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Eur J Clin Pharmacol (2010) 66:571–578
DOI 10.1007/s00228-010-0785-6

PHARMACOGENETICS

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Received: 13 October 2009 / Accepted: 11 January 2010 / Published online: 13 February 2010

Abstract
Purpose The main aim of the study was to investigate the distribution of cytochrome P450 2A6 (CYP2A6) and xanthine oxidase (XO) enzyme activities in the Serbian population. Secondly, we tested the influence of genetics (*CYP2A6* polymorphism), sex, and cigarette smoking on both enzymes.
Methods One hundred forty healthy Serbian volunteers were genotyped for common *CYP2A6* alleles. In 100 of them, CYP2A6 and XO activities were determined by the urinary 17U/17X and 1U/(1U+1X) ratios, respectively, after oral administration of 100 mg caffeine as a probe.
Results A 21-fold variation in the 17U/17X ratio was observed (range: 0.49–10.28, mean=1.65, 95% CI: 1.49–1.83). The urinary 1U/(1U+1X) ratios displayed four-fold variation, ranging from 0.17 to 0.71 (mean=0.43, 95% CI: 0.41–0.45). *CYP2A6* alleles **1A*, **1B1*, **9*, **4* and **1B1x2* were found with frequencies of 0.579, 0.307, 0.082, 0.029, and 0.004 respectively. *CYP2A6*5* was not detected. *CYP2A6* genotype influenced interindividual variability in CYP2A6 enzyme activity (P=0.04). Cigarette smoking inhibited CYP2A6 enzyme activity (P=0.02), but had no effect on activity of XO (P=0.16).There was no significant difference between men and women in terms of CYP2A6 or XO activity.
Conclusions Serbs displayed interindividual variations in CYP2A6 activity. *CYP2A6* genotype and cigarette smoking, but not sex, influenced CYP2A6 enzyme activity. Unimodal distribution of XO enzyme activity in Serbs implies the absence of subjects with low enzyme activity in this population. XO activity is not influenced by sex or cigarette smoking.

Keywords CYP2A6 · Xanthine oxidase · Serbian · Phenotype · Caffeine · Genotype

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Introduction

Cytochrome P450 2A6 enzyme (CYP2A6) is an important hepatic enzyme involved in the activation of many procarcinogens and the clearance of a number of pharmaceuticals [1, 2]. Wide interindividual variations in CYP2A6 activity, well documented both in vitro and in vivo [3, 4], have been associated with metabolism of several drugs, diseases, or environmental factors [1, 2, 5]. Previous studies, using nicotine and cotinine as probes, showed an inhibitory effect of cigarette smoking on CYP2A6 enzyme activity [5, 6]. Sex differences have been observed as well [4, 7–10], but the findings were not always consistent [11, 12]. Nevertheless, the major cause for the observed variability in enzyme activity seems to be genetic polymorphisms of the *CYP2A6* gene [1].

Xanthine oxidase (XO) is a multifunctional enzyme responsible for oxidation processes of a wide range of

substrates, including a number of endogenous compounds and several clinically important drugs [11, 13–17]. Previous studies, using radiochemical assay [13] or caffeine test [11, 14, 18, 19], showed that XO activity differs among individuals. Known functional genetic variations in the XO gene are rare [20]. Although environmental factors seem to be responsible for the described variability [15, 17, 19], it is generally accepted that cigarette smoking does not affect XO activity [11, 19, 21]. Sex differences have been extensively studied as well, but with contradictory results [11, 13, 14, 19, 21].

Both CYP2A6 and XO participate in the metabolism of caffeine: CYP2A6 is the major enzyme responsible for the conversion of caffeine metabolite paraxanthine (1,7-dimethylxanthine or 17X) into 1,7-dimethyluric acid (17U), while XO participates in conversion of paraxanthine metabolite 1-methylxanthine (1X) to 1-methyluric acid (1U) [12]. Because of its pharmacokinetics and noninvasive properties, caffeine as a probe drug represents the safest method for evaluation of CYP2A6 and XO in humans [11, 18, 22, 23].

Ethnicity is an important demographic variable affecting interindividual variability in drug metabolism and response, and a number of differences among populations have already been reported [14, 24, 25]. CYP2A6 exhibits marked interethnic differences [26], but data on its activity in Eastern European populations are scarce [11]. On the other hand, little is known about the interethnic variations in XO activity.

The Serbian population belongs to the South Slavonic group of Indo-European peoples, with its ethnic location in the central and western part of the Balkan Peninsula. There are around 6.2 million Serbs living in Serbia at present (http://webrzs.statserb.sr.gov.yu). Recently, we reported significant differences in CYP1A2 enzyme activity between Serbs and Swedes, although both populations are of Caucasian origin [14, 24, 25]. Nevertheless, data on activity of other drug metabolizing enzymes in Serbs are still lacking. Thus, in the present study we investigated CYP2A6 and XO enzyme activities in Serbs, using caffeine as a probe drug. In addition, we examined the correlation of *CYP2A6* genetic polymorphism with CYP2A6 activity, as well as the influence of sex and cigarette smoking on both CYP2A6 and XO enzyme activities.

Materials and methods

Study subjects One hundred forty unrelated healthy Serbian volunteers (72 men and 68 women) participated in the *CYP2A6* genotype analyses; of these 100 participated in the caffeine phenotyping. The subjects who were phenotyped included 55 men and 45 women 18–46 years old, with a median age of 27 years and a median weight of 73.8 kg (range 43.0–120.1 kg). Among them, 21 men and 17 women were smokers, smoking 2–40 cigarettes per day. None of the subjects had chronic or acute use of any medications, and none of the female participants were pregnant, breast-feeding, or using oral contraceptives. All subjects gave written informed consent for both phenotyping and genotyping analysis.

Study participants abstained from consuming caffeine-containing food or beverage for at least 24 h prior to and throughout the study period. After voiding the bladder, each subject received a 100 mg oral dose of caffeine (Koffein Recip; Recip, Årsta, Sweden), and urine was collected for 8 h in a plastic can without any additives. Volume and pH of collected urine were measured, and 20-ml aliquots were stored. In addition, a 20-ml venous blood sample was collected into EDTA-containing Vacutainer tubes (Sarstedt, Nümbrecht, Germany). All samples were frozen at −80°C and packed on dry ice. Blood samples were sent to Karolinska University Hospital-Huddinge, Sweden, for genotype analyses. Urine samples were sent to Extremadura University, Badajoz, Spain, for phenotyping. The study was approved by the ethics committees at the Medical Faculty, University of Kragujevac, Serbia, and at Karolinska Institutet, Stockholm, Sweden. The study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions.

CYP2A6 genotyping DNA was extracted from the whole-blood samples using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). All PCR reactions were performed on GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA), using the primers purchased from Invitrogen, Carlsbad, CA. All other reagents used for PCR amplification were from Roche Applied Science, Mannheim, Germany. Genotyping for gene deletion (*CYP2A6*4*), gene duplication (*CYP2A6*1x2*), 1436G>T (*CYP2A6*5*), and gene conversion in the 3′ region (*CYP2A6*1B1*) was carried out using the allele-specific PCR methods presented in Table 1 [2, 27]. For the detection of *CYP2A6*9* (−48T>G), we performed a two-step allele-specific PCR. PCR I was used to amplify a 335-bp-long region of *CYP2A6* exon 2, using the primers 2A6 F7 and 2A6 ex1R. The reaction mixture contained 1 μl genomic DNA, 0.25 μM of each primer, 0.8 mM of dNTPs, 1.5 mM $MgCl_2$, and 0.625 U of AmpliTaq DNA polymerase, and was carried out in 1× PCR buffer II, in a total volume of 25 μl. The initial denaturation was performed at 95°C for 1 min; followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 20 s, and extension at 72°C for 90 s; and with a final extension at 72°C for 7 min. In PCR II, forward primers TATA 16wt or TATA 16mut with reverse primer 2A6 ex1R were used for amplification of 243-bp-long

Table 1 SNPs and primers used in genotyping of *CYP2A6* gene by allele-specific PCR method

Nucleotide change	Allele variation	Reaction	Primer name	Sequence 5′ to 3′	PCR product (bp)
*CYP2A6*1B1*	Gene conversion in 3′ region	PCR I	2A6 ex8F2 2A6 R5	CTCCAACCCCCAGGACTTCAA GCACTTATGTTTTGTGAGACAT CAGAGACA	1300
		PCR II	2A6*1Bwt 2A6*1Bmut	ACTGGGGGCAGGATGGC AATGGGGGGAAGATGCG	300
			2A6 R2	TCTTAGCTGCGCCCCTCTCC	
*CYP2A6*4*	Gene deletion	PCR I	2A ex7F 2A6 R5	GGCCAACATGCCCTACATG GCACTTATGTTTTGTGAGACAT CAGAGACA	1,967
		PCR II	2A6 ex8F 2A7ex8F	CACTTCCTGAATGAG CATTTCCTGGATGAC	1,180
			2A6 R7	TCTTAGCTGCGCCCCTCTCC	
*CYP2A6*5*	1436G>T	PCR I	2A6 ex8F2 2A6 R5	CTCCAACCCCCAGGACTTCAA GCACTTATGTTTTGTGAGACAT CAGAGACA	1,300
		PCR II	2A6*5wt 2A6*5mut	CCCCAAACACGTGGG CCCCAAACACGTGGT	152
			2A6 R2	TCTTAGCTGCGCCCCTCTCC	
*CYP2A6*9*	−48T>G	PCR I	2A6 F7 2A6 ex1R	TGGCTGTGTCCCAAGCTAGGCA CTTCATGAGGGAGTTGTACATC	335
		PCR II	TATA 16 wt TATA 16 mut	CTTTTTCAGGCAGTAT CTTTTTCAGGCAGTAG	243
			2A6 ex1R	CTTCATGAGGGAGTTGTACATC	
*CYP2A6*1x2*	Gene duplication	One-step PCR	2A7 ex8F 2A6 ex8F	CATTTCCTGGATGAC CACTTCCTGAATGAG	1,258
			2A7 R1	GCACTTATGTTTTGTGAGACAT GAGATAGA	

product. The 25 -μl mixtures contained 0.25 μM of one of the forward primers, 0.25 μM of reverse primer, 0.4 mM dNTPs, 1.3 mM $MgCl_2$, 0.625 U of AmpliTaq DNA polymerase, and 1 μl of PCR I product in a 1× PCR buffer II. The reaction conditions were as follows: initial denaturation at 95°C for 1 min; 15 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 20 s, and extension at 72°C for 45 s; and final extension at 72°C for 7 min. Products of PCR I and PCR II were detected by gel electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Caffeine phenotyping The caffeine urinary test was performed according to the method described by Carrillo et al. [28], using high-performance liquid chromatography (HPLC) to determine molar concentrations of caffeine metabolites 17X (1,7-dimethylxanthine or paraxanthine), 17U (1,7-dimethyluric acid), 1X (1-methylxanthine), and 1U (1-methyluric acid) in urine samples. System Gold Microbore HPLC with 32 Karat Software (Beckman Instruments, Madrid, Spain) was used to process the HPLC data. Enzyme activity of CYP2A6 was assessed by the ratio of 17U/17X [4], and XO activity by the 1U/(1U+1X) ratio [19].

Statistical analysis Statistical analyses were performed with Statistica, version 7.1 (StatSoft, Tulsa, OK, USA). For all statistical procedures, $P<0.05$ was considered as significant. The 95% confidence interval calculations were according to the modified Wald method. Chi-squared test was used to compare observed with expected allele frequencies (Hardy-Weinberg equilibrium). The 17U/17X ratio was log-transformed before statistical analyses. The 1U/1X and 1U/(1U+1X) ratios were correlated using Pearson analysis. Consistency of the data with the normal distribution was assessed by Shapiro-Wilk test. The main effects ANOVA model followed by post-hoc analyses (the Fisher LSD test) was used to assess the effects of genotype, sex, and smoking on CYP2A6 and XO activities. The correlation between CYP2A6 activity and number of cigarettes smoked per day was tested using regression analysis.

Results

CYP2A6 enzyme activity was estimated by the urinary 17U/17X ratio in 100 healthy volunteers after administra-

tion of 100 mg caffeine as a probe. A 21-fold variation in the 17U/17X ratio was observed (range 0.49–10.28, mean= 1.65, 95% CI: 1.49–1.83), and the values displayed log-normal distribution (Fig. 1a; W=0.99, P=0.58). Comparison of 17U/17X ratio regarding the smoking habit indicated significantly lower enzyme activity among smokers (Fig. 1b, c; P=0.022; 95% CI for the mean difference 1.035–1.637). There were no differences in the 17U/17X ratio between men and women in the whole group or between nonsmokers and smokers (Table 2, 8.2% power). Using the allele-specific PCR, 140 healthy Serbian volunteers were genotyped for the following *CYP2A6* variant alleles: gene deletion (*CYP2A6*4*), gene duplication (*CYP2A6*1x2*), gene conversion in 3′ region (*CYP2A6*1B1*), the promoter SNP-48T>G (*CYP2A6*9*), and 1436G>T (*CYP2A6*5*). The frequencies of *CYP2A6* variant alleles and genotype combinations in the Serbian population are presented in Table 3. All the observed *CYP2A6* genotype frequencies were according to Hardy-Weinberg equilibrium (χ^2=1.3, P>0.05).

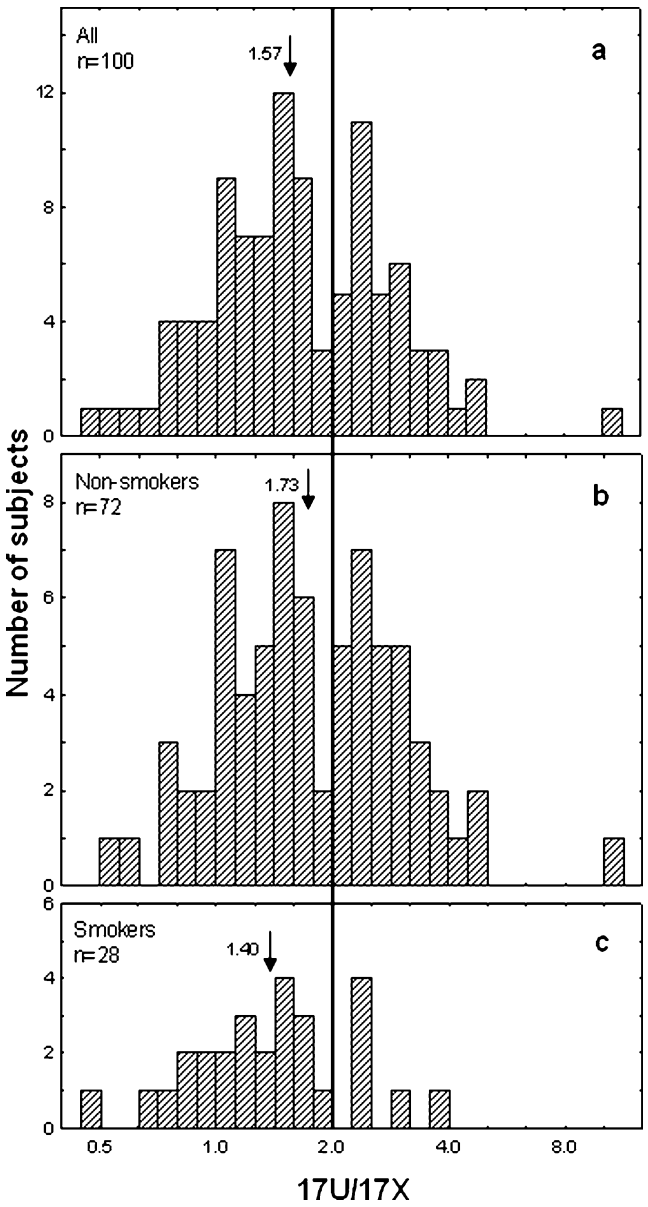

Fig. 1 The frequency distributions of the log-transformed 17U/17X ratio in Serbs. The *arrows* indicate the medians and the *adjacent numbers* are antilog values. The *vertical line* is shown at an arbitrary antilog value of 2.0 as reference

For the analysis of *CYP2A6* genotype-phenotype correlations, subjects were assigned to one of three genotype groups: group A (extensive metabolizers:**1A/*1A*, **1A/*1B1*, **1A/*1B1x2*, and **1B1/*1B1*), group B (intermediate metabolizers:**1A/*4*, **1A/*9*, **1B1/*4*, and **1B1/*9*) and group C (poor metabolizers: **4/*9* and **9/*9*). Assignments were performed according to the expected effects of specific *CYP2A6* genotypes on enzyme activity, based on a number of functional (**1A*, **1B1*, or **1B1x2*) or nonfunctional alleles (**9* and **4*). Frequency distribution of the 17U/17X ratio between the different genotype groups is displayed in Fig. 2. Comparison of mean 17U/17X ratios showed significant CYP2A6 genotype-phenotype association, the activity being higher in subjects having both functional alleles (Table 4; P=0.04). Further dividing the groups according to the cigarette smoking habit showed a similar tendency in nonsmokers (P=0.06) but not in smokers (P= 0.14, 39.6% power). In subjects carrying both functional alleles, the mean 17U/17X ratio remained significantly lower (P=0.04) among smokers (n=24, mean=1.46, 95% CI: 1.22–1.75) than nonsmokers (n=58, mean=1.89, 95% CI: 1.64–2.17). Among smokers, regression analysis indicated no significant correlation between 17U/17X ratio and number of cigarettes smoked per day (r^2=0.04, P=0.29).

XO activity was estimated in 100 healthy volunteers after oral administration of 100 mg caffeine as a probe. The urinary 1U/1X and 1U/(1U+1X) ratios were highly correlated (r=0.99), suggesting that either of the ratios can be used as an index, and the phenotype was determined on the basis of the latter. The frequency distribution of the 1U/(1U+1X) ratio is shown in Fig. 3. The individual metabolic ratios displayed fourfold variation, ranging from 0.17 to 0.71 (mean=0.43, 95% CI: 0.41–0.45), and the values were normally distributed (W=0.99, P=0.89). There were no differences in 1U/(1U+1X) ratio between men and women (P=0.17; men: n=55, mean=0.46, 95% CI: 0.43–0.48; women: n=45, mean=0.43, 95% CI: 0.40–0.46), or between nonsmokers and smokers (P=0.14; nonsmokers: n=72, mean=0.44, 95% CI: 0.41–0.46; smokers: n=28, mean=0.47, 95% CI: 0.43–0.51).

Discussion

In the present study, we examined CYP2A6 and XO enzyme activities in the Serbian population. Furthermore,

Table 2 Comparisons of mean 17U/17X ratios between Serbian men and women, based on the smoking habit

	Men		Women		*P* (95% CI of the mean difference)
	Number	Mean (95% CI)	Number	Mean (95% CI)	
All	55	1.65 (1.43, 1.90)	45	1.65 (1.41, 1.95)	0.97 (−0.09, 0.09)
					0.93 (0.02, 40.84)
Nonsmokers	39	1.78 (1.50, 2.12)	33	1.77 (1.46, 2.16)	0.98 (−0.11, 0.11)
Smokers	16	1.36 (1.06, 1.75)	12	1.37 (1.01, 1.85)	0.99 (−0.16, 0.15)
					0.93 (0.02, 40.84)

we investigated *CYP2A6* genotype-phenotype correlation, as well as the influence of sex and cigarette smoking on CYP2A6 and XO enzyme activities. The results designated *CYP2A6* genotype and cigarette smoking as determinants of interindividual variability in CYP2A6 enzyme activity. To the best of our knowledge, this is the first study to investigate CYP2A6 and XO enzyme activity, as well as CYP2A6 genetic polymorphism, in Serbs, and to report the influence of cigarette smoking on enzyme activity using caffeine as a probe drug while controlling for the effect of *CYP2A6* genotype.

CYP2A6 plays an important role in the activation of many procarcinogens, including aflatoxin B_1, *N*-nitrosodiethylamine, and 1,3-butadiene, as well as in the clearance of some pharmaceuticals [1, 29]. As observed in other populations [4], log-transformed 17U/17X ratios in Serbs were normally distributed. The level of interindividual variations in CYP2A6 activity found in Serbs corresponds to Caucasian data [11], while larger variability up to more than 100-fold has been reported in other populations [4, 30, 31]. Disease conditions and other modifying factors may

Table 3 Allele and genotype frequencies of *CYP2A6* gene in the Serbian population

	Observed frequency	95% CI
Allele[a]		
*CYP2A6*1A*	0.579 (162/280)	0.520, 0.635
*CYP2A6*1B1*	0.307 (86/280)	0.256, 0.364
*CYP2A6*1B1x2*	0.004 (1/280)	0.000, 0.022
*CYP2A6*4*	0.029 (8/280)	0.014, 0.057
*CYP2A6*5*	0.000 (0/280)	0.000, 0.017
*CYP2A6*9*	0.082 (23/280)	0.055, 0.121
Genotype		
*CYP2A6*1A/*1A*	0.336 (47/140)	0.263, 0.418
*CYP2A6*1A/*1B1*	0.336 (47/140)	0.263, 0.418
*CYP2A6*1A/*1B1x2*	0.007 (1/140)	0.000, 0.044
*CYP2A6*1A/*4*	0.036 (5/140)	0.013, 0.084
*CYP2A6*1A/*9*	0.107 (15/140)	0.065, 0.171
*CYP2A6*1B1/*1B1*	0.114 (16/140)	0.071, 0.179
*CYP2A6*1B1/*4*	0.014 (2/140)	0.001, 0.055
*CYP2A6*1B1/*9*	0.036 (5/140)	0.013, 0.084
*CYP2A6*4/*9*	0.007 (1/140)	0.000, 0.044
*CYP2A6*9/*9*	0.007 (1/140)	0.000, 0.044

[a] *CYP2A6*1A* Wild type, *CYP2A6*1B1* gene conversion in 3′ region, *CYP2A6*1B1x2* gene conversion in 3′ region, gene duplication, *CYP2A6*4* gene deletion, *CYP2A6*5* 1436G>T, *CYP2A6*9* −48T>G

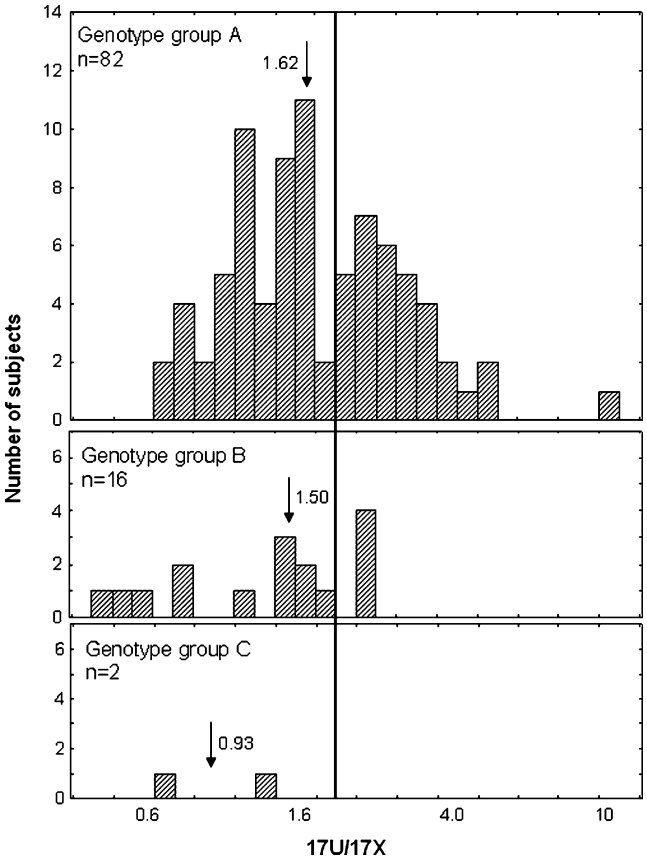

Fig. 2 Comparison of the frequency distribution of log-transformed 17U/17X ratio in different genotype groups of Serbs. The *arrows* indicate the medians and the *adjacent numbers* are antilog values. The *vertical line* is shown at an arbitrary antilog value of 2.0 as reference. Genotype group A: *CYP2A6*1A/*1A*, *CYP2A6*1A/*1B1*, *CYP2A6*1A/*1B1x2*, *CYP2A6*1B1/*1B1*; genotype group B: *CYP2A6*1A/*4*, *CYP2A6*1A/*9*, *CYP2A6*1B1/*4*, *CYP2A6*1B1/*9*; genotype group c: *CYP2A6*4/*9*, *CYP2A6*9/*9*

Table 4 Comparisons of mean 17U/17X ratios among different genotype groups[a] in Serbs, based on the smoking habit

	Group A		Group B		Group C	
	Number	Mean (95% CI)	Number	Mean (95% CI)	Number	Mean (95% CI)
All	82	1.75 (1.56–1.96)	16	1.31 (0.97–1.76)	2	0.93 (0.02–40.84)
Nonsmokers	58	1.89 (1.64–2.17)	14	1.39 (1.02–1.88)	0	-
Smokers	24	1.46 (1.22–1.75)	2	0.87 (0.00–1209.65)	2	0.93 (0.02–40.84)

[a] Group A: subjects carrying two functional alleles (*CYP2A6*1A/*1A*, *CYP2A6*1A/*1B1*, *CYP2A6*1A/*1B1x2*, and *CYP2A6*1B1/*1B1*); group B: subjects carrying one functional allele (*CYP2A6*1A/*4*, *CYP2A6*1A/*9*, *CYP2A6*1B1/*4*, and *CYP2A6*1B1/*9*); group C: subjects carrying no functional alleles (*CYP2A6*4/*9* and *CYP2A6*9/*9*

partly account for the observed wider interindividual variations in CYP2A6 activity [32]. Besides other nongenetic factors, *CYP2A6* genetic variation has been suggested to cause the interindividual variability in enzyme activity [33]. Carriers of gene deletion allele *CYP2A6*4* completely lack the CYP2A6 enzyme [2], while *CYP2A6*5* and *CYP2A6*9* both result in decreased enzymatic activity [1]. On the other hand, CYP2A6 activity in subjects having *CYP2A6* duplication, corresponding to the presence of three copies of the gene, was reported to be 1.4-fold higher than in wild-type homozygous [26, 27]. A gene conversion with *CYP2A7* in the 3′-untranslated region (3′-UTR), described as *CYP2A6*1B1* [34], is reported to be associated with either full [5, 34] or even increased [9] enzyme activity. In Serbs, the frequencies of described *CYP2A6* genetic variations, as well as of corresponding alleles and genotypes, were comparable with the results previously published for other Caucasians [27, 35, 36].

To investigate the influence of *CYP2A6* genotype on enzyme activity, subjects were grouped based on the number of functional variant alleles and the expected effect on phenotype [3]. Comparison of CYP2A6 enzyme activity among the different genotype groups showed significant differences. As expected, subjects homozygous for the functional alleles displayed higher enzyme activity than heterozygous subjects, while the lowest enzyme activity was observed in subjects homozygous for defective alleles. We further investigated genotype-phenotype correlation based on the cigarette smoking habit. In nonsmokers, CYP2A6 activity still tended to be higher among extensive metabolizers. However, we were not able to show the same effect in smokers due to the low number of subjects and hence sample power in this group.

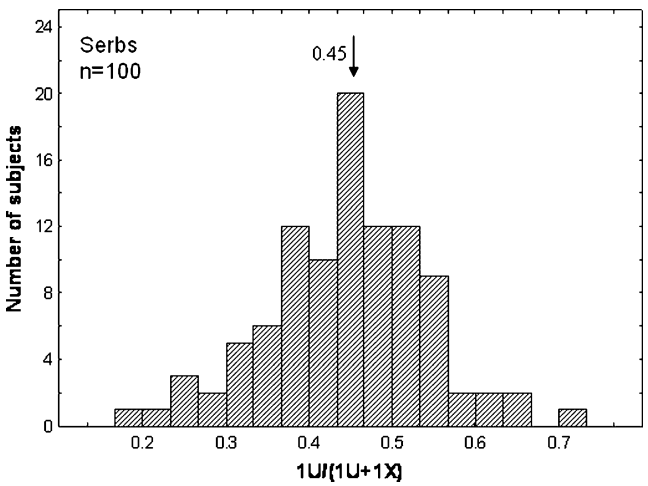

Fig. 3 The frequency distribution of the 1U/(1U+1X) ratio in Serbs. The *arrow* indicates the median and the *adjacent number* is the antilog value

Interestingly, in addition to *CYP2A6* genetic polymorphism, we observed significantly lower 17U/17X ratio in smokers compared to nonsmokers. It is known that cigarette smoking increases 17X formation by CYP1A2 induction [37–39]. In the present study, we showed that 17U/17X ratio, depicting CYP2A6 activity, was decreased in cigarette smokers. If metabolism of 17X to 17 U were highly saturable, we could assume that the reduction in the 17U/17X ratio in smokers was due to increased formation of 17X. However, as metabolism of 17X to 17U is not saturable in physiological conditions [40, 41], it is highly unlikely that CYP1A2 induction affects 17U/17X ratio in smokers. On the other hand, it has been suggested that CYP1A2 catalyzes metabolism of 17X to 17U to a minor extent [39]. The involvement of CYP1A2 in the formation of 17U from 17X is concentration dependent. At a lower concentration (0.1 mM) that may reflect the in vivo conditions, CYP2A6 selectively catalyzes the conversion of 17X to 17U [4]. Both in vitro and in vivo investigations demonstrated that CYP2A6 is the principal enzyme in the formation of 17U from 17X in humans [3]. In the present study, we observed a decreased 17U/17X ratio in cigarette smokers, indicating that CYP1A2 does not have a considerable role in 17X to 17U hydroxylation and hence cigarette smoking does influence CYP2A6 activity in Serbs. Since we observed significant genotype-phenotype correlation in the whole group, we further analyzed the effect of smoking habit on enzyme activity controlling for the effect of genotype. Among subjects carrying both functional alleles, significantly lower enzyme activity was

observed in smokers than in nonsmokers. In contrast, previous phenotyping studies using caffeine as a probe drug did not find influence of smoking status on CYP2A6 activity [4, 11, 12]. Thus, to the best of our knowledge, this is the first report of smoking-mediated inhibition of 17U formation from 17X in smokers. During the study period, subjects refrained from drinking coffee and other caffeine-containing beverages, but not from smoking. It is well known that nicotine is almost exclusively metabolized by the CYP2A6 enzyme [42]. Therefore, competitive inhibition by nicotine present in the cigarette smoke is likely to be responsible for the observed lower CYP2A6 enzyme activity. This finding might have clinical implications in patients treated with drugs metabolized by CYP2A6, such as halothane, valproic acid, and disulfiram [1, 29].

Most of the previous studies observed sex differences in CYP2A6 activity [4, 7–10]. Benowitz et al. [7], using plasma trans-3′-hydroxycotinine/cotinine ratio, reported faster nicotine metabolism in premenopausal (but not postmenopausal) women compared to men. At the same time, women taking oral contraceptives metabolized nicotine faster than those not taking oral contraceptives, suggesting the effect of sex hormones on CYP2A6 activity. Nevertheless, other studies reported higher 17U/17X ratios [4], as well as nicotine and cotinine clearances [10], in women compared to men regardless of oral contraceptive use. In the present study, women did not use oral contraceptives. Controlling for the effects of smoking, we did not detect any difference in CYP2A6 activity between men and women. Yet, this could be attributed to the small sample size. Similar to our result, some other groups also did not observe any sex difference using the urinary 17U/17X ratio [11] or 17U/(17U+17X+1U+1X+AFMU) ratio [12] as indices. Apparently, the influence of sex on CYP2A6 activity remains controversial, and future studies with more participants and higher sample power are needed to resolve this issue.

Xanthine oxidase is involved in a number of constitutional reactions of both physiological and pathological significance [17]. Furthermore, this enzyme participates in the metabolism of several clinically important drugs, such as acyclovir, mercaptopurine, azathioprine, methotrexate, doxorubicin, daunomycin, and mitomycin C [16]. Therefore, the variation in XO activity carries a potential for both lack of therapeutic effect and adverse drug reactions in treating seriously ill patients.

Several different populations have been investigated in terms of XO activity, using caffeine as a probe and either 1U/1X [14, 22, 43] or 1U/(1U+1X) urinary metabolic ratio [11, 18, 19, 44]. Some of the studies reported bimodal distribution of XO activity, suggesting the presence of poor metabolizers ranging from 4% of the population in Spaniards and Ethiopians [18, 19] up to 11% in Japanese [44]. However, in other populations, such as Danish [22] or Greek [11], XO activity appeared to be unimodally distributed. Our study clearly showed normal and unimodal distribution of XO activity, implying population homogeneity and the absence of Serbian subjects with low enzyme activity.

Because of the rarity of functional genetic variations in the XO gene [20], sex and cigarette smoking have been widely investigated as a possible determinants of XO activity. Most of the previous studies did not observe any correlation between sex and enzyme activity [19, 21, 22, 43, 44]. Opposite observations were presented by Relling et al. [14] and Guerciolini et al. [13], who reported higher and lower enzyme activity in women, respectively. On the other hand, it is generally accepted that cigarette smoking does not affect XO activity [19, 22, 43, 44]. In the present study, we did not detect any effect of sex or cigarette smoking on XO enzyme activity in Serbs. The results suggest that, in treating Serbian patients with drugs metabolized by xanthine oxidase, neither sex nor smoking habit needs to be considered as an important factor for dose adjustments.

In conclusion, Serbs display large interindividual variations in CYP2A6 activity. Lower CYP2A6 activity in smokers could be due to competitive inhibition of CYP2A6 by nicotine during cigarette smoking. *CYP2A6* genotype, but not sex, influenced CYP2A6 enzyme activity. Unimodal distribution of XO activity in Serbs implies the absence of subjects with low enzyme activity in this population. XO activity was not influenced by sex or cigarette smoking.

Acknowledgments The authors would like to thank all volunteers who participated in the study. We are very grateful to Lilleba Bohman for technical support in the laboratory. The study was financially supported by the Swedish Research Council, Medicine, 3902; the Medical Faculty, University of Kragujevac, Republic of Serbia, JP 1/05; the Swedish Institute; Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica (I+D+I), Instituto de Salud Carlos III, Subdirección General de Evaluación y Fomento de la Investigación, PI071152; and Ayudas para la consolidación y apoyo a grupos de investigación de Extremadura, GRU09015 (Orden de 17 de diciembre de 2008, DOE 5 de enero de 2009). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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