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Analysis of urinary caffeine metabolites by HPLC-DAD: the use of metabolic ratios to assess CYP1A2 enzyme activity

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Abstract

An improved extraction procedure and a new RP-HPLC method were developed for selective and rapid analysis of caffeine and 14 of its metabolites in urine. Analytes were isolated by solid-phase extraction and separated on an Eclipse XDB-C18 column. Recoveries ranged between 83 and 99%. Precision, linearity and accuracy of the chromatographic method were found to be within required limits. Using this procedure, caffeine metabolic ratios were determined in 20 subjects with characteristic CYP1A2 activities, relatively to smoking habit and contraceptives intake. The method might be useful to point out induction and inhibition of CYP1A2 activity. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Caffeine; Caffeine metabolites ratios; Phenotyping; CYP1A2

1. Introduction

Given the involvement of CYP1A2 (isoform of the CYP1A cytochrome P450 subfamily) in drug metabolism and its potentially important role in the induction of chemical carcinogenesis, there has been a real interest in the identification of substrates which can be used as in vivo probes for assessing the activity of this enzyme in humans. Caffeine, 1,3,7-trimethylxanthine (137X), is used world-wide in many kind of beverages as well as a drug and has been applied as a metabolic probe for determining CYP1A2 activity. This enzyme is involved in the liver biotransformation of many drugs and xenobiotics [1–3].

As shown in Fig. 1, CYP1A2 catalyses the N-1, N-3 and N-7 demethylations of caffeine (137X) to form theobromine (37X), paraxanthine (17X) and theophylline (13X), respectively, accounting for about 80, 11 and 4% of caffeine metabolism [4]. Each of these xanthines undergoes a N-monodimethylation reaction to give the corresponding monomethylxanthine, 1-methylxanthine (1X), 3-methylxanthine (3X) and 7-methylxanthine (7X). Caffeine and xanthines are hydroxy-

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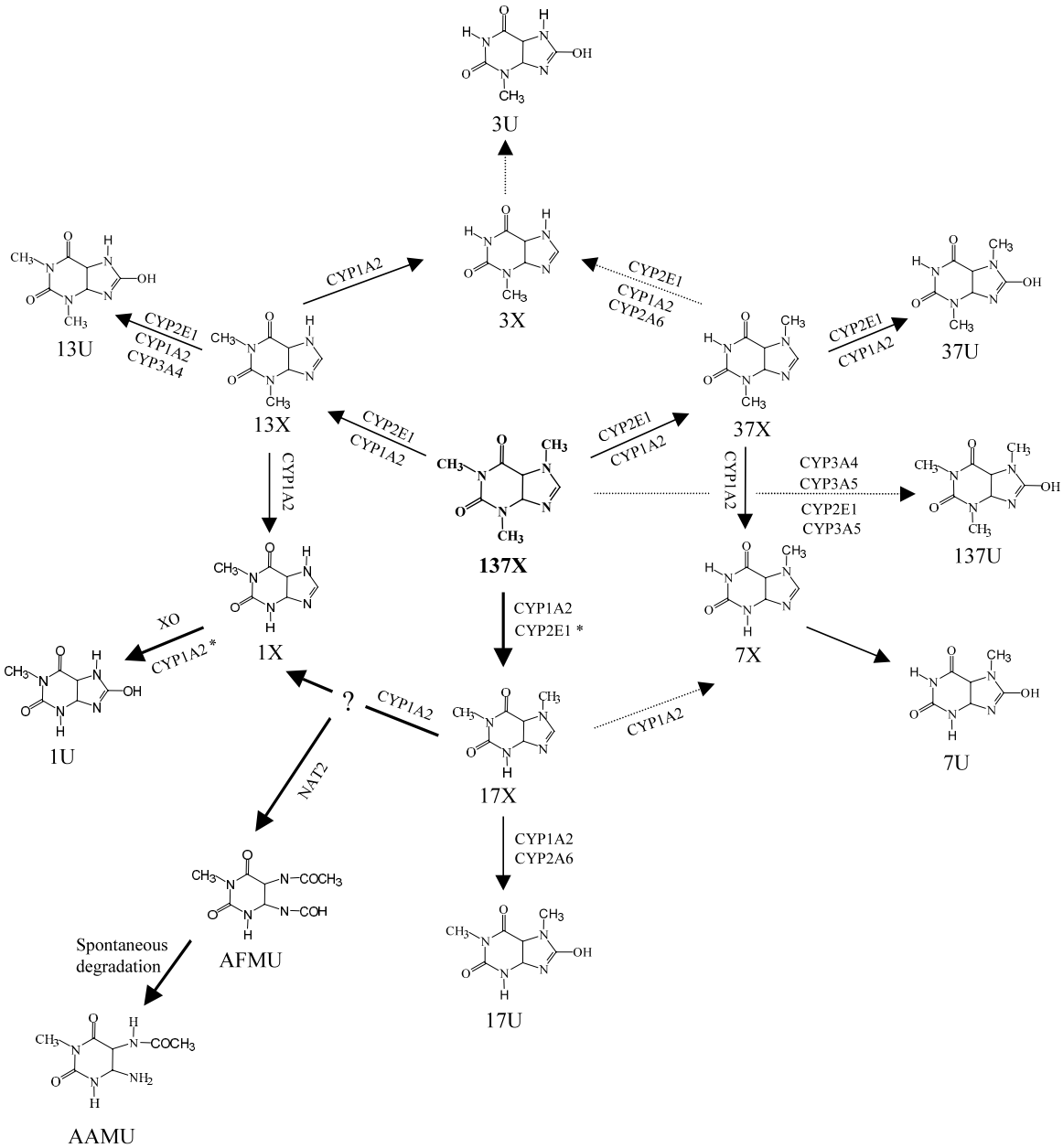

Fig. 1. Caffeine metabolism pathways in human liver. Bold arrows point out main pathways and dotted arrows mark minor pathways, which do not contribute to any of the ratios used to mark CYP1A2 or NAT2 activity. Symbols beside arrows indicate enzymes: NAT2, *N*-acetyltransferase; XO, xanthine oxidase. 137X is caffeine or 1,3,7-trimethylxanthine; 13X is theophylline or 1,3-dimethylxanthine; 17X is paraxanthine or 1,7-dimethylxanthine; 37X is theobromine or 3,7-dimethylxanthine; 1X is 1-methylxanthine; 3X is 3-methylxanthine; 7X is 7-methylxanthine; 137U is 1,3,7-trimethyluric acid; 13U is 1,3-dimethyluric acid; 17U is 1,7-dimethyluric acid; 37U is 3,7-dimethyluric acid; 1U is 1-methyluric acid; 3U is 3-methyluric acid; 7U is 7-methyluric acid; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; AAMU, 5-acetylamino-6-formylamino-3-methyluracil.

lated into their corresponding uric acids, 1,3,7-trimethyluric acid (137U), 1,3-dimethyluric acid (13U); 1,7-dimethyluric acid (17U); 3,7-dimethyluric acid (37U); 1-methyluric acid (1U); 3-methyluric acid (3U) and is 7-methyluric acid (7U). Other non-microsomal enzymes participate in caffeine metabolism [5]. The polymorphic *N*-acetyltransferase, NAT2, catalyses the C8–N9 bond scission and the acetylation of paraxanthine to produce 5-acetylamino-6-formylamino-3-methyluracil (AFMU) which is then spontaneously transformed into 5-acetylamino-6-amino-3-methyluracil (AAMU) in urine. Xanthine oxidase (XO) is responsible for the conversion of 1X into 1U.

Several chromatographic methods, especially HPLC methods, have been reported for determining caffeine and its metabolites in human biological fluids. Some of these methods focused on the determination of caffeine and several methylxanthines [6–8] while others separated and identified few xanthines and uric acids [9,10]. Furthermore, these methods used a liquid-phase extraction. Solid-phase extraction (SPE) is progressively substituting for liquid–liquid extraction, because it is more easy and solvent saving. However, literature reports assays that do not extract more than six or seven metabolites, generally of the xanthines group [6,11,12].

A new method using a solid-phase extraction from urine and able to analyse and quantify caffeine and 14 of its metabolites by reversed phase liquid chromatography-diode array detector (RP-HPLC-DAD) is reported. The method has been applied to the measurement of caffeine and its metabolites in urine samples taken from 20 healthy subjects, men and women, smokers (S) and non-smokers (NS), and women taking or not taking oral contraceptives (OC).

Molar ratios of the different caffeine metabolites have been calculated to assess the characteristic CYP1A2 activity of these groups of subjects. Among the various metabolic ratios reported in the literature, the following ratios were chosen as being not influenced by renal clearance and not affected by the contribution of the other enzymes involved in the caffeine metabolism, (17X + 17U)/137X; (13X + 17X + 37X)/137X; (3X + 13U + 13X)/137X and (7X + 37U + 37X)/137X [3,9].

2. Materials and methods

2.1. Chemicals

Caffeine, its xanthine and uric acid derivatives and β-hydroxyethyltheophylline (βHT) were obtained from Sigma-Aldrich Ltd (Oakville, Ont., Canada). Methanol, tetrahydrofuran (THF) and acetonitrile were purchased from V.W.R. Scientific Ltee (Montreal, Que., Canada). Glacial acetic acid and water HPLC reagent were from Moquin scientific Inc (Lachenaie, Que., Canada). Sodium hydroxide, hydrochloric acid, sodium acetate and sodium chloride were from Sigma-Aldrich Ltd (Oakville, Ont., Canada).

Solvents and chemicals were all of analytical grade.

2.2. Calibration and reagent solutions

Individual stock solutions containing 1 mg ml^{-1} of methylxanthines and uric acids were prepared in water/methanol (70:30, v/v). Metabolites needed addition of 1 N sodium hydroxide for complete dissolution, except for 13X, 17X, 137X and 137U. As 1U and casually 3U have been found to be unstable in basic or acidic solvent, in particular at room temperature, basic solutions were buffered at pH 7 with 1 N hydrochloric acid. A standard solution containing 100 μg ml^{-1} of all the metabolites was prepared. This standard mixture was diluted to 1, 10, 20 and 40 μg ml^{-1} with blank urine. Since it was really difficult to obtain urine free of all xanthines and uric acids, quantitative urine controls (Fisher Scientific, Montreal, Canada) were used for blank urine samples.

β-hydroxyethyltheophylline was chosen as internal standard because it is an analogue of theophylline which has chemical properties similar to those of caffeine metabolites. A stock solution containing 0.2 mg l^{-1} of β-hydroxyethyltheophylline was prepared in water/methanol (70:30, v/v). The concentration of the internal standard was set at 20 μg ml^{-1} in the blank urine. Solutions were kept stable when stored at 4 °C for several months.

2.3. Instrumentation and chromatographic conditions

An HPLC-DAD system, series 1100, from Hewlett-Packard (Kirkland, Que., Canada) was used. The chromatographic system consisted of a vacuum degasser on line with a binary pump linked to a thermostatted column compartment via an autosampler. The detection was carried out using a diode array UV detector (200–400 nm). Metabolites were separated on an Eclipse XDB-C18 reversed phase column (250 × 4.6 mm, 5 μm particle size) coupled with a Eclipse XDB-C18 guard column (12.5 × 4.6 mm, 5 μm particle size), from Hewlett-Packard (Kirkland, Que., Canada). The mobile phase A was a mixture of water/acetic acid/THF (996.5:1:2.5, v/v/v) (pH 3.3) and the mobile phase B was acetonitrile [6]. Both of them were filtered through a 0.45 μm membrane from Gelma Sciences (Ann Arbor, MI, USA). The elution program was chosen as follows, B was set at 3.5% for 11 min, then increase linearly to 12% from 11 to 18 min and then held at 12% for 7 min; with a post run of 10 min in order to equilibrate the column between injections. The flow rate was set at 1 ml min^{-1}. The column compartment was kept at a controlled temperature of 23 ± 0.5 °C. The pressure was 11 000 kPa. Compounds were detected at their maximum absorbance wavelength, 270 nm for xanthines and the internal standard and 290 nm for uric acids.

2.4. Sample preparation: solid phase extraction (SPE)

Before treatment, urine samples were filtered through a 0.45 μm nylon membrane from Millipore (Neapan, Ont., Canada). 500 μl of urine was spiked with 50 μl of internal standard (100 μg ml^{-1}) and then acidified with 50 μl of 1 N HCl. The solution was loaded onto a SPE column Oasis C18 from Waters Corporation (Milford, MA, USA) which had been previously conditioned with 2 × 1 ml of methanol, 2 × 1 ml of 0.02 N HCl and 2 × 1 ml of water. The column was dried under vacuum for 10 min and then washed with 0.1 M sodium acetate in methanol/acetonitrile (80:20, v/v) adjusted to pH 7.5 with glacial acetic acid. The eluate was collected and dried in a Rapidvap™ evaporator from Labconco (Kansas City, MO, USA) at 45 °C under vacuum, at 95% of the maximum vortex speed. Finally, the sample was reconstituted into 500 μl of water/methanol (70:30, v/v) and a 5 μl aliquot was injected into the HPLC system for analysis.

2.5. Analytical parameters

2.5.1. Precision and recovery

Within run precision was calculated from repeated analysis ($n = 4$) during one working day.

Day to day precision was calculated from repeated analysis of quality control samples on four successive working days. The efficiency of the extraction procedure was evaluated by comparing peak area ratios to internal standard, with and without extraction.

2.5.2. Linearity

Calibration curves were constructed by plotting peak area ratios (compound/internal standard) versus standard concentrations and the best relationship was determined by a least-squares linear regression analysis.

2.5.3. Limit of detection and quantification

The limits of detection (LOD) were calculated as a three-fold signal to noise ratio at the baseline. The limit of quantification (LOQ) was set as the lowest concentration of the calibration function.

2.5.4. Accuracy

The accuracy was tested using a Student's t-test, comparing the mean value Y_m ($n = 4$) of the peak area ratio of a urine standard of known concentration, injected four times ($n = 4$), with the peak area Y_R measured from the equation of the linear regression. Accuracy was verified with a threshold of tolerance of 5% when $t = (Y_m - Y_R)/(\text{S.D.}/2) < t_{table}(\alpha = 5\%,\ n - 1)$, where S.D. is the standard deviation of Y_m.

Accuracy was checked with concentrations of 1 and 40 μg ml^{-1}.

2.6. Subject and drug administration

A clinical trial was carried out in five groups of four healthy adults, aged between 18 and 40 years, men NS (group A); men S (group B); women NS taking no OC (group C); women NS taking OC (group D) and women S taking OC (group E). These subjects were part of 125 healthy volunteers who participated in a CYP1A2 phenotyping study with metabolic probes labeled with ^{13}C. Characteristic groups were chosen to evaluate the induction of the CYP1A2 (group B), the inhibition of the CYP1A2 (group D) and the simultaneous exposition to both an inducer and an inhibitor of this enzyme (group E).

The pre-requisites were a 7 day period without any medication, 48 h without alcohol and charbroiled meat and a 10 h fast before the trial. All subjects ingested an oral dose of labeled ^{13}C caffeine in aqueous solution, depending of the body weight. Doses were either 155, 200 or 245 mg for body weights ranging between 45 and 60 kg, 60 and 75 kg and 75 and 90 kg, respectively. These doses, corresponding to 3 mg about per kg of the body weight, were chosen according the doses previously used in the literature for caffeine breath test [13].

A urine sample, U_0, was collected just before the beginning of the test. All the urines were collected from the ^{13}C caffeine administration up to the end of the test, divided in two samples, U_1 and U_2 for, respectively, the time period between 0 and 3 h and the time period between 3 and 6 h.

Samples were stored at -20 °C until analysis.

2.7. Data analysis

The concentrations of caffeine and metabolites were measured in the three urine samples for the 20 subjects. The following molar ratios were calculated in U_1 and U_2 and used as an index for CYP1A2 activity, $(7X + 37U + 37X)/137X$, $(17X + 17U)/137X$, $(3X + 13U + 13X)/137X$ and $(13X + 17X + 37X)/137X$, for measuring, respectively, the N-1-demethylation, N-3-demethylation N-7-demethylation and the first three N-demethylations of caffeine. Mean ratios were compared between groups using a Student's t-test.

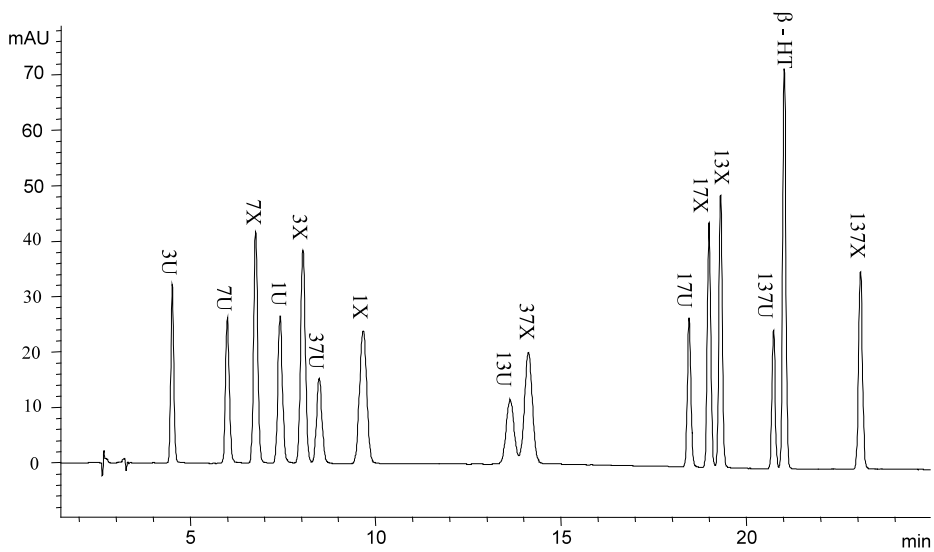

Fig. 2. Chromatogram for blank urine spiked with standard solution at 10 μg ml^{-1} at 270 nm. For compounds abbreviations refer to Table 1. Retention times are 4.51, 6.00, 6.76, 7.42, 8.04, 8.48, 9.67, 13.63, 14.12, 18.46, 18.99, 19.31, 20.73, 21.02, 23.08 min for 3U, 7U, 7X, 1U, 3X, 37U, 1X, 13U, 37X, 17U, 17X, 13X, 137U, βHT, 137X, respectively.

Table 1
Recovery and within-day and between-day precision ($n = 4$)

Compound	Recovery (%)	Coefficient of variation (%)							
		Within day				Between day			
		1 μg ml^{-1}	10 μg ml^{-1}	20 μg ml^{-1}	40 μg ml^{-1}	1 μg ml^{-1}	10 μg ml^{-1}	20 μg ml^{-1}	40 μg ml^{-1}
137X	97 ± 1	0.1	0.3	0.1	0.2	3.1	1.2	0.3	0.2
13X	99 ± 3	1.3	0.4	0.1	0.4	4.8	4.5	0.3	0.1
17X	99 ± 2	1.8	0.3	0.1	0.4	4.9	5.0	0.2	0.2
37X	97 ± 1	0.7	0.6	0.1	0.4	2.6	0.6	0.1	0.7
1X	98 ± 2	1.8	0.2	0.1	0.2	0.9	5.0	1.2	0.4
3X	96 ± 3	0.4	0.2	0.1	0.1	2.9	4.5	0.3	0.7
7X	90 ± 6	0.2	1.2	0.1	0.2	2.5	4.9	0.1	1.7
137U	95 ± 2	0.2	0.2	0.1	0.2	3.1	1.8	1.7	0.2
13U	96 ± 1	1.1	0.2	0.1	0.0	1.2	1.7	1.7	0.2
17U	95 ± 0	0.5	0.2	0.0	0.3	0.7	1.8	1.6	0.1
37U	96 ± 2	0.4	1.8	0.1	0.2	4.7	1.7	1.6	1.3
1U	86 ± 5	1.0	1.6	0.4	0.1	4.0	2.6	1.0	0.2
3U	33 ± 7								
7U	83 ± 7	0.4	0.5	0.0	0.1	0.7	1.9	1.6	2.7
βHT	98 ± 1								

3. Results and discussion

3.1. Method validation

In Fig. 2 is shown a chromatogram of extracted blank urine sample spiked caffeine and its metabolites, according to the method described above. Peak's resolution factors were superior to 1.5. No interference in blank urine control and no matrix effect were observed.

3.1.1. Precision and recovery

Coefficients of variation and the recoveries are listed in Table 1. For all metabolites, within day reproducibility values were below 1.8% for the concentrations of 1 and 10 μg ml^{-1} and below 0.4% for the concentrations of 20 and 40 μg ml^{-1}. Day-to-day coefficients of variation never exceeded 5%. Recovery for methylxanthines were higher than 90% and those for uric acids ranged between 83 and 96%, except for 3U (33%). 3U was not taken into account in the rest of the study because it is a minor metabolite of caffeine, which does not contribute significantly to any of the ratios used to check CYP1A2 activity.

The advantages of the SPE compared with liquid–liquid extraction has been described extensively [14,15]. SPE is simple, safety, solvent saving and low cost. Furthermore, our SPE method permitted to obtain recoveries similar to those reported when liquid extraction is used [8,10]. The method improved the determination of caffeine and its metabolites in terms of number of metabolites and efficiencies, as compared with the previously published SPE procedures. Recoveries using the current method were the same or greater than those archived previously by Z.Y. Zhang et al. [11] and I.N. Papadoyannis et al. [6].

3.1.2. Linearity

The values of the correlation coefficients (R^2) of the calibration functions were mostly higher than 0.999. Therefore, the response of the detector showed a good linearity for quantification of caffeine and its derivatives in the studied range of concentrations.

The LOD was evaluated at 3 ng for 1X, 37X, 37U and 13U and 2 ng for caffeine and the other metabolites. LOQ was set at 5 ng.

3.1.3. Accuracy

Accuracy was calculated for two concentrations within the calibration range (1–40 μg ml^{-1}). T was inferior to t_{table} (0.95, 3) = 3.18, except for 37X at 1 μg ml^{-1} and 37U and 1U at 40 μg ml^{-1}, thus the accuracy of the method can be considered as acceptable.

In conclusion, the results show that this new method allows to separate and quantify rapidly (23 min) the metabolites of caffeine, with high precision and accuracy, and that the parameters of quality of the method are similar to those reported in the literature [9,10,16]. Moreover, The solid-phase extraction procedure described here provides an easier and solvent saving sample preparation that with traditional liquid–liquid procedure. This SPE method is also the first method reported able to extract almost all caffeine metabolites with high recoveries (> 90%).

3.2. Human samples analysis

3.2.1. Urinary profile

Fig. 3 shows the chromatographic urinary profile of a U_1 sample and the various concentrations measured from U_1 and U_2, for one of the subjects included in the trial. It can be observed that caffeine and nearly all its metabolites were present in urine, 3 h after the ingestion of labeled caffeine (U_1). Concentrations were generally lower in U_2 than in U_1.

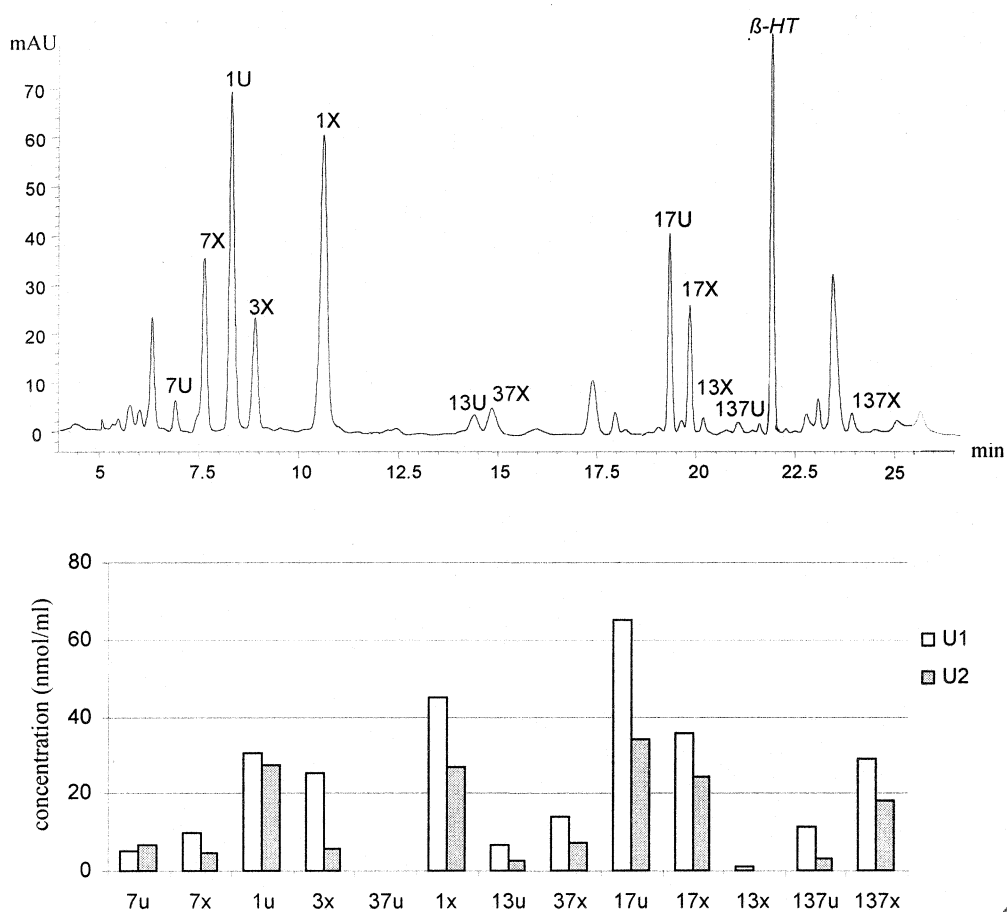

Fig. 3. Chromatogram for urine U_1 at 270 nm and concentrations in urine U_1 and U_2, for a women non-smoker taking no oral contraceptive steroids. U_1 and U_2 are the urines collected between 0 and 3 h and 3 and 6 h following the caffeine administration, respectively. For compounds abbreviations refer to Fig. 1.

	A		B		C		D		E	
ratio	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
(13X+17X+37X)/137X	2.8	2.0	3.2	1.4	3.4	1.3	2.0	1.6	2.5	1.9
(17X+17U)/137X	4.5	2.9	7.6	5.7	2.7	1.9	2.4	2.0	3.6	1.6
(3X+13U+13X)/137X	2.0	1.0	2.6	1.8	2.3	0.7	1.0	1.9	5.8	7.4
(7X+37U+37X)/137X	5.0	7.9	3.0	2.7	5.0	3.5	1.9	3.2	9.0	11.1

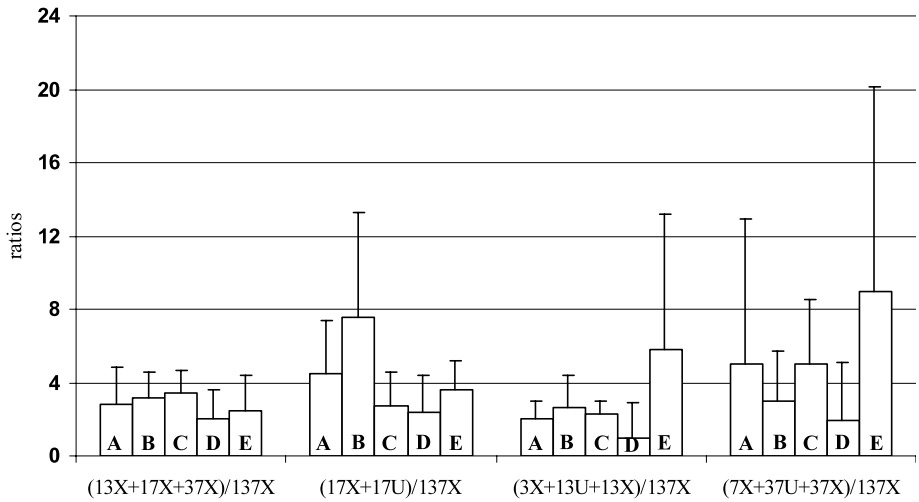

Fig. 4. Caffeine metabolite ratios used for marking CYP1A2 activity, calculated in U_1 for the groups of subjects A, B, C, D and E. U_1, urine collected between 0 and 3 h following the caffeine administration. A, four men non-smokers. B, four men smokers. C, four women non-smokers taking no oral contraceptive. D, four women non-smokers taking oral contraceptives. E, women smokers taking oral contraceptives.

3.2.2. Metabolic ratios

Figs. 4 and 5 gather the mean and the S.D. of various metabolic ratios in U_1 and U_2, respectively, for the five groups of subjects. Statistics differentiated significantly group C and group D for the ratio $(17X + 17U)/137X$ calculated in U_2, but failed to differentiate the five groups for the other ratios. Nevertheless, the values of metabolic ratios and their relative variations among groups show trends to induction and inhibition relative to smoking habits or OC intake.

The comparison of the ratios between group A and B showed that the values obtained from men NS were lower, but insignificantly, than those from men S, except for the ratio $(7X + 37U + 37X)/137X$ where it was the reverse. This observation is in accordance with an induction on the expression of the CYP1A2, due to the polycyclic aromatic hydrocarbons contained in smoke [17].

Comparisons between groups C and D seemed to show that the intake of OC inhibits the activity of the CYP1A2, as mentioned in other studies reporting also lower ratios in OC users [1,18]. The combination of the smoking habits and the OC intake increased slightly the activity of the CYP1A2 (group E vs. D). Moreover, the metabolic ratios observed in group E seemed to be higher than those observed in group C. Thus, the overall effect of smoking combined with the OC intake results preferentially in an induction of the CYP1A2.

The results obtained, in terms of metabolic ratios, from U_1 and U_2, brought nearly the same information about the activity of the CYP1A2 for the various groups. Nevertheless, the quality of measurement was improved when working with U_2.

These observations are in accordance with the ^{13}C caffeine breath test results [19]. However, the presence of metabolites observed in all samples of U_0 (even after a 10 h fast period) might have generated potential errors. Under these condi-

	A		B		C		D		E	
ratio	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
(13X+17X+37X)/137X	2.7	3.0	4.2	1.7	2.8	1.1	1.2	0.9	2.3	0.4
(17X+17U)/137X	3.9	4.2	8.9	5.9	2.9	1.0	1.1	0.6	3.2	1.6
(3X+13U+13X)/137X	1.5	2.2	3.0	2.0	1.4	1.1	0.3	0.3	1.8	1.2
(7X+37U+37X)/137X	4.8	8.7	3.2	2.0	3.6	2.5	0.6	0.5	3.0	2.1

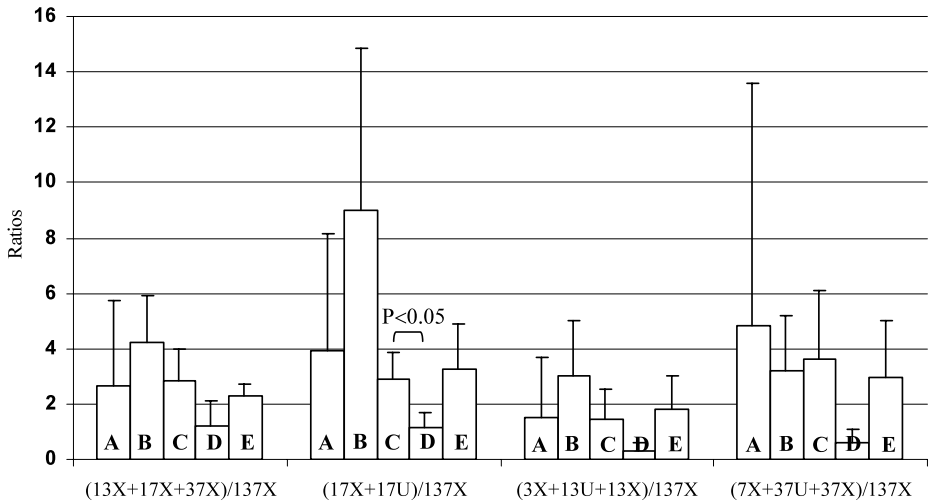

Fig. 5. Caffeine metabolite ratios used for marking CYP1A2 activity, calculated in U_2 for the groups of subjects A, B, C, D and E. U_2: urine collected between 3 and 6 h following the caffeine administration. A, four men non-smokers. B, four men smokers. C, four women non-smokers taking no oral contraceptive. D, four women non-smokers taking oral contraceptives. E: women smokers taking oral contraceptives.

tions, the validation of the comparisons between reference groups would require a higher number of subjects and a stricter xanthine-free diet, at least 2 days before the test.

Moreover, if ^{13}C caffeine is used as the metabolic probe, mass spectrometry could be used to detect more specifically the labeled substrate and its metabolites. Hence, labeled xanthines and uric acid metabolites could be differentiated from the unlabeled ones present in urine prior to the test.

4. Conclusion

The analytical method described above can easily be applied for the evaluation of the individual variations of the CYP1A2 activity in longitudinal study, for example for the evaluation of the drug metabolizing liver function during either normal or pathological pregnancy, for the study of drug–drug interaction or drug–environmental interaction, for assessing liver metabolic function during hepatic diseases, etc. In the context of these kinds of studies, the inter-individual variations do not interfere in the comparison of metabolic ratios, as each subject acts as its own standard.

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