Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(11):231-234

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Determination of caffeine and its metabolites simultaneously in vivo by HPLC and used for enzyme assays on pharmacological study

Jian Xiao[b], Dong-Sheng Ou-Yang[a], Zhi-Rong Tan[a], Wei Zhang[a], Dong-Li Hu[a] and Yao Chen*[a]

[a]*Institute of Clinical Pharmacology, Central South University, 110 Xiang-Ya road, Changsha, Hunan, China*
[b]*Department of Pharmacy, Xiangya Hospital, Central South University, Hunan, China*

ABSTRACT

A precise, accurate and validated HPLC method is developed for the determination of caffeine and its metabolites in vivo and used for enzyme assays. The separation is performed on a Hypersil BDS C_{18} column. The mobile phase composed by phase A (acetonitrile) and phase B (10mM ammonium formate/formic acid (998/2, v/v)) pumped according to a gradient elution program at a flow rate of 1ml·min^{-1} with detection at 280nm. Chloroform/isopropanol(9:1,v/v) is used as extractant, acetaminophen is selected as internal standard(IS). AFMU, 1U, 1X, IS, 17U, 17X and caffeine elute at 3.3, 7.3, 8.9, 10.7, 11.7, 12.1, 15.0 respectively, linear response (r>0.99) is observed for samples ranging from 1.25 to 160μM and the method provides recoveries of 81-94% in the concentration range of 1.25-80μM for these substances, intra- and inter-day variation in <10% R.S.D. The limit of detection (LOD) is 0.62μM for AFMU, 1X, 1U and 0.31μM for 17U, 17X, 137X.

Keywords: caffeine, HPLC, gradient elution, enzyme assay

INTRODUCTION

Caffeine is the drug most widely used in worldwide, it has been implicated in various clinical conditions, including arrhythmias[1], coronary artery disease[2] and cancer[3]. Caffeine is commonly used in the treatment of asthma[4] and neonatal apnea[5], because of the narrow therapeutic index, monitoring its concentration in biological samples is usually recommended, and accurate analytical methods for use are required[6]. Except that, caffeine has also been used as pharmacological probes to determine the actions of CYP1A2, CYP2A6, NAT2 and XO in vivo simultaneously, for they are the main enzymes involved in the caffeine metabolic process[7,8]. About 84% of caffeine(137X) is metabolized to 1,7-dimethylxanthine(17X) by CYP1A2, nearly 90% of 17X is metabolized to 1,7-dimethylurate(17U) by CYP2A6 and 10% of 17X is metabolized to 1-methylxanthine(1X) by CYP1A2, 1X is further converted to 1-methylurate(1U) completely by XO, only a small part of 17X is metabolized to 5-acetylamino-6-formylamino-3-methyluracil(AFMU) by NAT2, so the activity of CYP1A2 ,CYP2A6, NAT2 and XO could be evaluated by the urinary caffeine metabolite ratios (UCMRs) of (AFMU+1U+1X)/17U, 17U/(17U+17X+X+1U+AFMU), AFMU/(AFMU+1U+1X) and 1U/(1X+1U) respectively[8,9], so the analytical method for caffeine and its metabolites is also needed for the application on pharmacological study. Here we have set up a precise, accurate HPLC for determination of caffeine and its metabolites simultaneously and apply it on pharmacological study in healthy volunteers.

EXPERIMENTAL SECTION

137X, 17X, 17U, 1X, 1U standard references and internal standard reference acetaminophen were obtained from Sigma Chemical Co.(St. Lous, MO, USA), AFMU was a generous gift received from Prof. Xiao-dong Liu(China Medicine University). The Agilent 1100 series HPLC system(Agilent technologies Inc. America) consisted of a vacuum degasser(G1379A), a high pressure quatpump(G1311A), an autosampler(G1329) and an UV

detector(G1314A) controlled by HP ChemStation. The chromatographic separation was accomplished on a Hypersil BDS C_{18} column (4.6mm×200mm I.D.,5μm particle size), The mobile phase composed by phase A (acetonitrile) and phase B (10mM ammonium formate/formic acid (998/2, v/v) pumped according to a gradient elution program at a flow rate of 1ml·min^{-1} with an injection volume of 20μL. Typical conditions for elution were 98% B (0-4 min), 98-90% B (4-9 min), 90% B (9-15 min), 90-70% B (15-18 min), 70% B (18-21min), 70-98% B (21-25min) and 98% B (25-30 min). The detection wavelength was set at 280nm.

Sample Preparation

1.0 mL of urine samples were mixed with 100 μL 10% formic acid, 50 μL acetaminophen(I.S, 10μg·mL^{-1}) and 5 mL chloroform/isopropanol (9:1, v/v) in 10mL glass tubes. The mixture was vortexed for 5 min then centrifuged at 2000 rpm for 10min, then 4mL of supernatant was removed to another batch of glass tubes and evaporated under a gentle stream of nitrogen at 40℃, the residue was re-dissolved in 100μL mobile phase and 20 μL volume of aliquots were injected onto the analytical column.

Application to Pharmacological Study

Ethical approval for the study protocol was given by the Xiang-ya Ethics Committee of Central South University, Changsha, Hunan, China. The informed consent was provided by each participant. Twenty four Chinese male healthy volunteers were enrolled for the study without the history of cigarette-smoking and alcohol-drinking. All the subjects were informed to empty their bladders firstly and then administered 100 mg caffeine. Then total urine samples of 0-12 h were collected in plastic jars containing 100mg ascorbic acid, and the total amount of urine samples were counted after collection. Urine aliquots (10mL) were acidified to pH 3.5 and stored at -20 ^{o}C until HPLC analysis. During the experiment, no drug adverse effects happened.

RESULTS AND DISCUSSION

HPLC Method validation

Specificity was determined by comparing the retention times of each analyte against the chromatogram of extracted from six different sources of drug-free urine pooled samples without internal standard(five replicates). AFMU, 1U, 1X, IS, 17U, 17X and caffeine elute at 3.3, 7.3, 8.9, 10.7, 11.7, 12.1, 15.0min respectively. Chromatograms of caffeine and its metabolites were shown in Fig.1. Complete separation was obtained by present HPLC method and no endogenous peaks interfered with these substances. The lowest bias over the calibration curve range of 1.25-160μM·L^{-1} was calculated by a regression analysis of the data to a linear fit for the ratio of the peak area of analytes and the IS against the nominal concentration. The calibration curve was linear over the range tested yielding correlation of coefficients from 0.991 to 0.999 for all the compounds. LOD is 0.62μM for AFMU, 1X, 1U and 0.31μM for 17U, 17X, 137X. The intra-day and inter-day precisions were in the ranges of 94.5-104.5% and 98.0-102.7% respectively. CV values for intra-day analysis were between 2.6 and 9.5% and for inter-day analysis they were between 5.7 and 10.9%. Recoveries for the analytes were in the ranges of 81.4-94.7%. The post-preparative, freeze-thaw, short term and long term stability of the analytes were investigated and no significant degradation was found in these specific conditions. The data indicate that all the compounds are stable during three freeze and thaw cycles and the prepared samples for analysis are stable for at least 48 h at 5 ◦C. Moreover, no stability related problems are to be expected if urine samples are stored at room temperature for up to 8 h or when they are stored in −20 ◦C for 2 months.

Human Urine Samples Analysis

Metabolite ratios for CYP2A6, CYP1A2, NAT2 and XO were shown in table 1 respectively, the individual difference were 28.0%, 28.7%, 37.9% and 13.5% respectively, these values are similar to those reported previously in male population [8-10].

In conclusion, the present HPLC method was specific, sensitive and accurate. The method was successfully applied for evaluation the activity of CYP1A2, CYP2A6, XO and NAT2 in healthy volunteers.

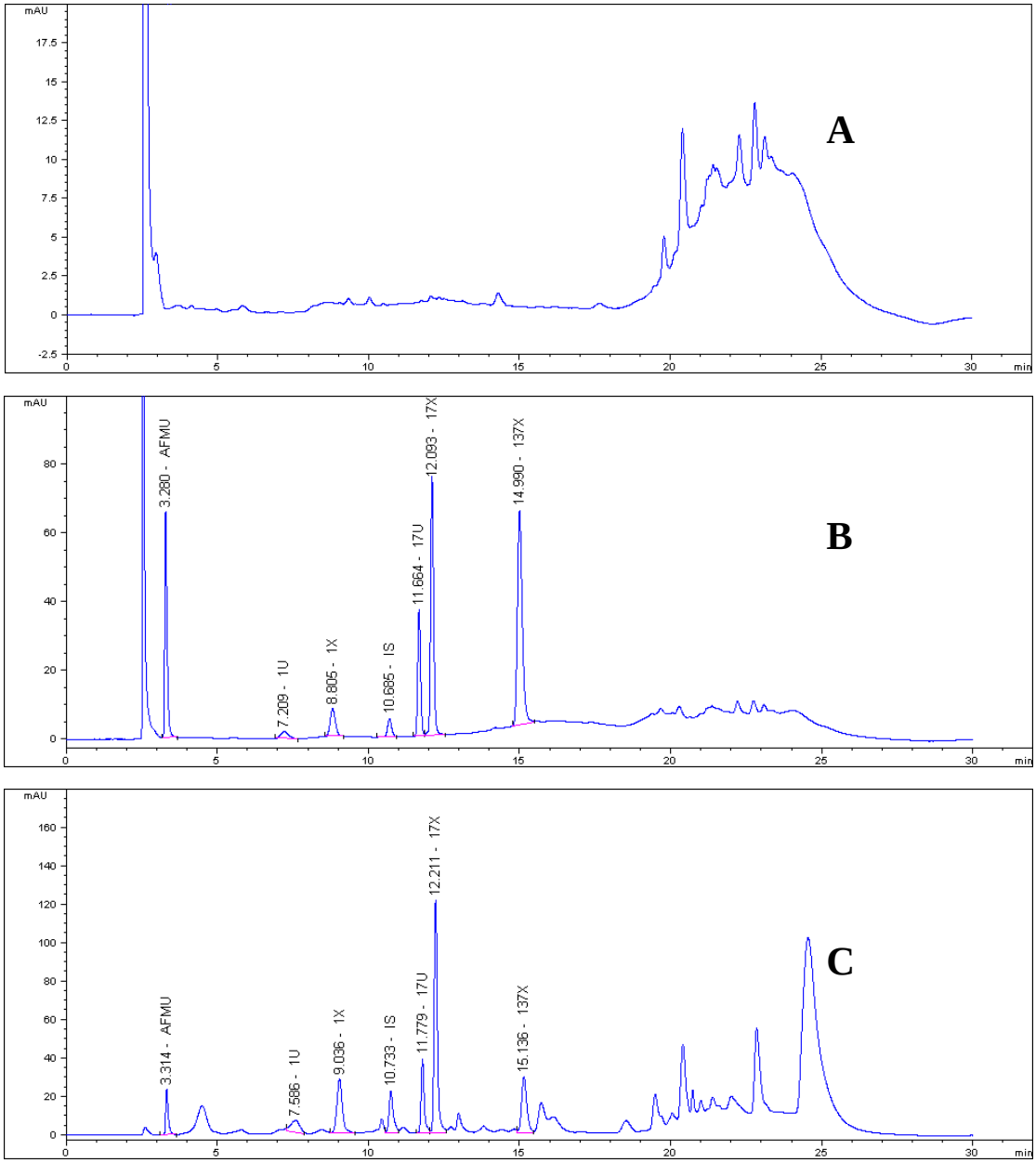

Fig.1. (A) Chromatogram of a blank urine sample; (B) Chromatogram of a pure standard mixture of caffeine and five caffeine metabolites and IS; (C) Chromatogram of an urine sample of a male healthy subject 0-12h after 100mg caffeine intake

Table 1. Biotransformation enzyme activities of healthy, non-smoking males, as assessed by HPLC analysis of urinary caffeine metabolites

Enzyme	Metabolite ratios	Krul C *et al.* study[8]	Begas E *et al.*study[9]	Schneider H *et al.* study[10]	In present study
CYP1A2	(AFMU+1U+1X)/17U	4.87±0.47 (2.79-7.55)	3.55 ± 0.30 (3.07-3.99)	4.4±0.7	4.1±1.18 (5.24-8.33)
CYP2A6	17U/(17U+17X+X+1U+AFMU)	0.16±0.01 (0.11-0.23)	2.15 ± 1.28 (0.85-4.37)	0.34±0.05	0.22±0.06 (0.14-0.39)
NAT2	AFMU/(AFMU+1U+1X)	1.59±0.32 (0.21-3.12)	Not provided	0.20±0.03	1.63±0.62 (0.15-3.2)
XO	1U/(1X+1U)	0.74±0.03 (0.63-0.88)	0.53 ± 0.05 (0.47-0.63)	1.2±0.2	0.65±0.09 (0.36-0.63)

Acknowledgements

We are very grateful to the grants support from National Scientific Foundation of China (No. 81302850, 81300204，81202515), General Financial Grant from the China Postdoctoral Science Foundation (No. 2013M531817), 863 Project (No. 2012AA02A518)， Young Teachers Boost Plans of Chinese Central College (grant

#201012200047)，and The natural science foundation of Hunan Province(No. 12JJ4085)

REFERENCES

[1] Myers MG.. *Ann Intern Me*d, **1999**, 114(2), 147-150
[2] Cornelis MC, El-Sohemy A. C*urr Opin Clin Nutr Metab Care*, **2007,** 10(6):745-751
[3] Tworoger SS, Gertig DM, Gates MA, Hecht JL, Hankinson SE. *Cancer*, **2008,** 112(5):1169-1177
[4] Taylor ES, Smith AD, Cowan JO, Herbison GP, Taylor DR. *Am J Respir Crit Care Med*, **2004,** 169(9):1019-1021
[5] Aranda JV, Outerbridge EW, Trippenbach T. *Am J Perinatol*, **1983,** 1(1):43-46
[6] de Wildt SN, Kerkvliet KT, Wezenberg MG, Ottink S, Hop WC, Vulto AG, van Den Anker JN. *Ther Drug Monit*, **2001,** 23(3):250-254
[7] Caubet MS, Elbast W, Dubuc MC, Brazier JL. *J Pharm Biomed An*al, **2002,** 27(1-2):261-270
[8] Krul C, Hageman G. *J Chromatogr B Biomed Sci Appl*, **1998,** 709(1):27-34
[9] Begas E, Kouvaras E, Tsakalof A, Papakosta S, Asprodini EK. *Biomed Chromatogr*, **2007,** 21(2):190-200
[10] Schneider H, Ma L, Glatt H. *J Chromatogr B Analyt Technol Biomed Life Sci* **,2003,** 789(2):227-237
[11] Nyéki A, Biollaz J, Kesselring UW, Décosterd LA. *J Chromatogr B Biomed Sci Appl*, **2001,** 755(1-2):73-84
[12] McQuilkin SH, Nierenberg DW, Bresnick E. *Cancer Epidemiol Biomarkers Prev* **,1995,** 4(2):139-146
[13] Bendriss EK, Markoglou N, Wainer IW. *J Chromatogr B Biomed Sci Appl* ,**2000,** 746(2):331-338
[14] Tanaka E. *J Chromatogr*, **1992,** 575(2):311-314
[15] Butler MA, Lang NP, Young JF, Caporaso NE, Vineis P, Hayes RB, Teitel CH, Massengill JP, Lawsen MF, Kadlubar FF. *Pharmacogenetics* , **1992,** 2(3):116-127