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Analysis of urinary caffeine metabolites to assess biotransformation enzyme activities by reversed-phase high-performance liquid chromatography

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Abstract

An isocratic high-performance liquid chromatography procedure was developed for the analysis of five urinary metabolites of caffeine; caffeine or 1,3,7-trimethylxanthine (137X), paraxanthine or 1,7-dimethylxanthine (17X), 1,7-dimethylurate (17U), 1-methylxanthine (1X), 1-methylurate (1U) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU). A standardized procedure was used for oral intake of caffeine and for urine collection. Conditions for sample storage and preparation were optimized, resulting in no detectable loss of caffeine metabolites after storage of the urine samples for four months. Urine samples were extracted with chloroform–2-propanol (4:1, v/v) and separated on a reversed-phase column with acetic acid (33%)–tetrahydrofuran–acetonitrile–water (1:2.5:44:925.5, v/v) as the eluent. Peaks were monitored at 280 nm. Peak heights were measured and the five metabolites were quantified using calibration curves. Cytochrome P4501A2 (CYP1A2) activity was calculated from the molar ratio (AFMU+1X+1U)/17U, *N*-acetyltransferase (NAT) from the ratio AFMU/1X, XO from the ratio 1U/1X+1U and cytochrome P4502A6 (CYP2A6) from the ratio 17U/(17U+17X+1U+1X+AFMU). The inter-assay coefficients of variation ranged from 1.7% for 17U to 5.7% for 1X. The intra-individual variation in metabolite ratios determined in two people, with intervals of a few days to several weeks between measurements, ranged from 2.1% for XO to 11.0% for CYP2A6. Using this procedure, metabolic ratios were determined for four groups of subjects; healthy, non-smoking females using oral contraceptives (OC users, $n=5$) and non-users ($n=5$), healthy non-smoking males ($n=9$) and children ($n=7$). Results found in this study were comparable to results reported in the literature for subjects with similar characteristics. A significantly higher CYP1A2 ratio was found for males (4.87 ± 0.47) compared to females (3.62 ± 0.91; $p=0.005$, Mann-Whitney). For the other enzyme activities, no significant differences were found between the groups of subjects in this study.

Keywords: Enzymes; Caffeine; Paraxanthine; 1,7-Dimethylurate; 1-Methylxanthine; 1-Methylurate; 5-Acetylamino-6-formylamino-3-methyluracil

1. Introduction

Caffeine, 1,3,7-trimethylxanthine (137X), is used world-wide as a drug and has been applied as a probe for the assessment of the phenotype of several liver biotransformation enzymes [1,2]. As a metabolic probe for liver biotransformation enzymes, caffeine has several important advantages, namely, its rapid and complete absorption, its distribution throughout the total body water, its low plasma

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protein binding, its complete biotransformation in the liver (short half life, $t_{1/2}$) and its renal excretion is negligible. In addition, caffeine is a relatively safe and known drug for human subjects [3]. In the human body, thirteen different metabolites of caffeine have been identified. First, caffeine undergoes three oxidative N-demethylations, mostly 3-demethylation (84%) to form 1,7-dimethylxanthine (17X) [4]. The metabolite 17X is further metabolized by three reactions; 8-hydroxylation by cytochrome P4502A6 (CYP2A6) to form 1,7-dimethylurate (17U), 7-demethylation by cytochrome P4501A2 (CYP1A2) to form 1-methylxanthine (1X) and, thirdly, the formation of the open ring product 5-acetylamino-6-formylamino-3-methyluracil (AFMU), which is catalyzed by *N*-acetyltransferase (NAT). AFMU is an unstable product that may be deformylated nonenzymatically to 5-acetylamino-6-amino-3-methyluracil (AAMU). The 8-hydroxylation of 17X to form 17U is mainly metabolized by the polymorphic enzyme CYP2A6 (90%) and about 10% is metabolized by CYP1A2. Part of 1X is metabolized to 1-methylurate (1U) by xanthine oxidase (XO) [5]. In Fig. 1, the main pathways of caffeine metabolism are shown.

Molar ratios of the different caffeine metabolites

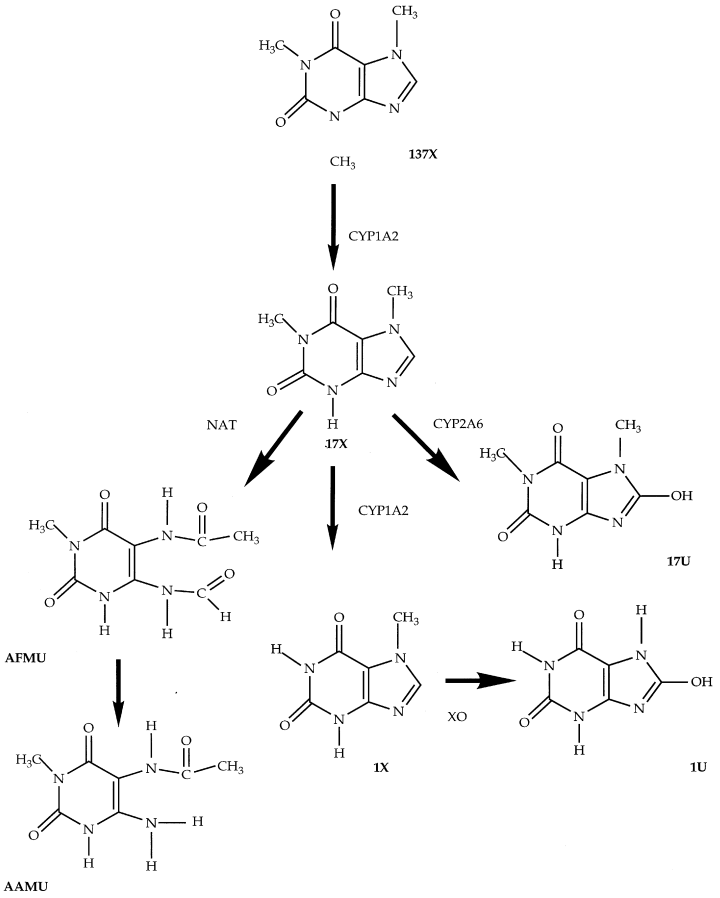

Fig. 1. Metabolism of caffeine by liver biotransformation enzymes. Caffeine, 1,3,7-trimethylxanthine (137X); paraxanthine or 1,7-dimethylxanthine (17X); 1,7-dimethylurate (17U); 1-methylxanthine (1X); 1-methylurate (1U); 5-acetylamino-6-formylamino-3-methyluracil (AFMU); 5-acetylamino-6-amino-3-methyluracil (AAMU); cytochrome P4501A2 (CYP1A2); *N*-acetyltransferase (NAT); xanthine oxidase (XO); cytochrome P4502A6 (CYP2A6).

have been used to assess the metaboliser phenotype of CYP1A2, NAT2, XO and CYP2A6 [4,6,7]. CYP1A2 activity can be assessed by the ratio (AFMU+1X+1U)/17U. Although other metabolite ratios for the estimation of CYP1A2 activity have been applied, the ratio of (AFMU+1X+1U)/17U has been shown not to be influenced by renal clearance [7]. NAT activity has been assessed from the ratio AFMU/1X, XO from the ratio 1U/1X+1U and CYP2A6 or 8-hydroxylase activity from the ratio 17U/(17U+17X+1U+1X+AFMU) [6,7]. These enzymes are involved in the activation and/or detoxification of various xenobiotic compounds, including carcinogens. The activities of these enzymes are genetically determined, but environmental factors, diet, alcohol, exercise, smoking and exercise have been reported to modify the activities of biotransformation enzymes [8,9]. Assessment of the metabolic phenotype may be of relevance in determining whether an individual may be expected to develop adverse reactions to certain drugs or is at risk of developing specific exposure-related diseases [10]. For example, CYP1A2 and NAT may activate procarcinogens into ultimate carcinogens, such as aromatic amines. Consequently, individuals with an extensive metabolizer phenotype for substrates of CYP1A2 are considered to be at risk of developing bladder or colorectal cancers, due to exposure to arylamines or heterocyclic amines present in cooked food [11,12]. Slow acetylators are expected to be at higher risk for heterocyclic amine-induced bladder cancer than are fast acetylators [13].

Several high-performance liquid chromatography (HPLC) procedures have been published that apply either gradient elution [2,12] or two separate procedures, one for the analysis of AFMU after conversion to its deformylated form, 5-acetylamino-6-amino-3-methyluracil (AAMU), and one for the analysis of the other metabolites [14]. Based on the HPLC methods of Grant et al. [1] and Carillo and Benitez [5], we developed a simple procedure for the determination of five caffeine metabolites, which enables the assessment of the above-mentioned enzyme activities in a single HPLC run with isocratic elution and UV detection. Urine sampling, storage and extraction procedures were optimized. The intra-individual variability of the enzyme activities was evaluated in eleven subjects who performed the caffeine test on different occasions with intervals varying from one day to several weeks. Using this modified procedure, enzyme phenotypes were determined in healthy, non-smoking subjects comprising females who used oral contraceptives (OC) and those that did not, males and children.

2. Experimental

2.1. Chemicals

1-Methylxanthine (1X), 1-methyluric acid (1U), 1,7 dimethylxanthine (17X), 1,7-dimethyluric acid (17U), *N*-acetaminophen and ammonium sulfate were purchased from Sigma (St. Louis, MO, USA). 5-Acetyl-6-formylamino-3-uracil (AFMU; purity >98%) was a gift from Dr. M.J. Arnaud and G. Philippossain, Nestec, Nestlé Research Centre, Lausanne, Switzerland. Tetrahydrofuran and acetonitrile were obtained from Biosolve (Barneveld, Netherlands). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Experimental protocol

The subjects were instructed to refrain from consumption of products containing methylxanthine, such as coffee, tea, cola and chocolate for 24 h before the test and for the duration of the test. No differences in the ratios of urinary caffeine metabolites were observed between samples from the same subject that were obtained after caffeine-free periods of 16 and 24 h. The subjects collected urine samples in the morning as a blank and drank one cup of coffee or two cups of very strong tea. The children drank a cola beverage (330 ml). The dose of caffeine for adults was between 80–140 mg and for the children, it was about 35 mg. Urine samples were not collected from the subjects during the first 4 h following ingestion of caffeine. At $t=5$ h after ingestion of the caffeine, urine samples were collected. Following collection, 100 mg of ascorbic acid (as an antioxidant) were added to urine samples (15 ml), which were then immediately stored at −20°C until analysis.

2.3. Analytical procedures

Various parameters, namely, the addition of ascorbic acid, adjustment of pH and the ratio of chloroform–isopropanol of the extraction solution, were evaluated to optimize the yield of caffeine metabolites. The definitive procedure was as follows: Urine samples were rapidly thawed, adjusted to pH 3.5 with 1 *M* HCl and kept on ice. After the addition of 120 mg of ammonium sulphate, a 200-μl volume of urine and 200 μl of the internal standard (I.S.), *N*-acetaminophen (150 mg/l), were placed in a 10-ml glass tube and vortex-mixed briefly. The samples were extracted with 6 ml of chloroform–isopropanol (4:1, v/v). After centrifugation for 5 min at 1000 *g*, the organic phase was removed, evaporated under a gentle stream of nitrogen at 40°C and dissolved in 1 ml of 0.05% acetic acid. A 20-μl aliquot was injected onto the HPLC column. All urine samples were analysed in duplicate. Urine collected shortly before caffeine consumption was analysed for caffeine metabolites or for possible interference by endogenous metabolites.

The chromatography system consisted of a Spectroflow 400 solvent delivery system, obtained from Separations Analytical Instruments (Hendrik Ido Ambacht, Netherlands), a Spectroflow 480 sample injector equipped with a 20-μl injection loop, from Separations Analytical Instruments, and a UV–Vis SPD-6AV Shimadzu detector obtained from Shimadzu ('s Hertogenbosch, Netherlands). The caffeine metabolites were separated on a Spherisorb S5 ODS2 column (25 cm×4.6 mm I.D.; particle size, 5 μm) from Phase Separations (Deeside, UK) at a flow-rate of 1 ml/min, with a mobile phase of acetic acid (33%)–tetrahydrofuran–acetonitrile–milli-Q water (1:2.5:44:952.5, v/v). The column effluent was monitored at 280 nm at 0.005 AUFS.

The following metabolites were measured: AFMU, 1U, 1X, I.S., 17U and 17X. Caffeine itself was not detected in the urine samples. The following biotransformation enzyme activities were calculated from the metabolites as follows: CYP1A2 activity from the ratio (AFMU+1X+1U)/17U, NAT activity from the ratio AFMU/1X, XO activity from the ratio 1U/1X+1U and CYP2A6 activity from the ratio 17U/(17U+17X+1U+1X+AFMU) [6,7]. Calibration curves of the five caffeine metabolites were made in the range of 59 ng/ml to 50 μg/ml. The metabolites 17X and 1U were dissolved in milli-Q water to which a drop of 1 *M* sodium hydroxide (NaOH) was added to facilitate dissolution. Stock solutions of 17U, 17X, 1U and 1X were kept at −20°C in the presence of ascorbic acid. AFMU was dissolved in a mixture of dimethylformamide and ethyl acetate (1:20, v/v) and it was also stored at −20°C. The metabolites were diluted to their final concentration with 0.05% acetic acid for preparation of the calibration curves. Since we were unable to obtain urine samples that were free of caffeine metabolites, even after the subjects had abstained from caffeine-containing food and drink for 36 h, calibration curves were made from metabolites in 0.05% acetic acid and extracted as described above for urine samples. Recovery of the metabolites was determined from water as well as from urine samples and was found to be >90%. The calibration curves obtained from the measurements of peak height ratios of analyte versus I.S. were linear over the concentration range applied, yielding correlation coefficients of between 0.994 and 1.000. In each series of analyses of urine extracts, a mixture of standard metabolites was also included.

2.4. Subjects

To determine the intra-individual variability, the caffeine test was performed on one female (using OC) and one male subject on three different days, with intervals of several weeks and days, respectively. In addition, the caffeine test was performed on nine male subjects on two separate days with a one-week interval.

The caffeine test was also performed with the following groups of subjects:

(1) Ten healthy, non smoking, female individuals (mean age, 25.8 years; range 21–34 years). The volunteers had no prior histories of liver and/or kidney disease and did not use medication, except for five women who were using OC.

(2) Nine healthy male individuals (mean age, 28 years; range 23–46 years), mean body weight, 72.7 kg (64–80 kg), non-smokers, moderate use of alcohol (<three units/day), no use of medication and mean physical exercise of 4 h/week were recruited. Seven healthy children (mean age, 12.3 years; range

seven–sixteen years; two males and four females) also performed the caffeine test with 330 ml of cola.

2.5. Statistics

Results are expressed as mean±S.E.M. for the different groups of subjects. The Mann-Whitney test for nonparametric, unpaired values was applied to evaluate the statistical significance of the observed differences.

3. Results

Before the definitive protocol was set up, the influence of the addition of ascorbic acid before storage, adjustment of the pH of the urine samples and the composition of the extraction mixture on the stability and extraction yield of the caffeine metabolites was evaluated. The addition of ascorbic acid to the urine before storage and adjustment of the pH to 3.5 with 1 *M* HCl were found to give the highest yield for all caffeine metabolites (data not shown). AFMU is considered to be unstable and may spontaneously deformylate to AAMU at neutral or basic pH values in the bladder. Therefore, complete conversion of AFMU to AAMU is often used to determine the concentrations of AFMU. However, when the urine was adjusted to pH 10 in vitro, we observed that AFMU was not completely converted to AAMU, but other metabolites or degradation products of AFMU could not be detected with our analytical procedure. For this reason, a hydrolysis step was omitted and the urine samples were stabilised by the addition of ascorbic acid, to determine AFMU for calculation of the different metabolic ratios. Under the conditions described in our procedure, no substantial decline in the levels AFMU was detected during storage at −20°C for up to four months, as well as during sample preparation and analysis. The urine was extracted with a mixture of chloroform–isopropanol at different ratios (9:1, 4:1 and 3:1, v/v). The optimal extraction mixture for recovery of caffeine metabolites was found to be chloroform–isopropanol (4:1, v/v). No differences in the ratios of metabolites were found when urine samples were extracted once or twice with chloroform–isopropanol (4:1, v/v). All caffeine metabolites could be accurately quantified at the lowest concentration studied, i.e., 59 ng/ml.

In Fig. 2, chromatograms of the five metabolites of caffeine that were analysed with our HPLC procedure are shown. Good separation was obtained with the standard mixture as well as with urine extracts (Fig. 3A). In Fig. 3B, a chromatogram of a urine sample after a methylxanthine-free period of 24 h is shown. We found that the caffeine-free period had to be a minimum of 16 h in order to obtain a control urine sample that was almost free of caffeine metabolites and that was not influenced by previous caffeine consumption. However, urine completely free of caffeine metabolites was not found, even after subjects had refrained from ingesting methylxanthine-containing products for three days.

Based on duplicate analyses, the intra-assay variation observed for AFMU was 8.3%, for 1U, it was 3.5%, for 1X, it was 8.6% and for 17U, it was 4.6%. Based on four series of experiments, an inter-assay variation of 5.2% was found for AFMU, 4.3% for 1U, 5.7% for 1X, and 1.7% for 17U. The coefficient of variation for three repeated assessments of the metabolic ratios ranged from 2.9% for CYP2A6 to 11.0% for NAT in the female subject, and for the male subject, the ratios ranged from 2.1% for XO to 9.0% for CYP1A2. For the nine male subjects that performed the caffeine test on two days, with a

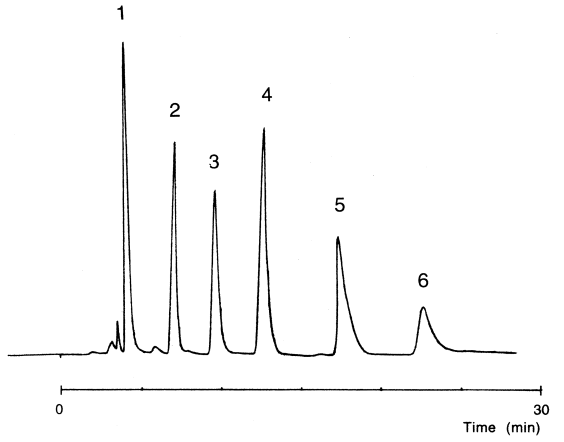

Fig. 2. HPLC chromatogram of a standard mixture containing 37.5 μg/ml of the five caffeine metabolites and the internal standard; the detector was set at 0.005 AUFS, and the recorder was set at 100 mV. 1=AFMU; 2=1U; 3=1X; 4=internal standard; 5=17U and 6=17X.

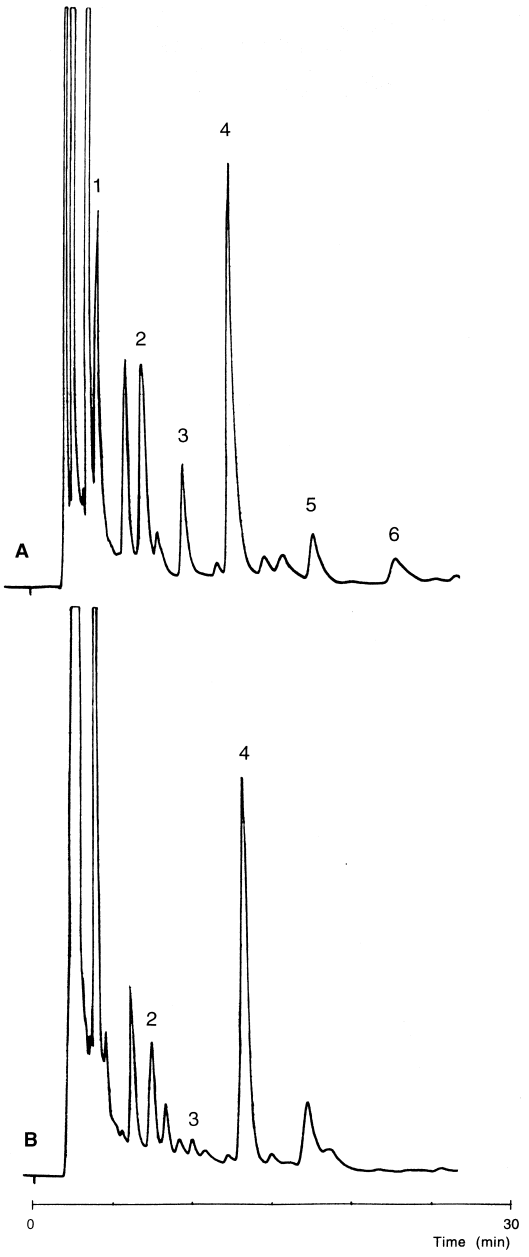

Fig. 3. Caffeine metabolites in a urine sample of a male subject (A) and a urine sample after a methylxanthine-free period of 24 h (B). The detector was set at 0.005 AUFS and the recorder at 50 mV. 1=AFMU; 2=1U; 3=1X; 4=internal standard; 5=17U and 6=17X.

one-week interval, the intra-individual variabilities were found to be 3.1, 10.2, 0.8 and 2.3%, respectively, for CYP1A2, NAT, XO and CYP2A6 ratios.

The mean ratios of the different enzyme activities for the different groups of subjects are presented in Table 1. None of the metabolic ratios of the OC users was found to be significantly different from the enzyme activities of non-OC users. The CYP1A2 activity of males, 4.87 ± 1.38 ($n=9$), was found to be significantly higher than the CYP1A2 activity of females, 3.62 ± 0.91 ($n=10$) ($p=0.005$, Mann-Whitney). No significant difference in the CYP1A2 ratio was observed between children and adults (males and females not using OC). None of the other metabolic ratios was significantly different between males, females and children. No correlation was found between age of the children and the four enzyme ratios. A strong negative correlation was observed, however, between metabolite ratios for CYP1A2 and CYP2A6; the coefficients of correlation between these ratios for males, females and children were 0.87, 0.90 and 0.82, respectively ($p=$ 0.007, 0.006 and 0.048, respectively; Spearman rank correlation coefficient). No significant associations were found between other enzyme ratios.

4. Discussion

Based on the HPLC methods of Grant et al. [1] and Carrillo and Benitez [5], a modified HPLC procedure was developed with optimization of the sampling and extraction procedures that enabled the analysis of five urinary caffeine metabolites. The modified method is simple and fast, because all caffeine metabolites required for the assessment of four different metabolic ratios can be detected in one HPLC run. Although environmental factors have been reported to influence CYP1A2 and, possibly, other enzyme activities, the intra-individual variations for NAT and CYP1A2 found in this study were relatively low and were comparable to variations found in other studies. Nevertheless, other research groups reported significantly higher intra-individual variation. McQuilkin et al. [12] observed an intra-individual variation 13.5% (range, 6.9 to 34.7%) for NAT in females ($n=9$), and a mean variation of 19.4% (range, 3.3 to 31.4%) for males ($n=9$). Butler et al. [2] reported an intra-individual variation for CYP1A2 that ranged from 12.9 to 48.0%.

The metabolic ratios used in our study have been

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

Enzyme	Females (non OC users) (n=5)	Females (OC users) (n=5)	Males (n=9)	Children (n=7)
NAT	2.01±0.61 (0.34–3.81)	2.41±0.95 (0.59–4.75)	1.59±0.32 (0.21–3.12)	1.57±0.60 (0.00–4.21)
CYP1A2	3.93±0.35 (2.58–4.48)	3.32±0.46 (1.62–4.42)	4.87±0.47 (2.79–7.55)	5.18±0.40 (3.85–7.15)
XO	0.75±0.02 (0.71–0.79)	0.79±0.04 (0.72–0.93)	0.74±0.03 (0.63–0.88)	0.72±0.02 (0.61–0.83)
CYP2A6	0.18±0.01 (0.16–0.23)	0.22±0.03 (0.17–0.36)	0.16±0.01 (0.11–0.23)	0.14±0.01 (0.11–0.19)

N-Acetyltransferase was assessed by the ratio AFMU/1X; cytochrome P4501A2 by (AFMU+1X+1U)/17U; xanthine oxidase by 1U/(1U+1X) and cytochrome P4502A6 by 17U/(17U+17X+1U+1X+AFMU).
Results are presented as mean±S.E.M. and the range is given in brackets

selected based on technical reasons and reproducibility. Although analysis of AFMU has been reported to lead to an underestimation of the CYP1A2 metabolic ratio [15], in our laboratory, no reproducible conversion of AFMU into AAMU could be achieved. In fact, after hydrolysis, the peak for AFMU disappeared, with no detectable peak emerging for AAMU. In spite of a possible underestimation of the metabolic ratio we used for CYP1A2 in our study (AFMU+1X+1U/17U), we found relatively small intra-individual variations that ranged from 3.1 to 9.0%, indicating that possible loss of AFMU during sample preparation and analysis did not result in substantial intra-individual variation for the CYP1A2 metabolic ratio. Other ratios, such as the ratio 17X+17U/caffeine, have been used to assess CYP1A2 activity, but these have been reported to be influenced by urine flow [7,15]. In addition, it has been reported that calculation of NAT activity with better reproducibility and less chance of misclassification could be achieved from the ratio AFMU/(AFMU+1X+1U) [4], however, when we compared both ratios, the ratio AFMU/1X was found to yield smaller intra-individual coefficients of variation and to have a better reproducibility. The metabolic ratio of NAT (AFMU/1X) has also been reported to be influenced by XO activity [9], however, in our study, no association could be detected between both ratios. However, we did find a negative association between CYP2A6 and CYP1A2 metabolic ratios, which can be explained by the fact that the denominator of the CYP1A2 metabolic ratio is the same as the numerator of the CYP2A6 ratio.

The mean CYP1A2 ratios for males, females and females using OC found in our study were comparable to the observations of other groups using the same metabolite ratios for calculation of biotransformation enzyme activities but with different HPLC procedures [8,9,16,17]. The significant difference observed in CYP1A2 ratios between males and females in our study appeared to be due to females using OC. When OC users were excluded, the difference between males and females was less pronounced and no longer statistically significant. After exclusion of one parous non-OC user with a low CYP1A2 ratio (2.58±0.12), a weakly statistically significantly lower CYP1A2 ratio was observed for females using OC compared to non-users in our study (p=0.06), which is in agreement with other studies that also reported a lower CYP1A2 ratio in OC users [8,15,16]. Although the CYP1A2 activity was recently reported to be relatively low in nulliparous, never breast-fed females, pregnant females and females using OC compared to parous females [17], in our study, the CYP1A2 ratio of one parous woman, who had breast fed and was not using OC, was found to be lower (2.58±0.12) than the CYP1A2 ratios of the four remaining nulliparous, non-OC users (4.3±0.1). More parous women will have to be studied, to clarify the possible postpartum changes in CYP1A2 activity. In addition to hormonal status, various life-style and environmental factors have been found to influence CYP1A2 activity [8,9]. For NAT and XO activity, differences between races have also been reported [9].

The CYP1A2 metabolic ratio has also been reported to be dependent on age. Children under the age of fourteen were reported to have higher

CYP1A2 metabolic ratios when compared to adults [4,8,16–18]. Growth hormones, estrogen and progestrogen have been reported to inhibit the activity of different cytochrome P450 enzymes, such as CYP1A2 [18]. Although children had higher CYP1A2 ratios, in our study, no significant difference was observed between CYP1A2 ratios of adults not using medication (4.40 ± 0.29) and the CYP1A2 ratios of children (5.18 ± 0.4). However, there was a tendency towards increased CYP1A2 ratios for children, which may become statistically significant when more subjects are studied.

A potential limitation of this procedure to assess biotransformation activities is that too low concentrations of caffeine metabolites are present in the urine samples of some subjects, thereby impeding the assessment of some enzyme activities. In those subjects, caffeine might be metabolized by minor pathways, resulting in metabolites that cannot be detected with our procedure. Advantages of our procedure are that all metabolites for the assessment of four different biotransformation enzyme activities in urine samples can be measured in one HPLC run within 30 min. Consequently, it is considered to be applicable in molecular epidemiologic studies to assess inter- and intra-individual variation in activation and/or detoxification of potentially toxic compounds, carcinogens and drugs in adults, as well as in children.

5. Abbreviations

137X	caffeine, 1,3,7-trimethylxanthine
17X	paraxanthine 1,7-dimethylxanthine
17U	1,7-dimethylurate
1X	1-methylxanthine
1U	1-methylurate
AFMU	5-acetylamino-6-formylamino-3-methyluracil
(CYP1A2)	cytochrome P450 1A2
NAT	*N*-acetyltransferase
(XO)	xanthine oxidase
CYP2A6	cytochrome P450 2A6

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