic pattern of methylxanthines and its clinical application in various fields, such as pulmonary diseases (treatment of asthma [6]), psychiatry (anxiety disorders [17]), paediatrics (treatment of apnoea in newborn [4,5]) and hepatology. Caffeine and dimethylxanthine metabolism in patients with different forms of liver disease is now under investigation using this HPLC method.

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