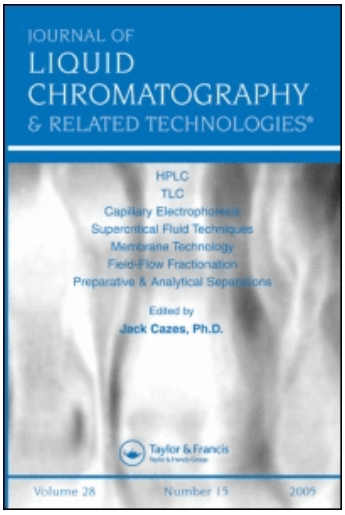

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A SENSITIVE METHOD FOR THE SIMULTANEOUS DETERMINATION OF CAFFEINE AND ITS DIMETHYLXANTHINE METABOLITES IN HUMAN PLASMA: APPLICATION TO CYP1A2 PHENOTYPING

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ABSTRACT

A sensitive high performance liquid chromatographic assay was developed for the determination of caffeine and its dimethylxanthine metabolites in human plasma. Caffeine, paraxanthine, theophylline, and theobromine were extracted from 0.5 mL of matrix with methylene chloride. Separation of the analytes was achieved using a mobile phase consisting of 0.1 M sodium acetate pH 4.5, methanol, tetrahydrofuran, and distilled water (10:6.5:1.4:82.1, v/v) and a Microsorb MV C_{18} analytical column with UV detection at 274 nm. Excellent linearity was observed for each analyte over the concentration range of 20 - 8000 ng/mL.

The within- and between-day coefficients of variation were less than 4.1% at all concentrations examined. This method is suitable for pharmacokinetic or pharmacogenetic studies utilizing caffeine as a probe for CYP1A2 activity whether given alone or in combination with other CYP probe drugs.

INTRODUCTION

Caffeine (CAF) is frequently used as an *in vivo* probe of the human cytochrome P450 (CYP) enzyme 1A2.[1] This enzyme metabolizes several clinically used drugs, such as theophylline and verapamil, as well as environmental chemicals such as polycyclic aromatic hydrocarbons.[2-4] CYP1A2 has also been implicated in the metabolic activation of several carcinogens, particularly heterocyclic aromatic amine compounds present in cooked foods.[5,6]

Since interindividual variability in the activity of 1A2 may alter the pharmacological response or susceptibility to these chemicals, methods to assess 1A2 activity *in vivo* have been developed for population phenotyping.[1]

CAF is predominately (80%) metabolized by 3-demethylation to form paraxanthine (PX); a reaction which is mediated by CYP1A2.[7,8] CAF also undergoes 1- and 7-demethylations to form theobromine (approximately 13%; TB) and theophylline (approximately 7%; TP), respectively. These demethylations are mediated by CYP1A2 and CYP2E1 (Figure 1).[8] The three dimethylxanthine metabolites (DMX) are measurable in plasma and saliva and ratios of PX to CAF at selected timepoints have been shown to highly correlate with the total plasma clearance of CAF, and thus may serve as surrogate measures of CYP1A2 activity.[9,10]

Several methods for the determination of CAF and DMX have been reported.[11-25] A common limitation of these methods is inadequate sensitivity for use in low dose CAF pharmacokinetic studies.

This paper describes a sensitive method for the determination of CAF and DMX in plasma. This method involves liquid-liquid extraction and uses an inexpensive C_{18} column.

Also discussed is an important modification of this method for use when CAF is simultaneously administered with other CYP probe drugs, which has become an important tool in clinical pharmacology research.[26]

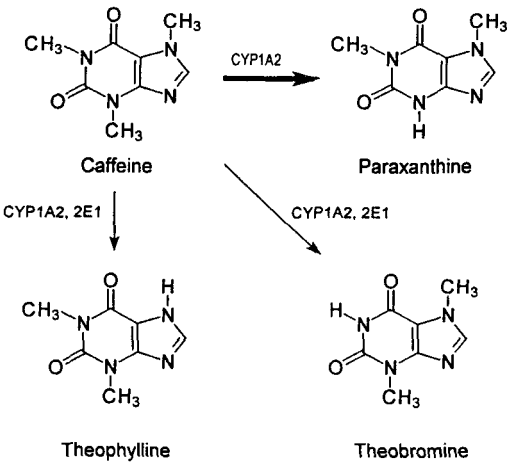

Figure 1. The metabolism of caffeine to the dimethylxanthine metabolites paraxanthine, theobromine, and theophylline by CYP enzymes.

MATERIALS AND METHODS

Reagents and Chemicals

CAF, PX, TB, TF, β-hydroxyethyltheophylline (internal standard) and reagent grade ammonium sulfate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade methanol, tetrahydrofuran, and methylene chloride were obtained from Baxter Scientific (McGaw Park, IL, USA). All water used in the analysis was purified with a four-bowl Milli-Q reagent water system (Millipore, Bedford, MA, USA). Reagent grade glacial acetic acid was obtained from Fisher Scientific (Pittsburgh, PA, USA). Polypropylene tubes (5 mL round bottom and 4.5 mL conical) were obtained from Sarstedt (Newton, NC, USA).

Equipment / Instrumentation

The HPLC system consisted of a Waters Model 600 solvent delivery pump, Model 715 Wisp automatic sample processor, and a Model 484 UV detector which was set at 274 nm. Signal output was captured with Waters Maxima 820 chromatography workstation (Waters Corporation, Milford, MA,

USA). Isocratic separation of caffeine and its dimethylxanthine metabolites was achieved with an Alltech Associates (Deerfield, IL, USA) direct-connect guard column (20 mm x 2 mm, I.D.) filled with pellicular C_{18} packing connected to a Rainin Microsorb MV 100 mm x 4.6 mm I.D. 3-μm C_{18} analytical column. The mobile phase used for the analysis consisted of 0.1 M sodium acetate pH 4.5, methanol, tetrahydrofuran, and water (10:6.5:1.4:82.1 v/v) delivered at a flow rate of 0.8 mL/min (130 bar). When CAF is simultaneously administered with other CYP probe drugs, a gradient wash is employed to hasten the elution of the other more nonpolar parent drugs and metabolite(s). A linear gradient at a flow rate of 0.8 mL/min was run using the following gradient profile: t = 0 to 13 min, 0% B; t = 13 to 14 min, 0 to 70% B; t = 19 to 20 min, 70 to 0% B; t = 20 to 25 min, 0% B. An additional 5 minutes is required for re-equilibration yielding an effective run time of 30 min. Mobile phase A is the same as for isocratic analysis, and mobile phase B is 100% methanol. The mobile phase(s) was degassed and filtered through a 0.22 μm Nylon 66 membrane before use. Sample preparation and analysis were performed at ambient temperature.

Preparation of Stock Solution and Spiked Standards

Stock solutions containing 0.05 and 0.5 mg/mL CAF and DMX were made in deionized water and stored at 4°C. These solutions were stable for at least 1 year when stored at 4°C. Standards and quality control samples were made by dilution of the stock solutions with deionized water and stored in 0.5 mL aliquots at - 20°C. Deionized water was used for standard and control solutions since caffeine-free plasma or saliva is generally unavailable and standard curves from water are identical to those obtained from plasma or saliva. The internal standard β-hydroxyethyltheophylline was prepared as a 1 mg/mL stock solution in water. A 0.015 mg/mL working internal standard solution was prepared by dilution of the stock solution with deionized water.

Preparation of Plasma Samples

Plasma (0.5 mL) was added to 5 mL round bottom tubes containing 200 mg ammonium sulfate. Working internal standard solution (50 μL) was added and the samples acidified by addition of 0.5 mL 0.2 M sodium acetate buffer (pH 4.5). Samples were vortexed briefly and then 3 mL of methylene chloride was added. The tubes were capped and shaken on low speed (85 cycles per min) for 10 minutes and centrifuged for 10 minutes at 2000 g. The upper aqueous layer was aspirated and discarded. The lower organic layer was transferred to a 4.5 mL conical tube and evaporated to dryness at 40°C under a

stream of nitrogen. The residues were reconstituted in 250 μL mobile phase, transferred to Wisp microinserts, and 50 μL aliquots were injected into the HPLC system.

Calibration and Linearity

Calibration curves using seven different concentrations of caffeine and each metabolite ranging from 20 to 8,000 ng/mL, were prepared and analyzed daily for three days. For each curve the peak height ratio of drug to internal standard was calculated and plotted against the respective concentrations of CAF or DMX. The concentrations of standards evaluated were 20, 40, 200, 800, 2000, 4000, and 8000 ng/mL. All standards were run as duplicates.

Precision and Accuracy

The precision and accuracy of the assay was determined through the analysis of caffeine and dimethylxanthine metabolites QC samples spiked at concentrations of 500 and 5000 ng/mL. Five QC samples at each concentration were analyzed daily for three days and the intra- and inter-day means, standard deviations and coefficients of variation were calculated.

Recovery

The extraction recoveries of CAF and each DMX from plasma were determined by comparing the peak height(s) obtained from extracted QC samples with the peak height(s) observed after direct injection of an aqueous solution containing the same analyte concentration(s). Percent extraction recovery was estimated as the difference between the individual peak heights of extracted QC samples and the unextracted standards. The means and standard deviations of percent extraction recovery were determined in each matrix from five replicates of the low and high QC concentrations.

Applicability to Pharmacogenetic Studies

The suitability of the assay for pharmacogenetic studies was evaluated in fourteen (14) normal healthy volunteers who received a single 100 mg oral dose (the typical dose used for phenotyping studies ranges from 50 to 250 mg). Blood samples were collected prior to and 4 and 8 hours after drug administration.

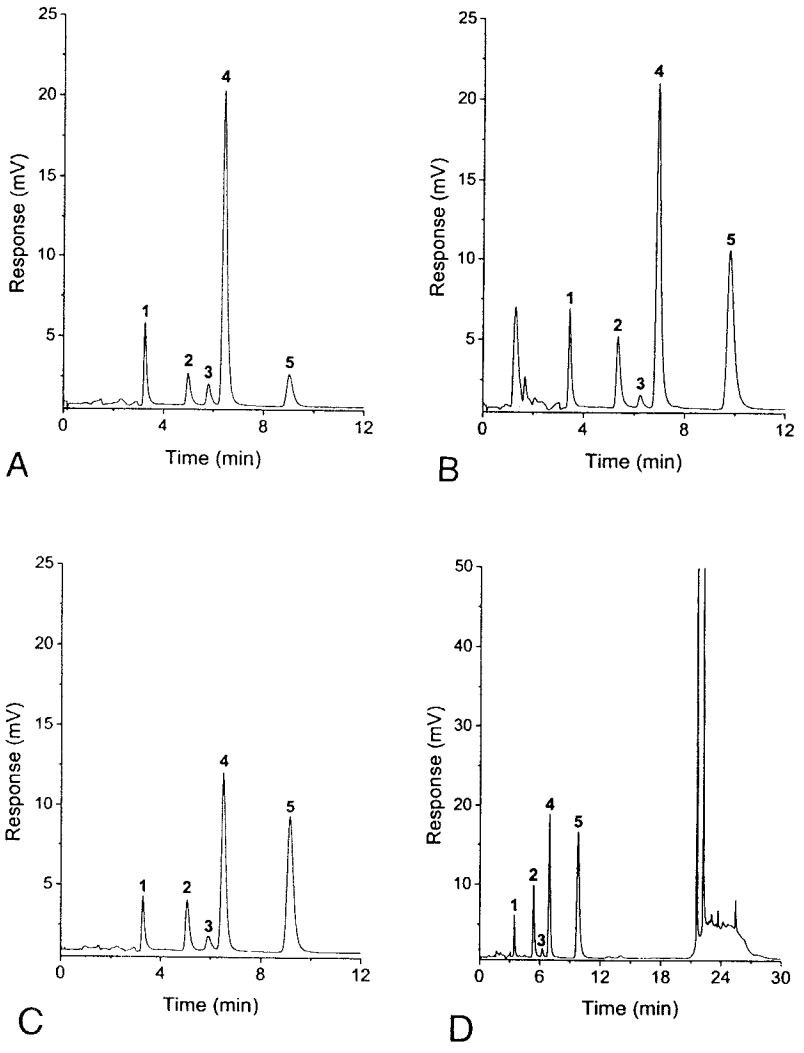

Figure 2. HPLC chromatograms of: (A) 250 ng/mL spiked plasma standard; (B) patient plasma sample at 8 hours; (C) patient saliva sample at 8 hours; (D) patient plasma sample at 8 hours after administration of multiple drug probes of CYP activity. The late eluting peaks in chromatogram (D) include chlorzoxazone, 6-hydroxychlorzoxazone, dapsone, and monoacetyldapsone. Peaks: 1 = theobromine; 2 = paraxanthine; 3 = theophylline; 4 = internal standard; 5 = caffeine.

Plasma was harvested by centrifugation for 10 minutes and stored at -20°C until analysis. A metabolic ratio indicative of CYP1A2 activity was calculated as the ratio of paraxanthine and caffeine concentrations at 4 and 8 hours.[9,10]

RESULTS AND DISCUSSION

Representative chromatograms of a spiked standard, a patient plasma sample, and a saliva sample each obtained at 8 hours following a 100 mg oral CAF dose, are shown in Figure 2. Also included in Figure 2 is a chromatogram of a patient sample obtained 8 hours after simultaneous administration of a multisubstrate cocktail that included CAF. The gradient wash was employed for this analysis. The retention times of TB, PX, TF, internal standard, and CAF were 3.3, 5.1, 5.9, 6.5, and 9.1 min, respectively. Calibration curves for CAF and each DMX were generated by weighted ($1/y^2$) linear regression analysis. Linear calibration curves were obtained for all compounds over the concentration range 20 - 8,000 ng/mL with correlation coefficients (r^2) greater than 0.99. The average extraction recoveries of CAF and each DMX metabolite from plasma ranged from 90 to 96% at the low (500 ng/mL) concentration and ranged from 89 to 92% at the high (5000 ng/mL) concentration. The limit of detection for each compound was approximately 5 ng/mL (signal to noise ratio of 4:1). Table 1 shows the intra- and inter-day precision and % CV for each QC concentration of PX, TB, TF, and CAF.

In addition to plasma, this method is also suitable for the determination of CAF and its DMX in serum or saliva without modification. Since the concentrations of CAF and DMX are comparable in plasma and saliva, the use of saliva samples offers a noninvasive method for use in epidemiological studies.[9,27] A method similar to the current method for determination of CAF and DMX in serum or plasma was reported by Tanaka, *et al.*[28] However, in our experience, the current method has improved, more consistent recovery of DMX by using a buffer instead of HCl addition and adding ammonium sulfate in the extraction procedure. The use of ammonium sulfate in the extraction procedure increased the mean recovery of DMX from approximately 40 percent to approximately 90 percent.

The method presented here is currently being used to support several investigations of drug and disease state effects on CYP activity. We are using CAF in addition to chlorzoxazone, dapsone, debrisoquine, and mephenytoin as simultaneously administered *in vivo* probes of the CYP isozymes 1A2, 2E1, 3A, 2D6, and 2C19, respectively.

Table 1

Intra- and Inter-Day Precision and Accuracy for Paraxanthine, Theobromine, Theophylline, and Caffeine

Compound	Concentration (ng/mL) Added	Found (Mean ± S.D.)	C.V. (%)	% Deviation
Intra-assay Reproducibility[a]				
Paraxanthine	500	503.2 ± 3.6	0.71	0.65
	5000	5022.8 ± 43.2	0.86	0.46
Theobromine	500	511.8 ± 15.0	2.9	2.4
	5000	5181.5 ± 99.7	1.9	3.6
Theophylline	500	501.4 ± 9.0	1.8	0.29
	5000	5003.5 ± 96.5	1.9	0.10
Caffeine	500	491.5 ± 16.5	3.4	-1.7
	5000	5079.8 ± 209.4	4.1	1.6
Inter-assay Reproducibility[b]				
Paraxanthine	500	501.1 ± 7.5	1.5	0.21
	5000	5013.7 ± 64.9	1.3	0.27
Theobromine	500	507.4 ± 201.9	4.1	1.5
	5000	5182.5 ± 157.9	3.1	3.7
Theophylline	500	498.9 ± 9.0	1.8	-0.22
	5000	4967.4 ± 109.0	2.2	-0.65
Caffeine	500	484.1 ± 18.6	3.8	-3.1
	5000	5073.7 ± 165.0	3.3	1.5

[a] Five samples per concentration.
[b] Five samples per day per concentration for three days.

The mean caffeine and paraxanthine concentrations following a single 100mg dose and the resulting metabolic ratios are shown in Table 2. Based on these concentration values, this assay would be suited for use with any of the typical caffeine doses used for pharmacokinetic or phenotyping studies. No analytical interferences were found from the co-administered drugs or their metabolites when using the gradient wash and no interferences have been

Table 2

Concentration of Paraxanthine and Caffeine in 14 Normal Healthy Volunteers Following a 100mg Oral Dose

	Predose	4 Hours	8 Hours
Caffeine (ng/mL)	94.0 ± 174.7 (<LLQ - 606.2)	1610.8 ± 420.4 (1152.4 - 2776.9)	882.8 ± 331.2 (417.3 - 718.9)
Paraxanthine (ng/mL)	109.5 ± 166.0 (<LLQ - 521.9)	556.7 ± 142.8 (358.9 - 876.5)	586.2 ± 116.4 (417.5 - 878.2)
Paraxanthine/Caffeine		0.36 ± 0.11 (0.20 - 0.59)	0.73 ± 0.21 (0.36 - 1.09)

LLQ = Lower limit of quantitation; Data are mean± SD (Range).

observed in plasma samples from different patient populations, including normal volunteers, patients with cancer, liver or renal disease, and transplant patients. The method described is suitable for use in pharmacokinetic or pharmacogenetic studies utilizing CAF.

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