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ORIGINAL RESEARCH

In vivo evaluation of CYP1A2, CYP2A6, NAT-2 and xanthine oxidase activities in a Greek population sample by the RP-HPLC monitoring of caffeine metabolic ratios

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ABSTRACT: A RP-HPLC method was developed for the assessment of caffeine and its metabolites in urine and was used for the evaluation of the CYP1A2, CYP2A6, xanthine oxidase (XO) and *N*-acetyl-transferase-2 (NAT-2) *in vivo* activities in 44 Greek volunteers (21 men, 23 women). Spot urine samples were analyzed 6 h after 200 mg caffeine consumption, following a 30 h methylxantine-free diet. The major urinary caffeine metabolites are 1-methyluric acid (1U), 5-acetylamino-6-formylamino-3-methyluracil (AFMU), 1-methylxanthine (1X), 1,7-dimethyluric acid (17U) and 1,7-dimethylxanthine (17X). CYP1A2, CYP2A6, XO and NAT-2 activities were estimated from the metabolic ratios (AFMU + 1U + 1X)/17U, 17U/17X, 1U/(1X + 1U) and AFMU/(AFMU + 1U + 1X), respectively. Metabolites and internal standard were extracted with chloroform/isopropanol (85:15, v/v) and separated on a C_{18} column by an isocratic HPLC system using a two-step elution with manual switch from solvent A (0.1% acetic acid–methanol–acetonitrile, 92:4:5 v/v) to solvent B (0.1% acetic acid–methanol, 60:40, v/v), and detected at 280 nm. The method exhibited adequate metabolite separation (resolution factors >1.48), accuracy (94.1–106.3%) and intraday and interday precision <8.02 and <8.78%, respectively ($n = 6$). Smoking affected only CYP1A2, whereas gender had no effect in any enzyme activity. NAT-2 exhibited bimodal distribution, 63.6% of volunteers being slow acetylators. The developed RP-HPLC method was fully validated and successfully applied for the evaluation of CYP1A2, CYP2A6, XO and NAT-2 activities. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: CYP1A2; CYP2A6; XO; NAT-2; HPLC; caffeine

INTRODUCTION

Caffeine is the drug with the largest consumption among humans, as it has become an almost universal component of the diet, either as a natural constituent or as a common additive in many different food products. Caffeine is commonly used as a probe drug for the simultaneous assessment of the phenotypes of various drug-metabolizing enzymes, including CYP1A2 (Grant *et al.*, 1983; Campbell *et al.*, 1987b; Kalow and Tang, 1993), CYP2A6 (Grant *et al.*, 1983; Bendriss *et al.*, 2000; Bechtel *et al.*, 2000; Nowell *et al.*, 2002), xanthine oxidase (XO; Grant *et al.*, 1983; Aklillu *et al.*, 2003) and *N*-acetyl-transferase-2 (NAT-2; Grant *et al.*, 1983; Carrillo and Benitez, 1994; Cascorbi *et al.*, 1995; Asprodini *et al.*, 1998).

Human CYP1A2 is involved in the metabolism of endogenous compounds. It activates metabolically a great number of procarcinogens to active intermediates and is responsible for the metabolism of many clinically used drugs such as theophylline and caffeine (Campbell *et al.*, 1987a), clozapine (Bertilsson *et al.*, 1994), propranolol (Masubuchi *et al.*, 1994) and imipramine (Lemoine *et al.*, 1993). CYP1A2 is one of the major CYP1A enzymes that catalyze 2-hydroxylation of estrogen (Yamazaki *et al.*, 1998), a sex hormone that plays a critical role in the etiology of breast cancer (Long *et al.*, 2006). Furthermore, CYP1A2-164A→C polymorphism (CYP1A2*1F) has been associated with high risk for colorectal adenomas in humans (Moonen *et al.*, 2005). Although previous studies have shown that CYP1A2 activity may be influenced by gender, menstrual cycle, race, age, coffee and alcohol intake, consumption of cruciferous vegetables and exposure to various contaminants (Kalow and Tang 1991; Tantcheva-Poor *et al.*, 1999; Le Marchand *et al.*, 1997), these findings have not been consistent across all studies. CYP1A2 activity has been reported to be also

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Abbreviations used: 1U, 1-methyluric acid; 1X, 1-methylxanthine; 17U, 1,7-dimethyluric acid; 17X, 1,7-dimethylxanthine; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; NAT-2, *N*-acetyl-transferase-2; XO, xanthine oxidase.

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impaired in liver cirrhosis (Denaro *et al.*, 1996; Bechtel *et al.*, 2000; Lelouet *et al.*, 2001). Conversely, a consensus in literature has identified smoking as a potent inducer of CYP1A2 activity (Campbell *et al.*, 1987b; Rasmussen and Brosen 1996; Tantcheva-Poor *et al.*, 1999). CYP1A2 activity exhibits a wide variability among individuals which is not associated with a specific nucleotide difference that explains the phenotypic variability (Jiang *et al.*, 2006). Conversely, there have been identified two mutations of the CYP1A2 gene that are related to increased inducibility of the enzyme (Castorena-Tores *et al.*, 2005).

The activities of the enzymes CYP2A6 and XO, using caffeine as a probe drug, have been studied less extensively than that of CYP1A2. The activity of CYP2A6 is not influenced by gender, use of oral contraceptives (Krul and Hageman 1998; Nowell *et al.*, 2002) and smoking (Nowell *et al.*, 2002), whereas the effect of age (Krul and Hageman 1998; Nowell *et al.*, 2002) and race (Grant *et al.*, 1983; Nowell *et al.*, 2002) has been controversial. Cytochrome CYP2A6 is the primary enzyme that catalyzes the biotransformation of nicotine (Messina *et al.*, 1997) and coumarin (Oscarson, 2001). CYP2A6 is also responsible for the metabolic activation of several procarcinogens and promutagens such as aflatoxin B1 (Aoyama *et al.*, 1990) and 3-methylindole (Thornton-Manning *et al.*, 1996). It is involved in the metabolism of nitrosamines, such as *N*-nitrosodiethylamine (Camus *et al.*, 1993), and drugs, such as chlormethiazole, methoxyflurane, halothane, valproic acid and disulfiram (Oscarson, 2001). CYP2A6 activity is induced by barbiturates (Nowell *et al.*, 2002). High CYP2A6 activity is associated with increased risk of liver and colorectal cancer (Nowell *et al.*, 2002). Furthermore, it has been shown that CYP2A6 activity is increased in liver cirrhosis (Bechtel *et al.*, 2000; Lelouet *et al.*, 2001).

XO is a cytoplasmic enzyme and is involved in the oxidation of endogenous purines and pyrimidines and in the metabolism of drugs, such as thiopurines and methyl-xanthines. The highest specific activities of XO are found in the liver and intestine (Guerciolini *et al.*, 1991). XO has been reported to catalyze organic nitrate reduction under anaerobic conditions (Li *et al.*, 2005). Although XO is not considered to be polymorphic, two- to fourfold ranges in activity have been reported among adults (Kashuba *et al.*, 1998). It is generally accepted that smoking (Aklillu *et al.*, 2003) and gender (Kalow and Tang, 1991; Aklillu *et al.*, 2003) do not affect XO activity. However, increased XO activity has been reported in smoking subjects (Vistisen *et al.*, 1991) and in females (Relling *et al.*, 1992). Furthermore, putative XO poor metabolizers have been reported in Ethiopians (4%; Aklillu *et al.*, 2003), Caucasians (20%; Guerciolini *et al.*, 1991), Japanese (11%; Saruwatari *et al.*, 2002) and Spanish (4%; Carillo and Benitez, 1994) populations. XO activity is increased in liver cirrhosis (Bechtel *et al.*, 2000; Lelouet *et al.*, 2001).

NAT-2 is a cytosolic enzyme with highest activity in the liver and intestine (Kashuba *et al.*, 1998). It is responsible for the detoxification from several carcinogen compounds (Gu *et al.*, 2005). NAT-2 is responsible for the acetylation of more than 15 drugs, such as isoniazide, hydralazine, procainamide, dapsone and sulfonamides (Evans, 1989). NAT-2 slow acetylator phenotype(s) infer a consistent and robust increase in urinary bladder cancer risk following exposure to aromatic amine carcinogens (Hein *et al.*, 2006). *In vivo* and *in vitro* studies have demonstrated that caffeine is metabolized mainly via *N*-3 demethylation to 1,7-dimethylxanthine (17X, paraxanthine). Hepatic *N*-3 demethylation of caffeine is almost exclusively catalyzed by CYP1A2. Furthermore, CYP1A2 contributes to *N*-1 and *N*-7 demethylations (Butler *et al.*, 1989; Gu *et al.*, 1992). Overall, CYP1A2 is responsible for more than 95% of the primary metabolism of caffeine, thus making caffeine a suitable substrate for the assessment of CYP1A2 activity (Kalow and Tang, 1993). Other important enzymes of the paraxanthine pathway are CYP2A6, XO and NAT-2 (Kalow and Tang, 1993). The major metabolites of caffeine in urine are 1-methyluric acid (1U), 5-acetylamino-6-formylamino-3-methyluracil (AFMU), 1,7-dimethyluric acid (17U) and 1,7-dimethylxanthine (17X; Fig. 1). Caffeine itself is a minor excretion product, accounting for 1–2% of the administered dose (Bonati *et al.*, 1992).

Among the urinary metabolic ratios of caffeine, implicating caffeine and its major metabolites that have been proposed as probes for their *in vivo* activity of CYP1A2, the metabolic ratio (AFMU + 1U + 1X)/17U seems to be the most reliable (Campbell *et al.*, 1987b; Rostami-Hodjegan *et al.*, 1996). This metabolic ratio is closely correlated to the systematic clearance of caffeine and it is affected neither by urine flow rate nor by sampling time, presuming that there is sufficient time interval between caffeine intake and urine collection (6–8 h; Campbell *et al.*, 1987b). Furthermore, this metabolic ratio has been reported to be decreased in patients with various types of liver disease (Denaro *et al.*, 1996; Bechtel *et al.*, 2000; Lelouet *et al.*, 2001) and may be used for monitoring the progress of liver disease. XO, CYP2A6 and NAT-2 activities have been assessed by the molar ratios 1U/(1U + 1X) (Kalow and Tang, 1991), 17U/17X (Grant *et al.*, 1983) and AFMU/(AFMU + 1U + 1X) (Rostami-Hodjegan *et al.*, 1996), respectively.

Diverse HPLC methodologies have been employed for the determination of caffeine and different number of its primary and secondary metabolites in urine. These involve gradient (Georga *et al.*, 2001; Caubet *et al.*, 2002) or isocratic (Rasmussen and Brosen, 1996; Krul and Hageman, 1998) elution of substances,

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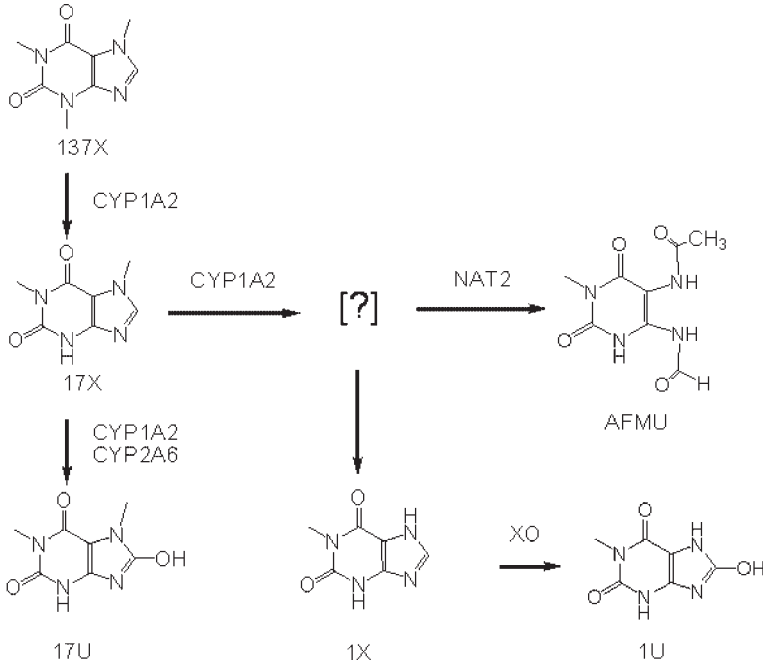

Figure 1. Caffeine metabolic pathways.

the conversion of AFMU to 5-acetylamino-6-amino-3-methyluracil (AAMU) and its separate quantification from the rest of the metabolites (Tang *et al.*, 1991; Baud-Camus *et al.*, 2001), and finally the simultaneous determination of AAMU and the primary and final metabolites of caffeine with minimum (Dobrocky *et al.*, 1994; Nyeki *et al.*, 2001) or no (Ambrose and Fritz, 1998) sample pretreatment. Interestingly, other methods employ metabolic ratios that do not include AFMU or AAMU and, thus, there is no need for analytical determination of these metabolites (Georga *et al.*, 2001; Caubet *et al.*, 2002). Finally, caffeine and its metabolites may be isolated in urine samples either by liquid–liquid extraction (LLE) using different organic phases (chloroform/isopropanol; Grant *et al.*, 1983; Krul and Hageman, 1998; Bendriss *et al.*, 2000, or ethylacetate/isopropanol; Rasmussen and Brosen, 1996) or by solid-phase extraction (SPE; Georga *et al.*, 2001; Baud-Camus *et al.*, 2001; Caubet *et al.*, 2002).

In the present study an RP-HPLC-based method for quantification of caffeine and its metabolites in urine samples was developed and was applied for the evaluation of the CYP1A2, CYP2A6, XO and NAT-2 *in vivo* activities by determining caffeine metabolite ratios (AFMU + 1U + 1X)/17U, 17U/17X, 1U/(1U + 1X) and AFMU/(AFMU + 1U + 1X), respectively, in urine of caffeine-probed patients. The simple method based on non-expensive isocratic HPLC system analysis permitted adequate separation of caffeine and its 14 metabolites as well as the quantification of AFMU, 1U, 1X, 17U and 17X.

EXPERIMENTAL

Chemicals and reagents

1-methylxanthine (1X), 7-methylxanthine (7X), 3-methylxanthine (3X), 3,7-dimethylxanthine (37X), 1,7-dimethylxanthine (17X), 1-methyluric acid (1U), 1,3-dimethyluric acid (13U), 1,7-dimethyluric acid (17U), 1,3,7-trimethyluric acid (137U), 7-methyluric acid (7U), 3-methyluric acid (3U) and 4-acetamidophenol were purchased from Sigma (Steinheim-Germany), 1,3-dimethylxanthine (13X) from Merck (Darmstadt, Germany) and caffeine (1,3,7-trimethylxanthine, 137X) and 3,7-dimethyluric acid (37U) from Fluka (Buchs, Switzerland). AFMU (5-acetylamino-6-formylamino-3-methyluracil, purity 98%) was kindly provided by R. Fumeaux (Nestle Research Centre, Lausanne, Switzerland). Acetonitrile and methanol were HPLC-grade and were purchased from Riedel-de Haen (Seelze, Germany). Hydrochloric acid, acetic acid, chloroform, isopropanol and ammonium sulfate were of analytical grade and were purchased from Riedel-de Haen (Seelze, Germany). HPLC-grade water was used throughout the analysis.

Chromatography instrumentation and conditions

Chromatographic analysis of caffeine and its metabolites was performed using a Marathon III pump (Rigas Labs, Greece) equipped with a model FASMA 500 UV–vis detector (at 280 nm, Rigas Labs, Greece) and a Rheodyne 7010 injection valve (Rheodyne, California, USA) with a 20 μL loop. Separation was achieved using a Kromasil 100 C_{18} column (5 μm, 250 × 4.6 mm i.d.; Macherey-Nagel, Germany) operated at ambient temperature. As eluent, two solvents were

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Table 1. HPLC flow program for the separation of caffeine and its metabolites

Time	Solvent A (%)	Solvent B (%)	Flow (mL/min)
0	100	0	0.7
5	100	0	0.7
5	100	0	1.1
15	100	0	1.1
15	0	100	1.1
19	0	100	0.7

Solvent A: 0.1% acetic acid–methanol–acetonitrile (92:4:5, v/v). Solvent B: 0.1% acetic acid–methanol (60:40, v/v).

applied in a step gradient mode described in Table 1 with solvent A, comprising 0.1% acetic acid–methanol–acetonitrile, 92:4:5 v/v, and solvent B, comprising of 0.1% acetic acid–methanol, 60:40, v/v. The mobile phase was degassed by an online degassing system (Alltech Associates Inc., Deerfield, IL, USA). Chrom & Spec software (Ampersand Ltd, Russia) was used for data acquisition and analysis.

Preparation of standard solutions

Stock solutions of caffeine and its metabolites (5 mM) were prepared as follows: caffeine (137X), 137U, 13X, 17X and 13U were made up in HPLC-grade water. 37X, 17U, 37U and 3U were made up in 2.5 mM NaOH solution. 1X, 3X, 7X, 1U and 7U were made up in 5.0 mM NaOH. AFMU (5 mM) and the internal standard (IS; 4-acetamidophenol, 10 mM) were prepared in 0.1% acetic acid. All stock solutions were stored at –20°C. A standard mixture consisting of 1U, 1X, 17U, 17X and AFMU (1 mM) was prepared from stock solutions and stored at –20°C. The mixture of 1U, 1X, 17U and 17X was adjusted to pH 3.5 with acetic acid before the addition of AFMU. This mixture was used to prepare the appropriate standards in urine of a healthy subject collected 4 days after xanthine-free diet.

Sample treatment

Urine aliquots (200 µL), were saturated with 250 mg ammonium sulfate; subsequently IS solution (20 µL) and 0.1% acetic acid (200 µL) were added to the sample. The samples were extracted with 6 mL chloroform–isopropanol solution (85:15, v/v) by vortex mixing for 30 s. After centrifugation at 5000 rpm for 2 min, the organic phase was removed by aspiration and evaporated under gentle stream of nitrogen at 40°C. The residue was re-suspended in 200 µL of 0.1% acetic acid, vortexed for 15 s and filtered through a nylon syringe filter 0.20 µm. Finally, an aliquot (20 µL) was injected into the column. In the case of spiked calibration standards or quality controls, 200 µL of xanthine-free urine were added to 200 µL of standard mixtures of the five metabolites at different concentrations and treated as above.

Method validation

Specificity. Specificity was determined by the analysis of xanthine-free urine samples. As can be seen from the chromatogram of blank urine extract, no endogenous peaks which interfere with the quantification of AFMU, 1U, 1X, 17U, 17X or the IS were detected [Fig. 2(B)].

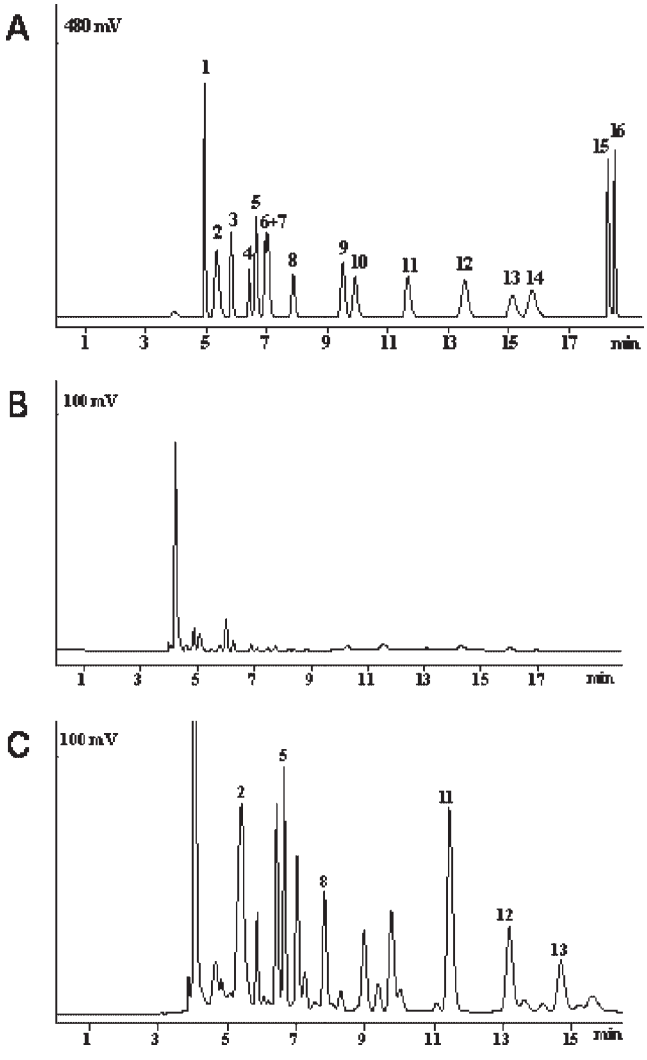

Figure 2. HPLC chromatograms. (A) Standard mixture of caffeine and 14 caffeine metabolites (100 µM) and the IS; (B) blank urine sample; (C) urine sample of a male healthy subject 6 h after caffeine intake. **1**, 3U; **2**, AFMU; **3**, 7U; **4**, 7X; **5**, 1U; **6**, 3X; **7**, 37U; **8**, 1X; **9**, 13U; **10**, 37X; **11**, IS; **12**, 17U; **13**, 17X; **14**, 13X; **15**, 137U; **16**, CA (137X).

Recovery. The absolute recovery of AFMU, 1U, 1X, 17U and 17X was determined by comparing peak-height of extracted spiked urine samples with that of the corresponding unextracted standards at concentrations 10, 20, 50, 100 and 200 µM for each of the five metabolites.

Linearity of calibration curves. Calibration curves were constructed in order to confirm the linear relationship between the ratio of peak-height of the compounds under investigation and that of IS and their concentration in the spiked sample. Matrix calibrators with metabolite concentrations 10, 20, 50, 100 and 200 µM were prepared by spiking xanthine-free urine samples. The calibrators were extracted as described previously and analyzed. After analysis, standard calibration curves were constructed by regression analysis.

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Precision and accuracy. Three quality control (QC) samples were prepared by spiking blank urine samples with a standard solution consisting of the five metabolites of interest and the IS at low (15 μM), medium (75 μM) and high (150 μM) concentrations of each metabolite. The intra- and inter-day accuracy and precision of the method were determined by analyzing six replicates of each QC sample, and are summarized in Table 3. For each QC the concentration was recalculated from the equation of the linear regression curve.

Limit of quantification (LOQ) and limit of detection (LOD). The LOQ of the method was determined as the lowest amount of the analyte which could be determined in a sample with a precision and accuracy better than 15%. The LOD, defined as three times the signal-to-noise ratio was determined by injecting diluted solutions of a solution consisting of the five metabolites.

Subjects and experimental protocol

All volunteers who participated in this study provided verbal informed consent and were judged to be healthy according to medical history, physical examination and routine laboratory analysis. None of the subjects was using any medications and none of the women were using oral contraceptives. Volunteers were divided into two groups according to their smoking status: smokers (>10 cigarettes/day) and non-smokers. The subjects abstained from ingesting any food or beverages containing methylxanthines for at least 30 h before the test and during the day of the test. On the day of the test the subjects were given a home-made gelatine lactose-free capsule containing 200 mg pure caffeine (Fluka, Switzerland). Spot urine samples were collected 6 h later in plastic jars containing 200 μL 6 M HCl. Urine aliquots were acidified to pH 3.5 to insure the stability of AFMU (Wong *et al.*, 2002) and stored at –20°C until HPLC analysis.

Statistical analysis

Data were inspected for normality and differences between medians were analyzed by Mann–Whitney test using the SPSS program package, version 9.0. Differences were considered significant at a p-value of <0.05. The linearity of the calibration curves were assessed by linear regression analysis.

RESULTS

Baseline separation and simultaneous quantification was obtained for the metabolites of interest (AFMU, 1U, 1X, 17U and 17X) and the internal standard (IS) within a single run without any interference from caffeine and all other major and secondary metabolites or matrix artefacts [Fig. 2(A, B)]. A step gradient method using two solvents with different elution strengths (Table 1) permitted adequate metabolite separation with a low-cost isocratic HPLC system. The composition of solvent A was optimized such that the resolution factors of the five metabolites of interest and the IS ranged from 1.48 to 6.31, indicating adequate separation of the compounds under analysis. At 5 min the flow rate was adjusted from 0.7 to 1.1 mL/min. The five caffeine metabolites of interest (AFMU, 1U, 1X, 17U and 17X) were eluted within approximately 15 min. At 15 min solvent B was used to elute the least polar 137U and caffeine and at 19 min the flow rate was readjusted to 0.7 mL/min [Fig. 2(A), Table 1]. During routine urine sample analysis, however, this last step could be omitted as 137U or caffeine was not used for metabolic ratio calculations. In this case the column was subsequently cleaned up by injecting 3 × 20 μL of 100% acetonitrile before the next sample analysis.

Approximately 20 min were required for the chromatographic separation of all caffeine metabolites and caffeine itself. Representative chromatograms of a blank urine sample and a spot urine sample from a healthy male subject are presented in Fig. 2(B, C). Minor impurities were observed in the chromatogram of the blank urine sample [Fig. 2(B)], which, however, did not interfere with the elution of any of the metabolites of interest.

The absolute recovery of the five metabolites at the different concentrations tested resulted in mean recoveries of AFMU, 1U, 1X, 17U and 17X corresponding to 73.6, 65.2, 94.3, 91.7 and 97.5%, respectively. The mean recovery of the IS was 91.6%.

Calibration curves of AFMU, 1U, 1X, 17U and 17X were linear over the concentration range investigated (10–200 μM). Correlation coefficients (r^2) were greater than 0.9989 for the five metabolites quantified (Table 2). The results of intra-day (n = 6) and inter-day (n = 6) method validation are presented in Table 3. The method was reproducible with coefficient of variation values (CV%) less than 8.02 and 8.78% for intra- and inter-day measurements, respectively. The accuracy values, at all concentrations, ranged from 94.1 to 106.3%. The overall intra- and inter-day accuracies of the estimated metabolic ratios (AFMU + 1X + 1U +)/17U, 17U/17X, 1U/(1U + 1X) and AFMU/(AFMU + 1U + 1X) from the spiked QC urine standards ranged from 99.2 to 107.2% with coefficients of variation between 0.77 and 9.66% (Table 4). The limit of detection was determined to be 0.08, 0.03, 0.09, 0.10 and 0.17 μM for AFMU, 1U, 1X, 17U and 17X respectively. The limit of quantification was determined to be 5 μM for the five metabolites with accuracy >86% and CV <5% (n = 5).

Table 2. Regression analysis results

		Least squares regression $y = a + bx$		
	Curve	R^2	a	b
1	AFMU	0.9997	0.0118	0.0117
2	1U	0.9995	0.0409	0.0096
3	1X	0.9997	0.0109	0.0049
4	17U	0.9994	0.0034	0.0047
5	17X	0.9989	0.0167	0.0026

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Table 3. Accuracy and precision of the five end-metabolites of caffeine in urine

	AFMU			1U			1X			17U			17X		
Added concentration (μM)	Measured concentration (μM), mean	CV (%)	Accuracy (%)	Measured concentration (μM), mean	CV (%)	Accuracy (%)	Measured concentration (μM), mean	CV (%)	Accuracy (%)	Measured concentration (μM), mean	CV (%)	Accuracy (%)	Measured concentration (μM), mean	CV (%)	Accuracy (%)
Intra-day, $n = 6$															
15	15.77	5.66	105.2	15.12	3.48	100.8	14.70	2.41	98.0	15.05	3.28	100.3	14.12	8.02	94.1
75	79.74	5.89	106.3	73.70	2.39	98.3	72.85	1.33	97.1	71.93	1.3	95.9	71.69	1.89	95.6
150	158.11	2.85	105.4	148.22	1.63	98.8	145.74	1.00	97.2	145.77	0.75	97.2	146.09	1.58	97.4
Inter-day, $n = 6$															
15	14.93	7.99	99.5	15.39	3.63	102.6	14.52	7.16	96.8	14.71	4.88	98.0	14.56	8.78	97.0
75	75.51	6.40	100.7	73.34	2.06	97.8	73.17	2.12	97.6	71.80	2.36	95.7	71.75	1.94	95.7
150	147.74	3.45	98.5	147.50	1.93	98.3	144.11	2.02	96.1	144.18	2.01	96.1	145.87	1.39	97.2

Table 4. Accuracy and precision of the metabolic ratios that reflect CYP1A2, CYP2A6, XO and NAT-2 activities

	Metabolic ratios															
	CYP1A2				CYP2A6				X.O.				NAT-2			
Added concentration (μm)	Expected	Measured (mean)	CV%	Accuracy (%)	Expected	Measured (mean)	CV%	Accuracy (%)	Expected	Measured (mean)	CV%	Accuracy (%)	Expected	Measured (mean)	CV%	Accuracy (%)
Intra-day ($n = 6$)																
15	3.00	3.03	2.77	101.0	1.00	1.07	9.66	107.2	0.50	0.51	1.34	100.8	0.33	0.35	3.91	105.0
75	3.00	3.15	2.97	104.9	1.00	1.00	1.86	100.3	0.50	0.51	0.77	100.8	0.33	0.35	2.99	105.0
150	3.00	3.10	1.28	103.4	1.00	0.99	1.49	99.8	0.50	0.51	0.37	100.8	0.33	0.35	1.46	105.0
Inter-day ($n = 6$)																
15	3.00	3.05	1.92	101.6	1.00	1.02	8.98	101.6	0.50	0.51	3.91	103.0	0.33	0.33	2.91	99.7
75	3.00	3.05	3.13	101.7	1.00	1.00	2.09	100.0	0.50	0.50	1.50	100.1	0.33	0.33	3.98	99.2
150	3.00	3.07	2.56	102.3	1.00	0.99	0.93	98.8	0.50	0.50	1.33	100.1	0.33	0.33	2.98	100.2

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Table 5. Effect of smoking and gender on the activity of CYP1A2, CYP2A6 and XO

	Non-smokers (n = 21)			Smokers (n = 23)		
	Mean ± SD	Median	Range	Mean ± SD	Median	Range
CYP1A2						
Total	3.61 ± 0.46	3.57	3.06–4.64	6.36 ± 1.98	5.83*	3.84–11.15
Men	3.55 ± 0.30	3.62	3.07–3.99	5.93 ± 1.28	5.54*	3.84–11.15
Women	3.66 ± 0.58	3.46	3.06–4.64	6.75 ± 2.45	6.12*	4.60–8.28
CYP2A6						
Total	1.98 ± 1.45	1.41	0.52–6.02	1.36 ± 0.80	1.20	0.42–3.80
Men	2.15 ± 1.28	1.56	0.85–4.37	1.87 ± 0.83	1.68	0.99–3.80
Women	1.84 ± 1.65	1.27	0.52–6.02	0.89 ± 0.41	0.79	0.42–1.69
XO						
Total	0.53 ± 0.05	0.53	0.47–0.63	0.51 ± 0.04	0.52	0.45–0.60
Men	0.53 ± 0.05	0.53	0.47–0.63	0.51 ± 0.04	0.52	0.45–0.57
Women	0.53 ± 0.05	0.52	0.47–0.62	0.52 ± 0.05	0.53	0.45–0.60

* $p < 0.001$, Mann–Whitney rank sum test.

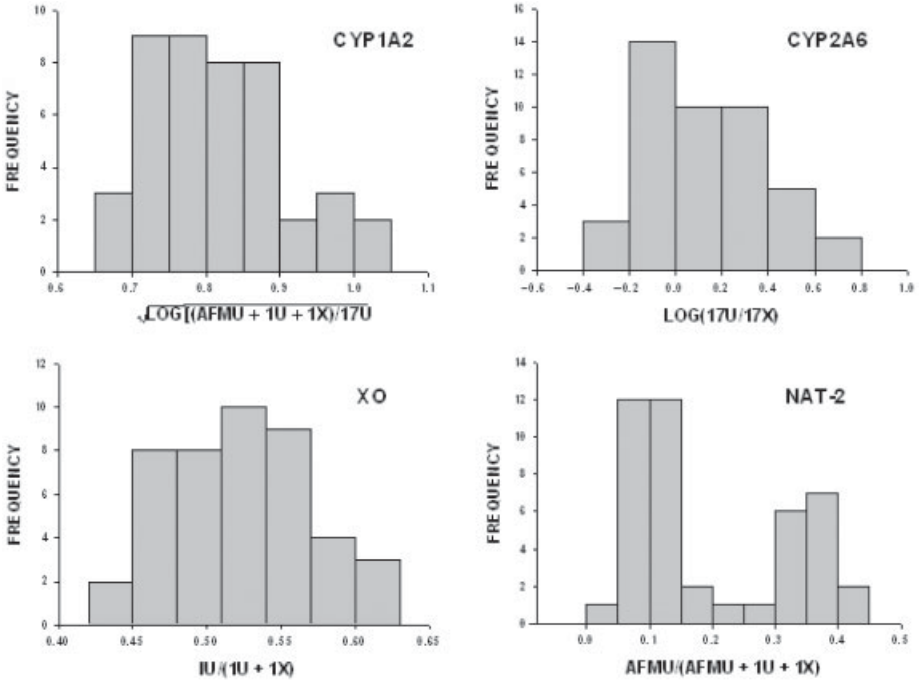

Figure 3. Frequency histograms of the metabolic ratios reflecting CYP1A2, CYP2A6, XO and NAT-2 activities.

Biological validation

The validated RP-HPLC method was applied to the biological monitoring of the population sample comprising 44 healthy volunteers including 21 men (11 cigarette smokers and 10 non-smokers; mean age 31.3 years, range 19–63) and 23 women (12 cigarette smokers and 11 non-smokers; mean age 32.5 years, range 19–52). To evaluate the effect of smoking on CYP1A2 activity, the ratio values obtained from 21 non-smokers (10 men and 11 women) and 23 smokers (11 men and 12 women) were compared. The median values of CYP1A2 activities were significantly different between non-smokers (metabolic ratio = 3.57) and smokers (metabolic ratio = 5.83; $p < 0.001$). When, however, the smoking habits were compared in relation to gender, no difference was found between men and women smokers (median 5.54 and 6.12, respectively, $p > 0.05$) and non-smokers (median 3.62 and 3.46, respectively, $p > 0.05$; Table 5).

The frequency histogram of the square root of the logarithm of the ratio (AFMU + 1U + 1X)/17U appeared to be unimodal with almost normal distribution of the values (Kolmogorov–Smirnov normality test, $p = 0.072$; Fig. 3).

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CYP2A6 (17U/17X) activity did not differ neither between non-smokers and smokers (median values 1.41 and 1.20, respectively, $p > 0.05$), nor between men and women who were non-smokers (median values 1.56 and 1.27, respectively, $p > 0.05$). The frequency histogram of the log-transformed values of the ratio appeared to be unimodal and normally distributed (Kolmogorov–Smirnov normality test, $p > 0.200$, Fig. 3).

XO activities [1U/(1U + 1X)] of 23 non-smokers and 21 smokers were compared; the median values of the ratios were not significantly different neither between non-smokers and smokers (0.53 and 0.52 respectively, $p > 0.05$; Table 5) nor between men and women who were non-smokers (median values 0.53 and 0.52, respectively, $p > 0.05$) or smokers (median values 0.51 and 0.53, respectively, $p > 0.05$). The frequency distribution of XO activity was unimodal approaching normality (Kolmogorov–Smirnov normality test, $p = 0.159$, Fig. 3).

The frequency distribution of NAT-2 activities [AFMU/(AFMU + 1U + 1X)] appeared to be bimodal (Fig. 3) with an apparent antimode at 0.25 separating slow from rapid acetylators. Subjects with metabolic ratios <0.25 were then classified as slow acetylators and those with metabolic ratios >0.25 as fast acetylators. Overall, our population sample included 28 (63.6%) slow and 16 (36.7%) rapid acetylators. The small sample size of the present study precluded any comparison between men and women or smokers and non-smokers in relation to acetylation status.

DISCUSSION

To the best of our knowledge this is the first report of the assessment of CYP1A2, CYP2A6, XO and NAT-2 *in vivo* activities, using caffeine as a metabolic probe, within the Greek population. The HPLC method used in the present study provides a relatively simple procedure for the simultaneous quantification of AFMU, 1U, 1X, 17U and 17X within a single run without any interference from caffeine, its major and secondary metabolites or matrix artifacts. Our method provides an analytically valid tool for the assessment of CYP1A2, CYP2A6, XO and NAT-2 activities in humans since it evidences good overall recovery, small variations and low detection limits for all metabolites. Caffeine metabolites were isolated from urine by liquid–liquid extraction using chloroform–isopropanol 85:15 (v/v) as it is described by Grant *et al.* (1983). This particular ratio of solvents gave the best results among other ratios tested (data not shown) concerning compounds of interest recovery and purification efficiency. The moderate mean recovery of 1U (65.2%) may have been due to the low solubility of 1U at low pH values (Tang *et al.*, 1991). The same mixture has been used by other researchers also at different ratios and with various percentages of recovery of caffeine metabolites (Krul and Hageman, 1998; Bendriss *et al.*, 2000). In order to avoid the use of toxic chloroform, Rasmussen and Brosen (1996) used an ethyl acetate–isopropanol mixture, but the extraction lasted 10 min, while, the chloroform–isopropanol mixture requires only 30 s of mixing/vortexing. Several methods of solid-phase extraction of caffeine metabolites have been reported (Georga *et al.*, 2001; Baud-Camus *et al.*, 2001; Caubet *et al.*, 2002). These methods reduce the use of organic solvents and give recovery percentages over 90% for the metabolites under study. However, a solid-phase extraction method for the simultaneous extraction of AFMU and other caffeine metabolites has never been reported. The HPLC method presented here allowed the quantitative determination of AFMU, 1U, 1X, 17U and 17X over a wide range of concentrations (5–200 μM). This range covered 88% of the examined samples, whereas the remaining 12% had one or more of the five metabolites slightly above the selected range and the concentrations were determined by diluting and reanalyzing these samples.

The separation time of the five caffeine metabolites of interest (AFMU, 1U, 1X, 17U and 17X) was approximately 15 min and it was the shortest time of all methods reported in the literature (Rasmussen and Brosen, 1996; Krull and Hageman, 1998; Bendriss *et al.*, 2000; Nyeki *et al.*, 2001; Schneider *et al.*, 2003).

In our study the CYP1A2 index was significantly increased in smokers. Smoking did not increase CYP2A6 or XO activities. It is generally accepted that smokers have higher mean values of CYP1A2 index than non-smokers (Campbell *et al.*, 1987b; Rasmussen and Brosen, 1996; Carrillo *et al.*, 2000). It has been reported that the inducing effect of smoking on CYP1A2 is dependent upon the number of the cigarettes smoked per day (Tantcheva-Poor *et al.*, 1999). Our small sample size of smoker volunteers precluded the evaluation of a dose-dependent inducing effect of smoking on CYP1A2. A maximal inducing effect of smoking on CYP1A2 was, therefore, insured by including in the present study only smoker volunteers who consumed more than 10 cigarettes per day.

The smoking habits were compared in relation to gender; however, no difference was found between men and women smokers and non-smokers. Earlier studies on the effect of gender on CYP1A2 activity by the use of caffeine metabolism have shown that the enzyme activity is lower in women than in men (Campbell *et al.*, 1987b; Kalow and Tang, 1991; Relling *et al.*, 1992; Rasmussen and Brosen, 1996; Krul and Hageman, 1998; Tantcheva-Poor *et al.*, 1999; Carrillo *et al.*, 2000). However, other studies have reported that the enzyme activity is not influenced by gender (Le Marchand *et al.*, 1997; Kashuba *et al.*, 1998). The use of oral contraceptives has been correlated with reduced values

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of CYP1A2 index as compared with that of non-users (Campell *et al.*, 1987b; Kalow and Tang, 1991; Rasmussen and Brosen, 1996; Krul and Hageman, 1998). It is noteworthy that no oral contraceptive users participated in the present study.

Gender and smoking had no significant effect in the CYP2A6 and XO activity. Only a few studies have used the caffeine metabolic ratio as a marker of CYP2A6 activity. An earlier study on the effect of gender and smoking on CYP2A6 activity using caffeine metabolism has shown higher activity in females compared with males and no difference in smokers compared with non-smokers (Nowell *et al.*, 2002). In another study, no significant influence of smoking and gender in the CYP2A6 and XO activity was found (Krul and Hageman, 1998). Aklillu *et al.* (2003) have reported that neither smoking nor gender affected XO activity. In contrast, XO activity appeared to be lower in males than in females in a study of 342 healthy subjects by Relling *et al.*, (1992).

In the present study the indices of CYP1A2 and CYP2A6 were normally distributed, implying population homogeneity and precluding the existence of any polymorphism affecting CYP1A2 and CYP2A6 activity. Normal distribution of CYP1A2 activity has been observed in several studies (Grant *et al.*, 1983; Kalow and Tang, 1991; Rasmussen and Brosen, 1996; Saruwatari *et al.*, 2002; Hong *et al.*, 2004). CYP2A6 activity was also normally distributed in the reports of Grant *et al.* (1983) and Nowell *et al.* (2002).

XO activity provides, in some cases, unimodal distributions with few subjects appearing with very low metabolic capacity (Guerciolini *et al.*, 1991: Relling *et al.*, 1992). In other studies, a bimodal distribution is suggested in which approximately 4% of the subjects are poor metabolizers (Carrillo and Benitez, 1994; Aklillu *et al.*, 2003). In our study, XO activity appeared to be unimodal and normally distributed, suggesting that there were no subjects with low enzyme activity.

A non-normal and bimodal distribution of NAT-2 activity has been observed. Within the population studied it has been found that 63.6% can be classified as slow and 36.7% as fast acetylators. The percentile distributions of slow and rapid acetylators within the population presented here are similar to those in previously published reports concerning several Caucasian populations. In Canadian (Tang *et al.*, 1991), French (Pontes *et al.*, 1993), Spanish (Carrillo and Benitez, 1994), Greek (Asprodini *et al.*, 1998) and German (Cascorbi *et al.*, 1995; Jetter *et al.*, 2004) populations the percentage of the rapid acetylators was 59, 61.3, 65.4, 66.3, 55.1 and 54.7%, respectively, and the percentage of the slow acetylators was 41, 38.7, 34.6, 33.7, 44.9 and 45.3%, respectively.

The inexpensive method based on isocratic HPLC system was developed and validated for quantification of caffeine metabolites in urine samples. The method was successfully applied for the assessment of CYP1A2, CYP2A6, XO and NAT-2 *in vivo* activities within the Greek population sample of 44 people by the mean caffeine metabolic test. The caffeine test is a non-invasive, well-accepted, easily accessible method and can give valuable information on the *in vivo* activity of the above mentioned enzymes; in turn, it may provide useful guidelines on cancer susceptibility, drug toxicity and drug interactions (Schneider *et al.*, 1996; Hong *et al.*, 2004) in population studies of healthy subjects. This information may also be used for the monitoring of the liver function in the therapeutic approach of different liver diseases.

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