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Original Article

Solid-phase extraction for determination of caffeine and its dimethylxanthine metabolites in rat urine using high performance liquid chromatography and its application

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Abstract

A solid-phase extraction (SPE) method for use with high performance liquid chromatography to measure caffeine (CF) and its metabolites in rat urine was developed and validated. Oasis HLB cartridges were used and washed with water, 1% acetic acid: 3% methanol-water (0.5:99.5 v/v), and 1% ammonium hydroxide: 3% methanol-water (0.5:99.5 v/v), respectively, and eluted with methanol:acetronitrile (80:20 v/v). Method validation showed good linearity (r>0.9990) over all calibration ranges for CF and its metabolites. The method was precise (1.0–17.6%RSD) and accurate (−13.8–(+) 16.0%DEV). The SPE method resulted in a high recovery for all analytes (72.9–98.9%). The lower limits of quantification were 0.1 µg/ml (PX), 0.25 µg/ml (CF and theophylline), and 0.5 µg/ml (theobromine and 1,3,7-trimethyluric acid). This method was successfully applied to determine the urinary profiles of CF and its metabolites, and the caffeine metabolic ratios in rats receiving a single oral dose of CF.

Keywords: caffeine, SPE, urine, rat

1. Introduction

Caffeine (CF) (Figure 1A) is a methylxanthine. It is primarily metabolized via demethylation to dimethylxanthines (i.e. theobromine (TB) by 1-N-demethylation) (Figure 1B), paraxanthine (PX) by 3-N-demethylation (Figure 1C), theophylline (TP) by 7-N-demethylation (Figure 1D), and oxidetion to 1,3,7-trimethyluric acid (137U) by C-8-hydroxylation (Figure 1E). Dimethylxanthines are further metabolized to mono-methylxanthines and uric acids. PX is a main metabolite in humans while 137U is the major product in rats. All metabolites and the parent compound (1-4%) are excreted in the urine (Donovan & DeVane, 2001; Kot & Daniel, 2008).

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Since cytochrome P-4501A2 (CYP1A2) contributes to primary metabolism of CF, CF is therefore accepted as a probe for evaluation of CYP1A2 activity (Kot & Daniel, 2008; Perera *et al.*, 2012). One of the accepted indices is the caffeine metabolic ratio (CMR) which is the concentration ratio of metabolite to CF in biological samples such as plasma, urine, and saliva. CMR has been widely used in several studies for CYP1A2 phenotyping in humans (Begas *et al.*, 2007; Butler *et al.*, 1992; Caubet *et al.*, 2004; Hakooz, 2009; Kalow & Tang, 1991; Nordmark *et al.*, 1999; Tang *et al.*, 1994; Zadoyan *et al.*, 2012) and rats (Jodynis-Liebert, & Matuszewska, 1999; Jorritsma *et al.*, 2000; Tang *et al.*, 2012).

To investigate the pharmacokinetic effects of any xenobiotics on CYP1A2 activity, an appropriate analytical method is necessary to measure CF and its metabolites in biological samples. Most studies used high-performance liquid chromatography and ultraviolet detection (HPLC-UV)

(Caubet *et al.*, 2002; Georga *et al.*, 2001; Grant *et al.*, 1983; Novitskaya *et al.*, 2013), ultra high performance liquid chromatography with mass spectrometric detection (Ogawa *et al.*, 2012), and liquid chromatography and mass spectrometry (Bianco *et al.*, 2009; Caubet *et al.*, 2004; Rybak *et al.*, 2014). However the biological samples were either not directly extracted (Bianco *et al.*, 2009; Rybak *et al.*, 2014; Schrader *et al.*, 1999) or were subjected to i) protein precipitation (Novitskaya *et al.*, 2013; Ogawa *et al.*, 2012), ii) liquid-liquid extraction (LLE) (Grant *et al.*, 1983; Zydron *et al.*, 2004) or iii) solid-phase extraction (SPE) (Caubet *et al.*, 2002, 2004; Georga *et al.*, 2001).

Urinary CMR is a widely used parameter for evaluation of CYP1A2 activity in both humans and rats (Butler *et al.*, 1992; Jorritsma *et al.*, 2000). Rats are a good *in vivo* model for assessing CYP1A2 activity by monitoring the excreted amount of 137U (Kot & Daniel, 2008). However, urine is a readily accessible complex matrix. The method to analyze 137U in rat urine using a universal HPLC-UV with simple sample preparation is limited. Therefore, the present study aimed to develop and validate a better SPE method for the HPLC-UV technique to determine CF and its metabolites (i.e. 137U, TB, PX, and TP in rat urine). The study also aimed to demonstrate the application of the method to determine the urinary profiles of the analytes and urinary CMR of rats given a single oral dose of CF.

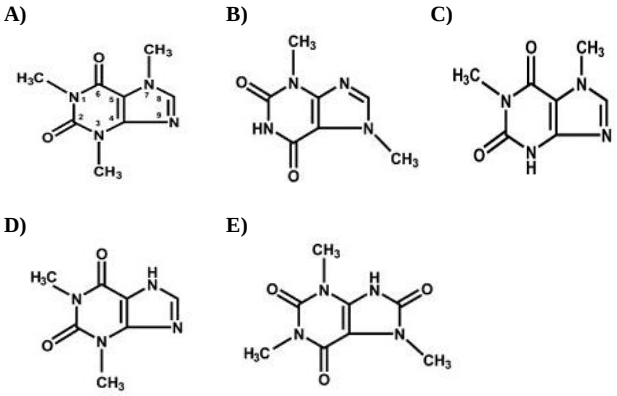

Figure 1. Chemical structures of CF and its primary metabolites: A) CF, B) TB, C) PX, D) TP, and E) 137U (Peri-Okonny *et al.*, 2005).

2. Materials and Methods

2.1 Chemicals and reagents

CF, TB, PX, and TP were from Sigma-Aldrich (MO, USA). 137U was from Suzhou Yacoo Chemical Reagent Corporation (Shanghai, China). Methanol and acetonitrile were from Mallinckrodt Baker Inc. (NJ, USA) and tetrahydrofuran was from Fisher Scientific UK Limited (Leicestershire, UK). Other chemicals were of analytical reagent grade.

2.2 Preparation of standard solutions

Individual stock solutions (1000 µg/ml) were prepared using these solvents: deionized water for CF, 0.1 M sodium hydroxide for TB and 137U, and 0.1 M ammonium hydroxide for PX and TP. Working standard solutions (5-500 µg/ml) were prepared as a mixture by diluting the stock solutions with deionized water. Calibration standard mixtures (0.1-10 µg/ml) were prepared using rat urine. Samples for quality control (QC) of CF and TP (0.25, 1, 5, and 10 µg/ml), TB, and 137U (0.5, 1, 5, and 10 µg/ml), and PX (0.1, 1, 5, and 10 µg/ml) were prepared in a similar way.

2.3 Chromatographic instruments and conditions

The chromatographic system consisted of a Waters 2695 Separation Module and a Waters 5487 Dual λ Absorbance detector (Milford, MA, USA). Data were processed using the Empower™ Software System. Urinary concentrations of CF and its metabolites were determined based on a previous method (Janchawee *et al.*, 2014). A C18 column controlled at 32 ºC was used. The gradient elution was performed using a mixture of water:acetic acid:tetrahydrofuran (996.5:1:2.5, v/v/v) as solvent A and acetronitrile as solvent B. The flow rate was 1.3 ml/min. A wavelength of 274 nm was used for detection.

2.4 Sample preparation

2.4.1 SPE study

Oasis HLB cartridges (30 mg, 1 cc, Waters, Milford, MA, USA) were used. The cartridges were sequentially preconditioned with 1x1 ml of water and then methanol prior to sample loading. Several methods for washing and eluting the analytes as well as sample dilution and pH adjustment were tried (Table 1). The eluates were evaporated to dryness under a stream of nitrogen at room temperature. The residue was reconstituted with the mobile phase. The supernatant was filtered through a 0.22 µm nylon membrane before HPLC injection. The optimum procedure was the one that resulted in the highest recovery with low interferences and good peak resolution.

2.4.2 Sample extraction

Each urine sample (200 µl) was diluted with 4% phosphoric acid (800 µl). After loading, the cartridges were washed with 1x1 ml of water, followed by 1% acetic acid:3% methanol-water (0.5:99.5 v/v), and then 1% ammonium hydroxide:3% methanol-water (0.5:99.5 v/v), respectively. The analytes were eluted with 1x1 ml of methanol:acetronitrile (80:20 v/v). The injection volume was 10 µl.

2.5 Method validation

The method of validation was in accordance with United States Food and Drug Administration guidance for the validation of bioanalytical methods (US Food and Drug Administration [USFDA], 2001). The validation parameters were linearity, intra-day and inter-day precision, accuracy, recovery, and the lower limit of quantification (LLOQ).

Linearity was determined from the calibration curves (n=3) between the different concentrations of the analytes against the peak areas. The concentrations were 0.25, 1, 2.5, 5, 7.5, and 10 µg/ml for CF and TP, 0.5, 1, 2.5, 5, 7.5,

Table 1. SPE procedures used in the study to extract CF and its metabolites from rat urine

Method	Sample (µl)	Dilution		Wash 1 (x1)		Wash 2 (x1)		Wash 3 (x1)		Elution		Recon-stitution (µl)	Injection (µl)
		Solvent	Vol. (µl)	Solvent	Vol. (ml)	Solvent	Vol. (ml)	Solvent	Vol. (ml)	Solvent	Vol. (ml)		
A	250	0.1N HCl	250	water	2	5% MeOH-water	1	-	-	MeOH	1	250	20
B	250	0.1N HCl	250	water	1	3% MeOH-water	1	-	-	MeOH	1	250	20
C	250	0.1N HCl	250	water	1	conc.CH_3COOH: 3% MeOH-water (1:99)	1	-	-	MeOH	1	250	20
D	250	0.1N HCl	250	water	1	1% NH_4OH: 3% MeOH-water (0.5:99.5)	1	-	-	MeOH	1	250	20
E	250	0.1N HCl	250	water	1	3% MeOH-water	1	1% NH_4OH: 3% MeOH-water (0.5:99.5)	1	MeOH	1	250	20
F	250	0.1N HCL	250	water	1	conc.CH_3COOH: 3% MeOH-water (1:99)	1	1% NH_4OH: 3% MeOH-water (0.5:99.5)	1	MeOH	1	250	20
G	250	0.1N HCl	250	water	1	1% CH_3COOH: 3% MeOH-water (0.5:99.5)	1	1% NH_4OH: 3% MeOH-water (0.5:99.5)	1	MeOH	1	250	20
H	250	4% H_3PO_4	250	water	1	1% CH_3COOH: 3% MeOH-water (0.5:99.5)	1	1% NH_4OH: 3% MeOH-water (0.5:99.5)	1	MeOH	1	250	10
I	250	4% H_3PO_4	250	water	1	1% CH_3COOH: 3% MeOH-water (0.5:99.5)	1	1% NH_4OH: 3% MeOH-water (0.5:99.5)	1	MeOH-ACN (80:20)	1	250	10
J	200	4% H_3PO_4	800	water	1	1% CH_3COOH: 3% MeOH-water (0.5:99.5)	1	1% NH_4OH: 3% MeOH-water (0.5:99.5)	1	MeOH-ACN (80:20)	1	200	10

and 10 µg/ml for TB and 137U, and 0.1, 1, 2.5, 5, 7.5, and 10 µg/ml for PX. The correlation coefficient (r) and the calibration equation were determined by linear regression analysis.

Precision and accuracy were evaluated by analyzing the QC samples. The intra-day precision was performed using five samples of each concentration on the same day under the same experimental condition, while the inter-day precision was evaluated by assaying of the samples for five consecutive days. Precision was expressed as the relative standard deviation (%RSD). Accuracy was expressed as the deviation (%DEV). The level of acceptance for precision was within 15%RSD except at the LLOQ, where 20%RSD was acceptable. The accuracy was required to be within ±15%DEV except at the LLOQ where ±20%DEV was acceptable.

Recovery of extraction was determined by comparing the peak areas of the extracted sample with those of directly injected sample (n=5). The LLOQ was the lowest concentration of the analyte that produced a signal-to-noise ratio (S/N) of 5:1.

2.6 Method application

Adult male Wistar rats (n=6, 200-220 g) were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Thailand. The experimental designs were approved by the Ethics Committee for Experimental Animal (Ref no. 09/55), Prince of Songkla University. Animal handling was in accordance with the guidelines of the National Research Council of Thailand based on the International Guiding Principles for Biomedical Research Involving Animals (Council for International Organiztions of Medical Sciences [CIOMS] &International Council for Laboratory Animal Science [ICLAS], 2012). The animals were maintained in a controlled environment with food and water *ad libitum*. Prior to the treatment, the rats were made to fast overnight with free access to water.

A single dose of 10 mg/kg CF was administered by gavage feeding. The animals were housed individually in a metabolic cage (Tecniplast, Italy) which had a specifically designed grid, funnel, and cone for separation of urine and feces. After urination, urine flowed into the collection tube.

Collection of the urine was accomplished without disturbing the animals. Urine samples were collected at baseline and at 0-1, 1-3, 3-6, and 6-9 h intervals post-dose. The samples were centrifuged (6000 rpm, 10 min, ca. 25 ºC). The supernatant was separated and kept at -20 ºC until analysis.

2.7 Data analysis

Concentrations of CF and its metabolites were determined based on peak areas. The CMRs are expressed as the total concentrations of metabolites divided by CF. Data are expressed as mean±SEM.

3. Results and Discussion

3.1 SPE Study

Sample extraction is very important during an analysis of any component in biological matrices. The appropriate method should efficiently remove interfering compounds while providing a high recovery of extraction. In the present work, several extraction methods were tried. Prior to the SPE study, a pilot study was initiated with the LLE technique using a mixture of chloroform-isopropanol (85:15, v/v) as the extracting solvent (Grant *et al.*, 1983). However, the recovery of extraction was quite variable (i.e. 53.5-96.5% for all analytes). In addition, the method did not effectively clean up the sample as noted by the remaining yellowish residues and the turbidity after reconstitution of the residue (data not shown).

Due to the poor cleansing efficiency of the LLE method, the SPE technique was used. The cartridges used for sample preparation to quantify the urinary CF and its metabolites in previous studies included C18 (Caubet *et al.*, 2002; Caubet *et al.*, 2004), Oasis HLB, and Abselut Nexus (Georga *et al.*, 2001). In the present work, Oasis HLB was selected for many reasons. The cartridge contains n-vinylpyrrolidone and divinylbenzene monomers providing a hydrophilic-lipophilic balance. It can bind acidic, basic, and neutral compounds whether they are polar or nonpolar and stable in a wide pH range (1-14). Since it is macroporous, less sorbent is needed and it has up to five times higher capacity compared to a silica-based cartridge. Additionally, it has a greater compatibility to organic solvents. Modification of the pH or solvent composition during washing and elution steps can easily clean the extract (Huck & Bonn, 2000).

Several wash-elute protocols modified from the generic method were carried out. The extraction efficiency was expressed as recovery and residue clearness (Table 2). Acidification of urine makes the analytes free from interacting with proteins that may be found in urine, especially from rats (Galaske *et al.*, 1979), and dilution improves sample flow. CF and its metabolites are alkaloids with heterogeneous physicochemical properties. Due to the high capability of the sorbent, the analytes, which are mixtures of the basic (CF, TB, TP, and PX) and acidic (137U) compounds and other unknown polar and less polar components in acidified urine, were retained in the cartridge. Initiation by washing with water was an essential step to remove highly polar interfering compounds. Washing with water followed by a mixture of 5% methanol-water (Method A) were obviously too strong to remove all polar compounds including all analytes. A mixture of 3% methanol-water (Method B) moderately removed some polar interfering compounds with good recovery. The 3% methanol wash was sufficiently weak that all analytes were not washed off.

Table 2. Extraction recovery (mean percentage) of CF and its metabolites from rat urine after undergoing different SPE methods in the pilot study.

Method	Recovery (%)					Residue clearness[a]
	TB	PX	TP	137U	CF	
A	0	0	0	0	0	++++
B	87.91	96.56	98.36	102.28	90.28	++
C	68.63	93.49	93.21	70.09	93.55	++
D	96.66	98.42	98.71	86.71	90.74	+
E	67.41	93.88	98.88	80.88	96.48	+++
F	39.32	103.71	101.14	160.02	89.21	++++
G	72.97	96.63	99.73	100.59	96.75	++++
H	64.06	96.30	110.23	197.52	90.33	++++
I	62.54	91.79	117.85	80.79	92.06	++++
J	79.35	99.26	99.17	98.25	99.04	++++

[a] +; poor, ++; fair, +++; good, ++++; very good; justified by color of residues and clearness of sample after reconstitution of the residue.

Washing with a mixture of 3% methanol-water containing either glacial acetic acid or 1% ammonium hydroxide (Methods C & D) was unable to effectively clean the sample because only polar basic or acidic interfering compounds were removed. More steps of washing with alkalized or acidified methanol-water mixture resulted in a cleaner residue (Methods E & F). Presence of concentrated acetic acid resulted in low TB recovery (Methods C & F). That may be due to an increase in the solubility of TB in concentrated acid (Spiller, 1997). Slight modifications using a more diluted acid and a smaller ratio of components (0.5:99.5 v/v) obtained a reasonably clean sample with good recovery (Method G).

Further methods were carried out to improve TB recovery. A smaller injection volume (10 µl) and dilution with a weaker acid (phosphoric acid) were tested. Although the residue was clear, some unknown peaks interfered with 137U causing an error in the calculation of the amount (Method H). An eluent mixture of methanol and acetonitrile, which has less polarity than pure methanol, resulted in acceptable recovery of PX and CF but not TB or TP (Method I). An increase in the sample dilution factor (1:4) reduced the peak interference for 137U and resulted in a high recovery of all analytes (Method J).

Acidified and alkalinized washing solvents improved the elimination efficiency of neutral, basic, and acidic polar interfering compounds, relative to the neutral methanol-water mixture. The mixture of 1% acetic acid:3% methanol-water (0.5:99.5 v/v) and 1% ammonium hydroxide:3% methanol-water (0.5:99.5 v/v) was found to be essential and effective in removing most interfering compounds from the urine sample. Organic solvents, such as a mixture of methanol-acetonitrile (80:20 v/v) with moderate polarity, were capable to elute CF and its metabolites which have poor lipophilicity but varied hydrophilicity.

3.2 Chromatographic profile

A chromatogram of a standard mixture of CF and its main metabolites that spiked in the urine sample is presented in Figure 2A. All analytes were well separated and eluted within 14 min. The retention times for all analytes obtained from this study were shorter than in a previous report (Caubet *et al.*, 2002) which were 14.12, 18.99, 19.31, 20.73 and 23.08 min, respectively. That was due to the higher column temperature (32 ºC vs. 23 ºC) and flow rate (1.3 ml/min vs. 1 ml/min).

3.3 Method validation

Regression analysis results showed that the calibration curves of the concentrations of CF and its metabolites against their peak responses were linear. The parameters of regression equations for CF, TB, PX, TP, and 137U are presented in Table 3. All showed good correlation coefficients (r>0.999).

Table 4 shows the LLOQs, precision, accuracy, and recovery of extraction for all analytes. The LLOQs were 0.25 µg/ml for CF and TP, 0.5 µg/ml for TB and 137U, and 0.1 µg/ml for PX. The precision (%RSD) and accuracy for both intra- and inter-assays were within the acceptable ranges for all analytes which indicated that this method of analysis was precise and accurate. The SPE method developed in this work produced good extraction recoveries that ranged from approximately 73 to 99% for all analytes.

3.4 Method application

The SPE method developed for the HPLC-UV technique was used to determine the levels of CF and its metabolites excreted in the urine of rats that received a single oral dose (10 mg/kg) of CF. The chromatogram of CF and its metabolites during a 3-6 h interval is presented in Figure 2B. The measured concentrations are expressed as urinary concentration-time profiles of CF and its metabolites (Figure 3A) and

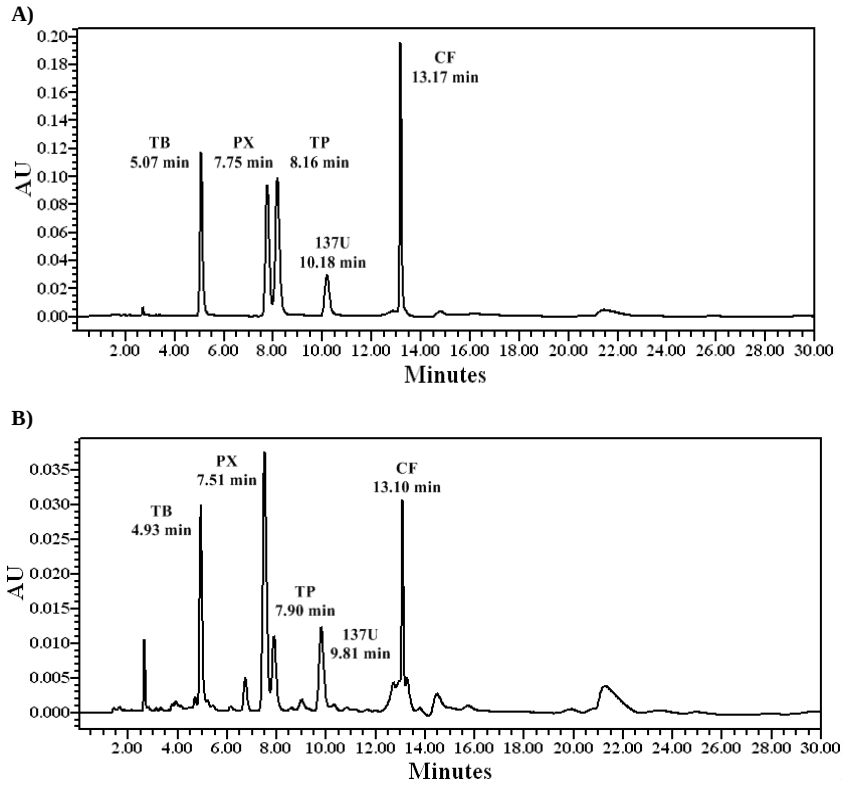

Figure 2. Representative chromatograms of the separation of CF, TB, PX, TP, and 137U in rat urine. A) urine blank sample spiked with a standard mixtures (10 µg/ml), B) urine sample collected during a 3-6 h interval following a single oral dose of CF (10 mg/kg).

Table 3. Parameters (mean±SD) for the linear equations of the concentrations of CF, TB, PX, TP, and 137U in rat urine (n=3).

Analyte Range (µg/ml)	CF 0.25–10	TB 0.5–10	PX 0.1–10	TP 0.25–10	137U 0.5–10
Slope	20439±823	16881±371	16199±371	18383±106	7690±38
y-intercept	–1895±383	965±410	–567±117	108±151	–1277±614
r	0.9999	0.9997	0.9997	0.9999	0.9997

Table 4. LLOQs, precision, accuracy, and extraction recovery of the method for determination of CF, TB, PX, TP and 137U from rat urine (n=5)

Analyte Range (µg/ml)	LLOQ (µg/ml)	Precision (%RSD)		Accuracy (%DEV)		Recovery (%)
		Intra-day	Inter-day	Intra-day	Inter-day	
CF 0.25–10	0.25	3.75–19.06	3.01–13.92	(–)0.43–(+)14.96	(–)1.00–(+)4.58	83.04–94.90
TB 0.5–10	0.5	3.00–4.14	2.36–4.71	(–)12.70–(+)0.94	(–)6.04–(+)0.90	72.91–75.81
PX 0.1–10	0.1	6.10–12.54	5.52–8.82	(–)0.55–(+)15.99	(–)12.13– (+)1.15	94.27–98.87
TP 0.25–10	0.25	6.27–10.51	4.29–6.57	(–)13.82–(+)0.37	(+)0.23– (+)2.48	90.54–98.59
137U 0.5–10	0.5	1.03–16.03	5.00–17.58	(–)1.08–(+)14.32	(+)0.30– (+)5.05	90.28–97.15

calculated to the CMR during different time intervals after dosing (Figure 3B). Since CF is the parent compound, it was found in the urine during the 9 h after drug administration. 137U and PX are metabolites which are found mostly in the 3-6 h interval. CF is rapidly eliminated with an elimination half-life of 1.4-3 h (Lau *et al.*, 1997; Noh *et al.*, 2015). The tendency to observe a decrease in urinary concentrations of CF during 3-9 h was due to its rapid excretion and conversion to metabolites. During 6-9 h, the level of CF was very low and the levels of PX, TB, and TP did not rise, which may be due to further metabolism to subsequent mono-methylxanthines and uric acids metabolites. Variation in the concentrations of CF and its metabolites resulted in a gradual increase in CMRs which reached the maximum value in the 6-9 h interval.

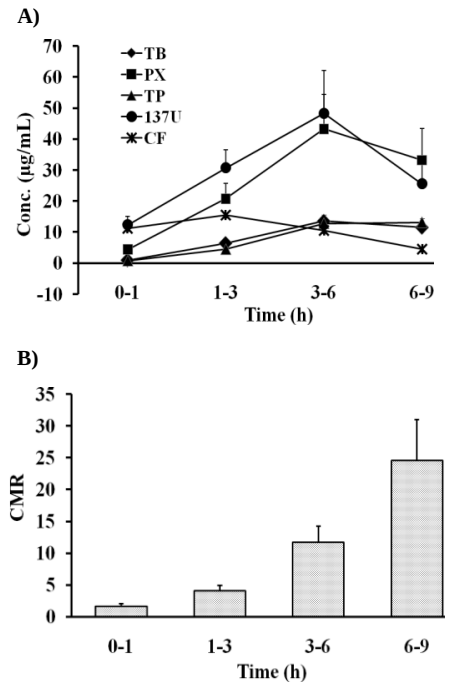

Figure 3. Method for application to a pharmacokinetic study of CF and its metabolites in rats (n=6) given a single oral dose of CF (10 mg/kg). A) urinary concentration-time profiles, B) urinary CMRs against time intervals. Data are expressed as mean±SEM, n=3.

4. Conclusions

The SPE method developed in this study was relatively simple for urine sample preparation. It was highly efficient for cleaning up samples and achieved high extraction recovery. Validation parameters which are acceptable according to the United States Food and Drug Administration guidance indicated a high sensitivity, precision, and accuracy of the method when it is used with the HPLC-UV method for simultaneously determining CF and its primary metabolites in rat urine, which is an accessible biological fluid from a non-human *in vivo* model. The method was applicable in describing the pharmacokinetics of CF, for instance the urinary profiles of CF and its metabolites and CMR, which is one of the indices for the activity or phenotype of CYP1A2 *in vivo*.

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References

Begas, E., Kouvaras, E., Tsakalof, A., Papakosta, S., & Asprodini, E. K. (2007). *In vivo* evaluation of CYP1A2, CYP2A6, NAT-2 and xanthine oxidase acti-vities in a Greek population sample by the RP-HPLC monitoring of caffeine metabolic ratios. *Biomedical Chromatography*, *21*, 190-200.

Bianco, G., Abate, S., Labella, C., & Cataldi, T. R. (2009). Identification and fragmentation pathways of caffeine metabolites in urine samples via liquid chromatography with positive electrospray ionization coupled to a hybrid quadrupole linear ion trap (LTQ) and Fourier transform ion cyclotron resonance mass spectrometry and tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 23*, 1065-1074.

Butler, M. A., Lang, N. P., Young, J. F., Caporaso, N. E., Vineis, P., Hayes, R. B., . . . Kadlubar, F. F. (1992). Determination of CYP1A2 and NAT2 phenotypes in human populations by analysis of caffeine urinary metabolites. *Pharmacogenetics,2*, 116-127.

Caubet, M. S., Comte, B., & Brazier, J. L. (2004). Determination of urinary 13C-caffeine metabolites by liquid chromatography-mass spectrometry: the use of metabolic ratios to assess CYP1A2 activity. *Journal of Pharmaceutical and Biomedical Analysis, 34*, 379-389.

Caubet, M. S., Elbast, W., Dubuc, M. C., & Brazier, J. L. (2002). Analysis of urinary caffeine metabolites by HPLC-DAD: the use of metabolic ratios to assess CYP1A2 enzyme activity. *Journal of Pharmaceutical and Biomedical Analysis, 27*, 261-270.

CIOMS & ICLAS. (2012). The International Guiding Principles for Biomedical Research Involving Animals. Retrieved from http://www.cioms.ch/images/stories/CIOMS/IGP2012.pdf

Donovan, J. L., & DeVane, C. L. (2001). A primer on caffeine pharmacology and its drug interactions in clinical psychopharmacology.*Psychopharmacology Bulletin, 35*, 30-48.

Galaske, R. G., Van Liew, J. B., & Feld, L. G. (1979). Filtration and reabsorption of endogenous low-molecular weight protein in the rat kidney. *Kidney International, 16*, 394-403.

Georga, K. A., Samanidou, V. F., & Papadoyannis, I. N. (2001). Use of novel solid-phase extraction sorbent materials for high-performance liquid chromatography quantitation of caffeine metabolism products methylxanthines and methyluric acids in samples of biological origin. *Journal of Chromatography B: Biomedical Sciences and Applications, 759*, 209-218.

Grant, D. M., Tang, B. K., & Kalow, W. (1983). Variability in caffeine metabolism. *Clinical Pharmacology and Therapeutics, 33*, 591-602.

Hakooz, N. M. K. (2009). Caffeine metabolic ratios for the *in vivo* evaluation of CYP1A2, N-acetyltransferase 2, xanthine oxidase and CYP2A6 enzymatic activities. *Current Drug Metabolism, 10*, 329-338.

Huck, C. W., & Bonn, G. K. (2000). Recent developments in polymer-based sorbents for solid-phase extraction. *Journal of Chromatography A, 885*, 51-72.

Janchawee, B., Wongsakul, A., Prutipanlai, S., & Ruangrut, P. (2014). Effect of betong watercress and phenethyl isothiocyanate on N-demethylation of caffeine in rats. *Tropical Journal of Pharmaceutical Research, 13*, 559-566.

Jodynis-Liebert, J., & Matuszewska, A. (1999). Effect of toluidines and dinitrotoluenes on caffeine metabolic ratio in rat. *Toxicology Letters, 104*, 159-165.

Jorritsma, U., Schrader, E., Klaunick, G., Kapitulnik, J., Hirsch-Ernst, K. I., Kahl, G. F., & Foth, H. (2000). Monitoring of cytochrome P-450 1A activity by determination of the urinary pattern of caffeine metabolites in Wistar and hyperbilirubinemic Gunn rats. *Toxicology, 144*, 229-236.

Kalow, W., & Tang, B. K. (1991). Caffeine as a metabolic probe: exploration of the enzyme-inducing effect of cigarette smoking. *Clinical Pharmacology and Therapeutics, 49*, 44-48.

Kot, M., & Daniel, W. A. (2008). Caffeine as a marker substrate for testing cytochrome P450 activity in human and rat. *Pharmacological Reports, 60*, 789-797.

Lau, C. E., Wang, Y., & Falk, J. L. (1997). Differential reinforcement of low rate performance, pharmacokinetics and pharmacokinetic-pharmacodynamic modeling: independent interaction of alprazolam and caffeine. *The Journal of Pharmacology and Experimental Therapeutics, 281*, 1013-1029.

Noh, K., Nepal, M. R., Jeong, K. S., Kim, S. A., Um, Y. J., Seo, C. S., . . . Jeong, T. C. (2015). Effects of baicalin on oral pharmacokinetics of caffeine in rats. *Biomolecules and Therapeutics, 23*, 201-206.

Nordmark, A., Lundgren, S., Cnattingius, S. and Rane, A. (1999). Dietary caffeine as a probe agent for assessment of cytochrome P4501A2 activity in random urine samples. British Journal of Clinical Pharmacology, 47, 397-402.

Novitskaya, Y. G., Litvin, A. A., Zherdev, V. P., Blynskaya, E. V., & Kondakov, S. E. (2013). Assay of caffeine and its metabolites in the rat blood plasma using HPLC as a method for determination of metabolic ratios. *Moscow University Chemistry Bulletin, 68*, 47-50.

Ogawa, T., Hattori, H., Iwai, M., Ito, K., Iwakura, K., Ishii, A., . . . Seno, H. (2012). A rapid and simultaneous analysis of theophylline and its metabolites in human whole blood by ultra-performance liquid chromatography-tandem mass spectrometry. *Forensic Toxicology, 30*, 142–148.

Perera, V., Gross, A. S., & McLachlan, A. J. (2012). Measurement of CYP1A2 activity: a focus on caffeine as a probe. *Current Drug Metabolism, 13*, 667-678.

Rybak, M. E., Pao, C. I., & Pfeiffer, C. M. (2014). Determination of urine caffeine and its metabolites by use of high-performance liquid chromatography-tandem mass spectrometry: estimating dietary caffeine exposure and metabolic phenotyping in population studies. *Analytical and Bioanalytical Chemistry, 406*, 771-784.

Schrader, E., Klaunick, G., Jorritsma, U., Neurath, H., Hirsch-Ernst, K. I., Kahl, G. F., & Foth, H. (1999). High-performance liquid chromatographic method for simultaneous determination of [1-methyl-14C]caffeine and its eight major metabolites in rat urine. *Journal of Chromatography B: Biomedical Sciences and Applications, 726*, 195-201.

Spiller, G. A. (1997). *Caffeine*. Boca Raton, FL: CRC Press.

Tang, B. K., Zhou, Y., Kadar, D., & Kalow, W. (1994). Caffeine as a probe for CYP1A2 activity: potential influence of renal factors on urinary phenotypic trait measurements. *Pharmacogenetics, 4*, 117-124.

Tang, J., Zhou, X., Ji, H., Zhu, D., & Wu, L. (2012). Effects of ephedra water decoction and cough tablets containing ephedra and liquorice on CYP1A2 and the pharmacokinetics of theophylline in rats. *Phytotherapy Research, 26*, 470-474.

USFDA. (2001). Guidance for Industry Bioanalytical Method Validation.Retrieved from http://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf

Zadoyan, G., Rokitta, D., Klement, S., Dienel, A., Hoerr, R., Gramatte, T., & Fuhr, U. (2012). Effect of *Ginkgo biloba* special extract EGb 761® on human cytochrome P450 activity: a cocktail interaction study in healthy volunteers. *European Journal of Clinical Pharmacology, 68*, 553-560.

Zydron, M., Baranowski, J., & Baranowska, I. (2004). Separation, pre-concentration, and HPLC analysis of methylxanthines in urine samples. *Journal of Separation Science, 27*, 1166-1172.