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Determination of urinary ^{13}C-caffeine metabolites by liquid chromatography–mass spectrometry: the use of metabolic ratios to assess CYP1A2 activity

Marie-Sophie Caubet [a], Blandine Comte [b,*], Jean-Louis Brazier [a]

[a] *Faculté de Pharmacie, University of Montreal, Montreal, Que., Canada H3C 3J7*
[b] *Department of Nutrition, University of Montreal, Montreal, Que., Canada H3C 3J7*

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Abstract

A method using liquid chromatography coupled with mass spectrometry with an atmospheric pressure electrospray source was developed for analysis of labelled caffeine and fourteen of its metabolites in urine. Caffeine metabolic ratios were determined after an oral bolus of labelled caffeine in 20 healthy subjects with different characteristic CYP1A2 activity, relative to smoking habit and oral contraceptive intake. The use of labelled caffeine for the calculation of metabolic ratios avoided taking into account the important background of endogenous caffeine metabolites, very difficult to eliminate even after a specific diet. The selectivity and high sensitivity of mass spectrometry detection allowed urine collections for only a 3 h period. Comparison between characteristic groups showed that labelled caffeine metabolic ratios were sensitive markers of changes in CYP1A2 activity.

Keywords: Labelled caffeine; LC–MS; CYP1A2; Caffeine metabolic ratios

1. Introduction

CYP1A2, an isoform of the CYP1A cytochrome P450 super-family, is involved in the metabolism of many drugs and plays a potentially important role in the induction of chemical carcinogens leading to 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 [1,2]. Caffeine has been used as a model probe for CYP1A2 liver function investigation [3]. As shown in Fig. 1, caffeine is metabolized via successive pathways mainly catalyzed by CYP1A2, xanthine oxidase or *N*-acetyltransferase-2 to give 14 different metabolites. Molar caffeine metabolites ratios (CMRs) have been used to assess the metabolic phenotype of those enzymes [4–6]. CYP1A2 activity shows an inter-individual variability among the population. Moreover, behavioural parameters such as alcohol consumption, exercise, smoking, contraceptive intake, etc. have been reported to modify

* Corresponding author. Present address: Centre de Recherche de l'Hôpital Sainte-Justine, Block 7, Room 1727, 3175 Côte Sainte-Catherine, Montréal, Que., Canada H3T 1C5.
Tel.: +1-514-345-4931x3983; fax: +1-514-345-4988.
E-mail address: bcomte@justine.umontreal.ca (B. Comte).

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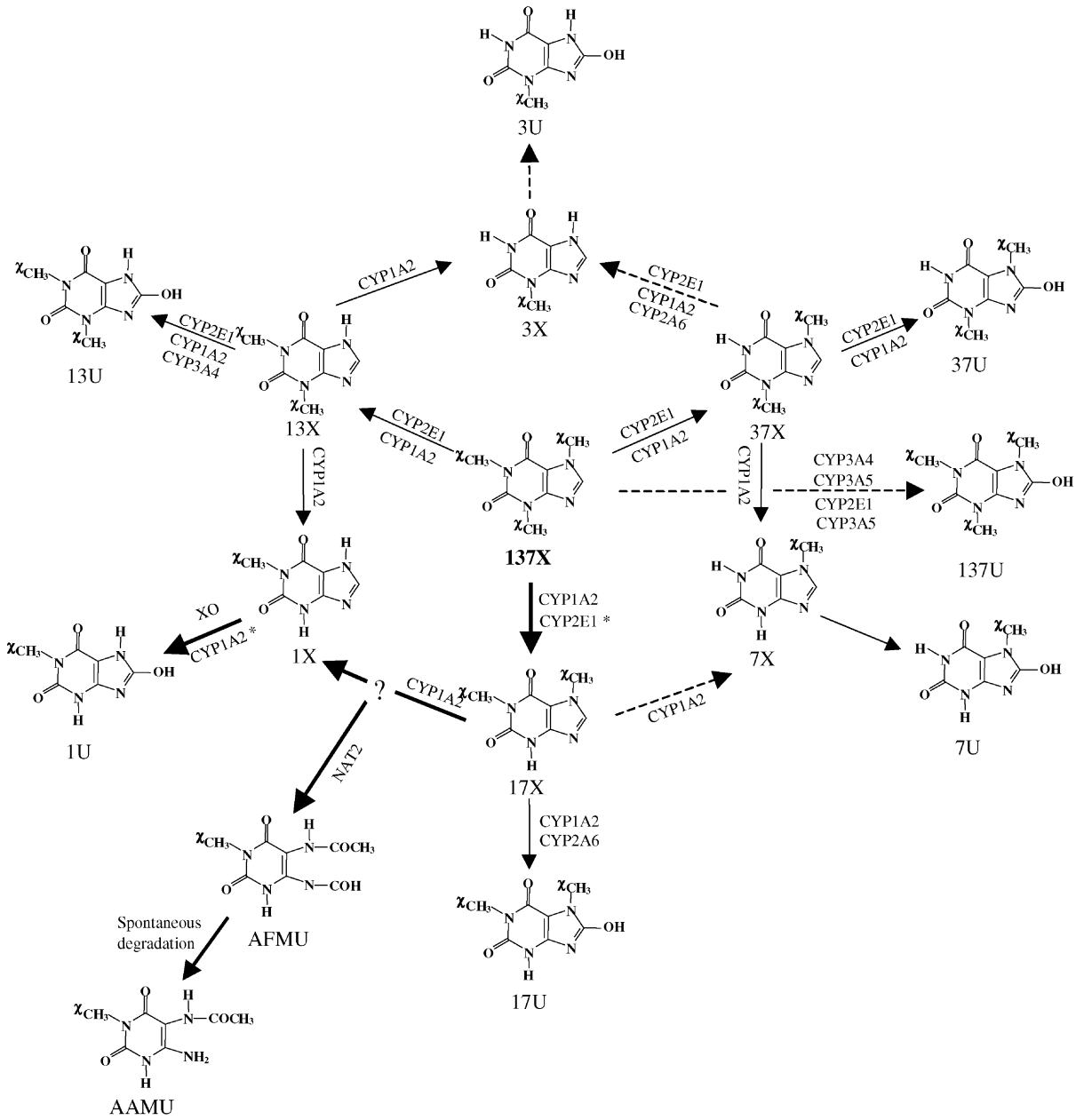

Fig. 1. Caffeine metabolic pathways in human liver [15]. Bold arrows point out main pathways and dotted arrows minor pathways, which do not contribute to any of the ratios used to study CYP1A2 or NAT2 activities. χ indicates the labelled carbon with the isotope ^{13}C. Symbols beside arrows indicate enzymes: NAT2: *N*-acetyltransferase; XO: xanthine oxidase. 137X: caffeine or 1,3,7-trimethylxanthine; 13X: theophylline or 1,3-dimethylxanthine; 17X: paraxanthine or 1,7-dimethylxanthine; 37X: theobromine or 3,7-dimethylxanthine; 1X: 1-methylxanthine; 3X: 3-methylxanthine; 7X: 7-methylxanthine; 137U: 1,3,7-trimethyluric acid; 13U: 1,3-dimethyluric acid; 17U: 1,7-dimethyluric acid; 37U: 3,7-dimethyluric acid; 1U: 1-methyluric acid; 3U: 3-methyluric acid; 7U: 7-methyluric acid; AFMU: 5-acetylamino-6-formylamino-3-methyluracil; AAMU: 5-acetylamino-6-formylamino-3-methyluracil.

the activities of these drug-metabolizing enzymes [6–8]. Metabolic phenotyping of CYP1A2 may be of relevance in determining whether patients may develop adverse reactions to certain drugs metabolized by CYP1A2 or risks for developing bladder or liver disorder [9,10]. Actually, caffeine metabolism is impaired in liver disease and the measurement of caffeine clearance is an indicator of the severity of liver cirrhosis [10].

High performance liquid chromatography (HPLC) coupled with ultra-violet (UV) detection is the most frequently used technique for the analysis of caffeine and its metabolites in biological samples [11–14]. However, poor selectivity and sensitivity are recorded with this method of detection, particularly when analyzing metabolites with very similar UV spectra. Moreover, biological fluids such as plasma or urine generally contain endogenous compounds absorbing UV radiations and being sources of interference. Mass spectrometry (MS) improves both selectivity and sensitivity and is a powerful tool for drugs analysis. The aim of the present study was to develop a new HPLC–MS method with an atmospheric pressure electrospray source (API-ES) for the analysis of ^{13}C labelled caffeine and its metabolites in urine to assess the CYP1A2 activity.

In the previous study [15], HPLC–UV was used for measuring caffeine metabolic ratios from 20 healthy subjects, males and females, including smokers and women taking oral contraceptive steroids (OCS), who received an oral bolus of ^{13}C labelled caffeine. These subjects were also evaluated with the ^{13}C-caffeine breath test. Results showed the presence of high concentrations of caffeine metabolites in urine samples taken before the beginning of the protocol and after a 10 h fasting period. In fact, as mentioned in other studies [16], it is really difficult to obtain urine free of any caffeine metabolites, even after a 3-day washout. This background is the main source of potential errors in the measurement of urine concentrations of caffeine metabolites. The interest and the power of the HLPC–MS, combined with the use of labelled substrate, are obvious in this context. In animal cells, the isotopic labelling does not generally induce isotopic effect and toxicity, except with deuterium [17–19]. For isotopes such as ^{13}C, ^{15}N, etc. it has been demonstrated that isotopic effects are negligible in enzymatic, physiologic and cell processes because the weighting difference between the element and its isotope is proportionally low [20,21]. The present study used mass spectrometry to quantify both labelled and unlabelled compounds in the urine samples of the same 20 healthy subjects previously studied [15], allowing us to determine how unlabelled residual caffeine derivatives in urine contribute to the total metabolites levels. The method was used for the measurement of labelled CMRs for evaluation of CYP1A2 activity: (7X + 37U + 37X)/137X, (17X + 17U)/137X, (3X + 13U + 13X)/137X, (13X + 17X + 37X)/137 X (for compound abbreviations, refer to Fig. 1).

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). Caffeine (1,3,7-trimethyl-$^{13}C_3$, 99%) was purchased from Isotec, Inc., OH, USA.

2.2. Subject and drug administration

The 20 subjects included in this study (18–40 years old, 68 ± 12 kg) were part of the 125 healthy adult volunteers who participated in a clinical trial for CYP1A2 phenotyping study with the ^{13}C-caffeine breath test [22]. The protocol was reviewed and approved by the Research and Ethical Committee of Algorithme Pharma, Inc. (Montreal, Que., Canada), in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Eight men, 4 non-smokers (group A), 4 smokers (group B), and 12 women, 4 non-smokers (group C), 4 non-smokers taking OCS (group D) and 4 smokers taking OCS (group E) were included. The induction of CYP1A2 activity was evaluated in the group B whereas its inhibition was studied in the group D. The simultaneous exposition to both an inductor and inhibitor of this enzyme was evaluated in the group E.

The pre-requisites for this study were a 7-day period without any medication, 48 h without alcohol and charbroiled meat consumption and a 10 h fast before the trial. Each volunteer received an oral bolus of labelled caffeine (1,3,7-trimethyl-$^{13}C_3$-caffeine, 99%) in aqueous solution, depending on the body

weight. Three different doses were used: 155 mg, 200 and 245 mg for body weights ranging between 45 and 60 kg, 60 and 75 kg and 75 and 90 kg, respectively (2.6–3.4 mg/kg of caffeine). These doses correspond to those usually used for a caffeine breath test in adult and can be found in about two cups of coffee. No adverse effect from caffeine was observed during the protocol. One urine sample was collected before the oral bolus and all the following urine volumes were collected and combined for the 3 h period of the study. Subjects were allowed to drink water ad libitum, 1 h after the caffeine administration. Samples were stored at −20 °C until further analysis.

2.3. Sample analysis

Analyses were performed on a 1100 series HPLC–MS, from Agilent Technologies (Kirkland, Que., Canada). Caffeine and its metabolites were extracted from urine by solid phase extraction (SPE) and separated by HPLC as previously described [15], with slight modifications for the mass spectrometry detection. Metabolites were separated on an Eclipse XDB-C18 reversed phase column (250 mm × 4.6 mm, 5 μm particle size) coupled with a Eclipse XDB-C18 guard column (12.5 mm × 4.6 mm, 5 μm particle size), from Hewlett-Packard (Kirkland, Que., Canada). The aqueous mobile phase was a mixture of water/acetic acid/THF (996.5:1:2.5, v/v/v) (pH 3.3) whereas the organic one was acetonitrile. The chromatographic elution program was as follow: 3.5% acetonitrile for 11 min, increased to 8% from 11 to 32 min and then increased to 13% from 32 to 36 min; with a post run of 20 min in order to re-equilibrate the column between injections. The flow rate was set at 0.8 ml/min and the column compartment was kept at a controlled temperature of 23 °C ± 0.5 °C.

Before treatment, urine samples were filtered through a 0.45 μm nylon membrane from Millipore (Neapan, Ont., Canada) to remove particles in suspension. Five hundred microliter of urine were spiked with internal standards 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 pre-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. Finally, the sample was reconstituted into 50 μl of water/methanol (70:30, v/v) and a 5 μl aliquot was injected into the HPLC system for analysis.

Compounds were introduced into the mass spectrometer by API-ES. In the spray chamber, drying gas temperature was set at 350 °C, drying gas flow at 10 ml/min and nebulization pressure at 55 psi. The mass spectrometer was operated in the positive and negative ion modes for the detection of xanthines and uric acids, respectively. Capillary voltage was set at 5000 V and fragmentor voltage at 70 eV. Quantification was achieved using selected ion monitoring (SIM). For each compound, appropriate ion sets were monitored to analyse both labelled and unlabelled molecules (Table 1), with a dwell time of 296 and 329 ms/ion for positive and negative mode, respectively. Calibrations with standard curves in urine were achieved using unlabelled compounds. Blank urine samples were spiked with standard solutions for final concentrations of 0.1, 0.5, 1, 5, 10, 20 and 40 ng/μl for caffeine and its metabolites and 5 ng/μl for internal standards (IS). β-Hydroxyethyltheophylline (β-HT) and 1,9-dimethyluric acid (19U) were chosen as IS for xanthines and uric acids, respectively. Peak areas from the non-spiked urine samples were subtracted

Table 1
Selected ions monitored for analysis of caffeine and its metabolites

Compound	*m/z*
Positive mode	
1X, 3X, 7X	167, 168
13X, 17X, 37X	181, 182, 183
137X	195, 196, 197, 198
β-HT (IS)	225
Negative mode	
1U, 7U	181, 182
13U, 17U, 37U, 19U (IS)	195, 196, 197,
137U	209, 210, 211, 212

The mass spectrometer was operated on selected ion monitoring (SIM) with a dwell time of 296 and 329 ms/ion for respectively positive and negative detection mode. The different ions for each compound correspond to $[M_0]$, $[M_0 + 1]$, $[M_0 + 2]$ or $[M_0 + 3]$ for the presence of 0, 1, 2 or 3 atoms of ^{13}C, respectively, on the molecule, where $[M_0]$ corresponds to $[M + H]^+$ for xanthines and $[M - H]^-$ for uric acids.

from the corresponding peak areas in the spiked samples and calibration curves were determined by a least-square linear regression analysis. The precision of the method was determined from four repeated assays. For measurement of the labelled metabolites, the peak areas were corrected for naturally occurring heavy isotopes as previously described by Fernandez and Des Rosiers [23].

2.4. Data analysis

Each caffeine metabolite excreted in the combined 3 h urine collection was expressed as a percentage of the administered dose of caffeine per weight (kg). The following CMRs were calculated and used as index of CYP1A2 activity: $CMR_1 = (7X + 37U + 37X)/137X$ for caffeine *N*-1-demethylation, $CMR_2 = (17X + 17U)/137X$ for *N*-3-demethylation of caffeine, $CMR_3 = (3X + 13U + 13X)/137X$ for *N*-7-demethylation of caffeine and $CMR_4 = (13X + 17X + 37X)/137X$ for the three *N*-demethylations of caffeine. Differences were analysed by the Student's *t*-test and correlations were calculated as Spearman's rank order correlation coefficient. A $P < 0.05$ was accepted as significant.

3. Results and discussion

3.1. Analytical method

Fig. 2 illustrates the ion chromatograms of labelled caffeine and its metabolites extracted from a representative urine sample obtained from a male smoker (group B). Most of metabolites were detected in both positive (Fig. 2a) and negative (Fig. 2b) conditions. However, higher sensitivity was achieved when analysing xanthines in the positive mode (ion $[M + H]^+$) and uric acids in the negative mode (ion $[M - H]^-$). The ions used for each metabolite are presented in Table 1. Small modifications were made to adapt the previous HPLC–UV method to MS detection. The time of analysis was lengthened in order to separate 17X and 13X in positive mode and the flow rate was reduced to fit with the API-EI source conditions. Modification could eventually be done in the chromatographic elution conditions to reduce the run time. However, the run time depends on the number of metabolites of interest to be analysed. The samples were concentrated 10 times compared to the previous method in order to be able to quantify very low concentrations of metabolites. Using the HPLC–UV method, both labelled and unlabelled compounds were not distinguished, leading therefore to the higher measured concentrations than with the HPLC–MS method.

Fig. 3 presents the ion chromatograms under positive (a) and negative (b) mode of a blank urine sample showing that before the beginning of the protocol, all metabolites, except caffeine, were detected. In most of the urine samples, unlabelled compound often represented more than 50% of the total amount of the assayed metabolite, particularly for monomethylxanthines (1X, 3X and 7X) and monomethyluric acids (1U, 3U and 7U). This confirms that a 10 h fasting period before the trial is not enough to obtain urine free of xanthines and uric acids. As reported in other studies [16], it is really difficult to obtain urine free of caffeine metabolites even after a 3-day fasting period. In fact, caffeine and xanthines are not only contained in coffee or tea, but also in numerous foods such as chocolate and soft drinks [24]. Moreover, caffeine is metabolized via several parallel and successive pathways (Fig. 1), leading to the production of different metabolites with more or less rapid metabolism, even if the half-life of caffeine is only about 4 h. Therefore, the use of labelled caffeine as a metabolic probe for the assessment of CYP1A2 activity is particularly relevant to differentiate labelled caffeine derivatives from residual endogenous metabolites.

The lower limit of detection (LOD), defined as a three-fold signal to noise ratio at the baseline was 50 pg for all compounds. The limit of quantification was set at the lowest concentration of the calibration curves. Compared to the UV detection [15], the sensitivity of the MS analysis increased about sixty times in terms of their LOD (LOD decreased from 3 ng to 50 pg). A similar LOD was obtained in the only publication reported in the literature for the determination of caffeine and its metabolites in plasma by HPLC–MS [25].

Calibration functions were linear through the studied range of concentrations, with correlation coefficients ranging from 0.97 to 0.99, the same order as those presented in the study of Kanazawa et al. [25]. Correlation coefficients from HPLC–MS methods are

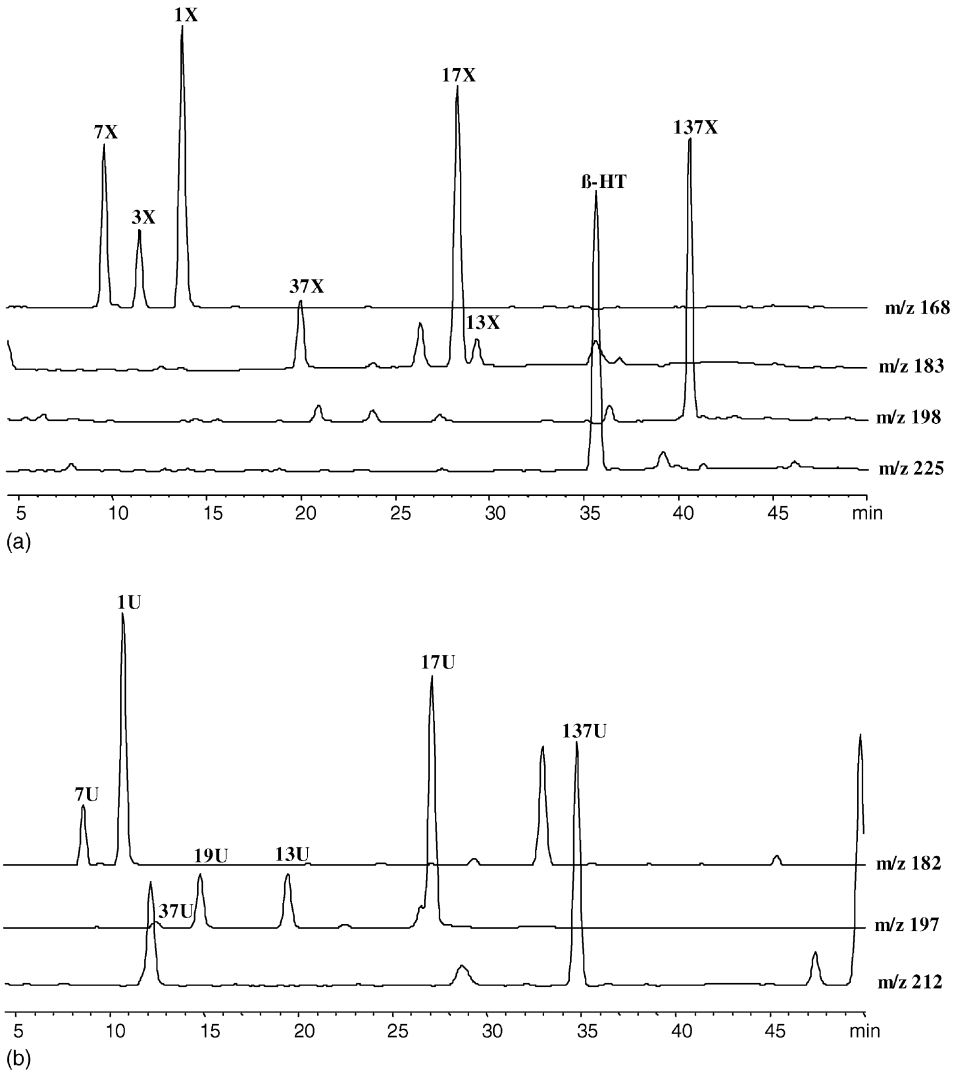

Fig. 2. Ion chromatograms of labelled caffeine and its metabolites analysed under (a) positive and (b) negative ion detection modes, extracted from a representative urine sample coming from a smoking men (group B). For compound abbreviations, refer to Fig. 1 and text.

slightly lower than those observed in conventional HPLC–UV methods [15,16]. The ionisation process in MS detection (API-ES or APCI sources) is probably the main source of distortion in the linearity. Coefficients of variation for the method precision were less than 6%. Standard curves were generated using unlabelled standards and after subtraction of the important background from blank urine. Utilization of ^{2}H or ^{13}C labelled standards ($M + 4$ or above to not interfere with the metabolism of the ingested labelled caffeine) would have been preferable and more specific. However, these compounds were unfortunately not commercially available and their utilisation would have increased considerably the cost of the study.

Fig. 4 presents the quantities of labelled caffeine and its various metabolites assayed in urine and expressed as a percentage of the amount of the labelled caffeine ingested per kg. The negative values measured for few metabolites were due to the variability of the method (the standard deviation presented on the

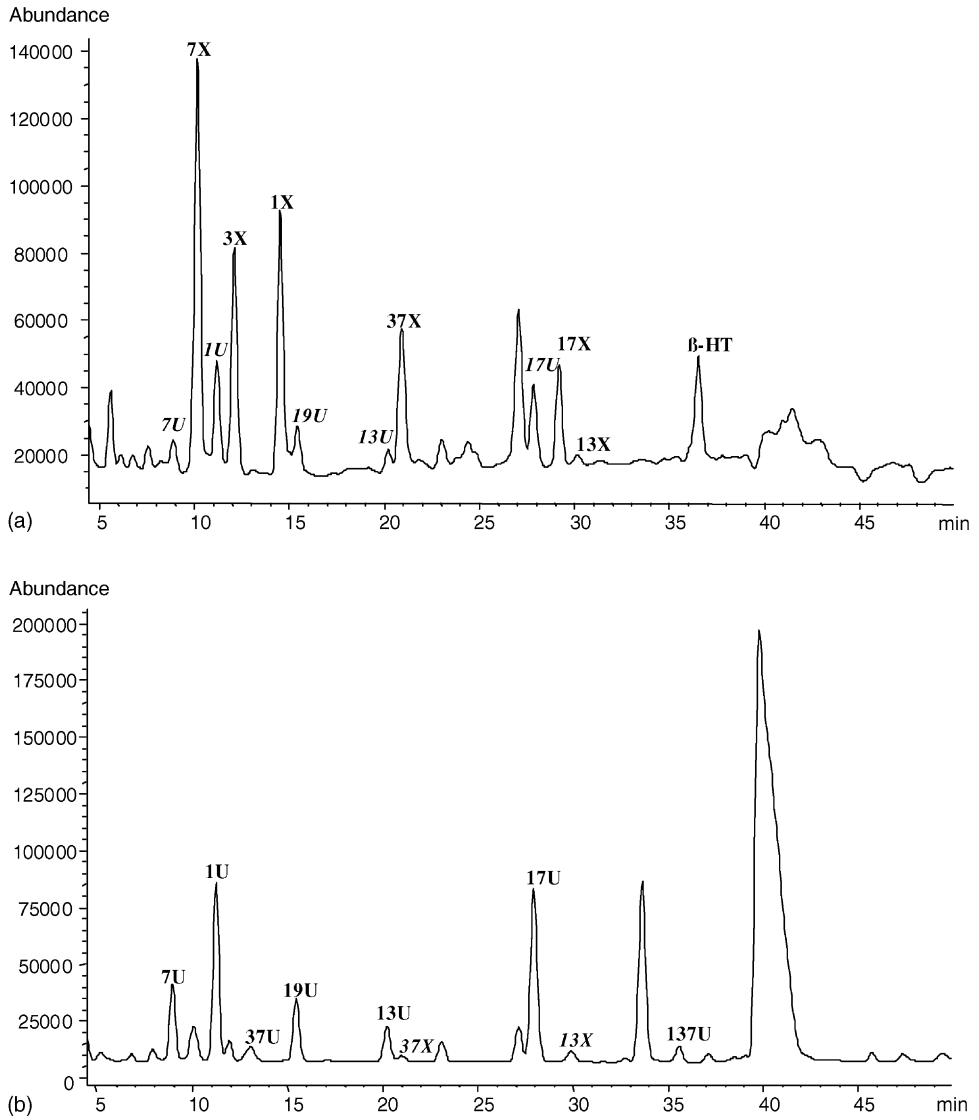

Fig. 3. Total ion chromatogram of caffeine and its metabolites detected under (a) positive and (b) negative modes in a blank urine sample spiked with internal standards. For compound abbreviations, refer to Fig. 1 and text.

graph represents the variability of the percentage measured in a group). The main metabolites eliminated 3 h after caffeine administration were 17X and 1X and their corresponding uric acids. These results confirm that caffeine is mainly *N*-3-demethylated into 17X for about 80% and 17X is then *N*-1-demethylated mainly into 1X for about 12% [3]. In a 3 h urine collection, 0.01–0.16% of the individual dose/kg (or 0.7–13% of the individual dose) was recovered, depending on the subjects. These recovery coefficients are low compared to those generally used for CMRs measurement (from 30 to 50% of recovery depending on the time of urine collection, 8–24 h after the caffeine administration) [3,26]. Nevertheless, our results show that LC–MS, by allowing measurements of very low amounts of metabolites with high sensitivity, shortens the time of urine collection, without compromising the validity (instead of sensitivity) of CMR tests.

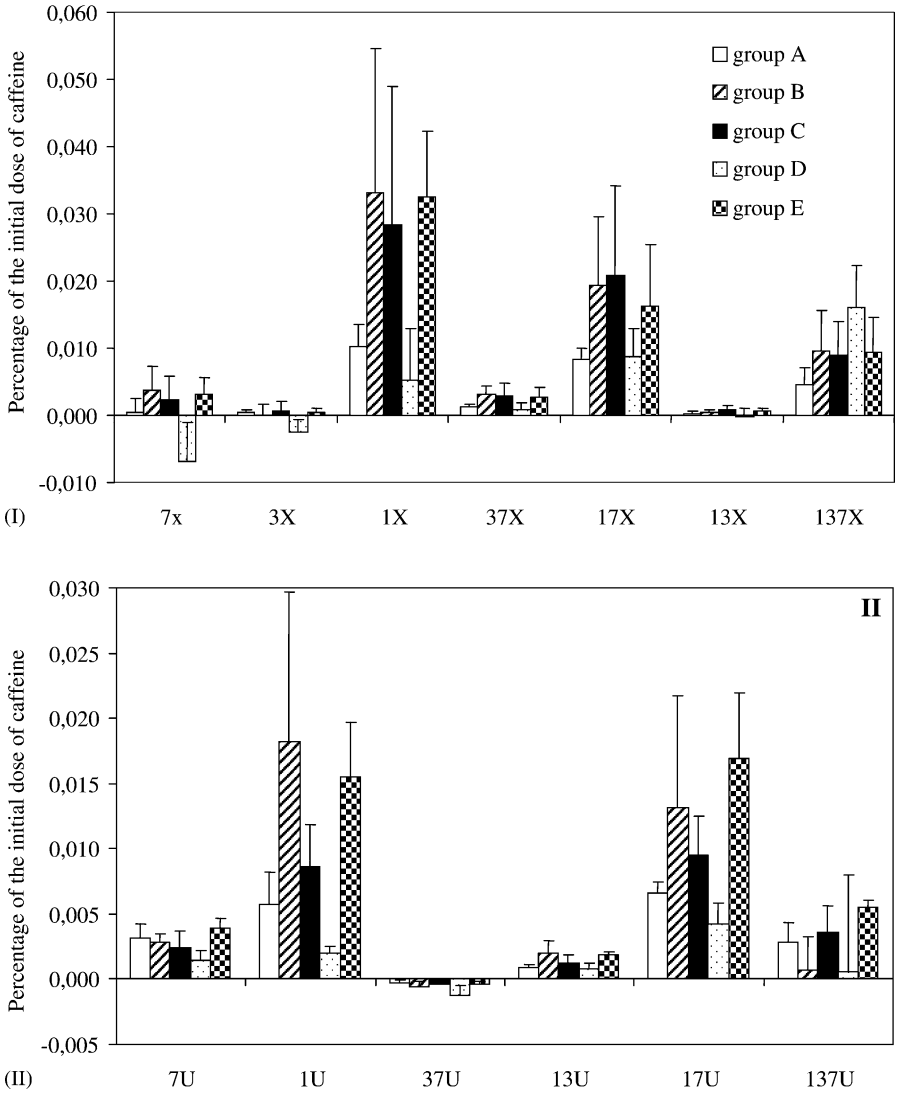

Fig. 4. Caffeine metabolites (xanthine (I) and uric acid (II)) in urine samples, expressed as percentages of the initial dose of labelled ingested caffeine (molar quantity of metabolites/molar quantity of caffeine/kg). For compound abbreviations, refer to Fig. 1. (A) non-smoking men; (B) smoking men; (C) non-smoking women; (D) non-smoking women taking oral contraceptives; (E) smoking women taking oral contraceptives. Data are expressed as means ± standard deviations; number of patients per group: $n = 4$.

3.2. Metabolic ratios

Four ratios were chosen among the different CMRs previously reported as being probes for the in vivo CYP1A2 activity [26,27]. Fig. 5 summarises CRM calculations in the different groups of subjects.

Inter-individual coefficients of variations were on average, 50% in each group and for each ratio. The variability was slightly higher than in previous published studies (15–40%) and was probably related to the small number of subjects analysed (four per group) [4,26]. Inter and intra-individual differences in the renal clearances or the excretion rates of the various

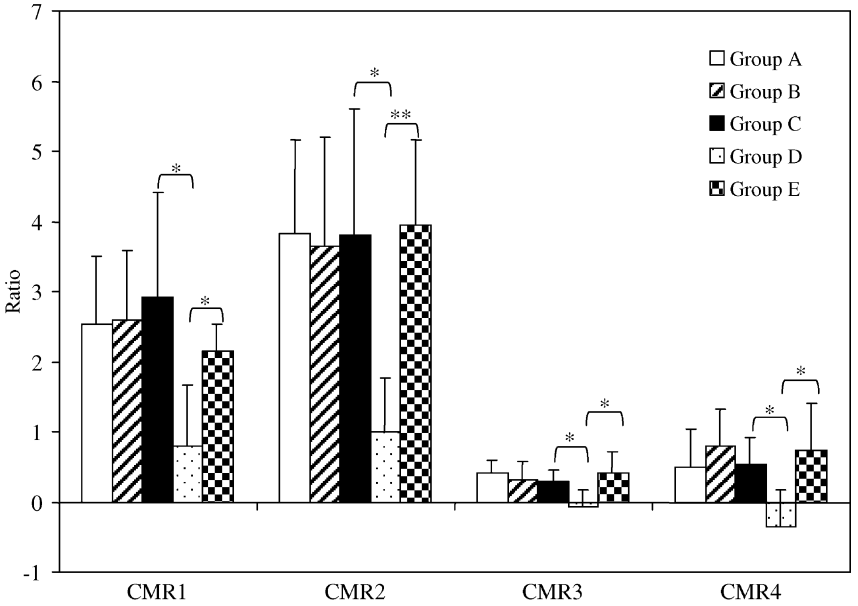

Fig. 5. Caffeine metabolic ratios (CMRs) used for phenotyping CYP1A2 activity. CMR_1 = (7X + 37U + 37X)/137X, CMR_2 = (17X + 17U)/137X, CMR_3 = (3X + 13U + 13X)/137X, CMR_4 = (13X + 17X + 37X)/137X. For compound abbreviations, refer to Fig. 1. (A) Non-smoking men; (B) smoking men; (C) non-smoking women; (D) non-smoking women taking oral contraceptives; (E) smoking women taking oral contraceptives. Data are expressed as means ± standard deviations; number of patients in each group: $n = 4$; $^{*}P < 0.05$, $^{**}P < 0.001$ using Student's *t*-tests.

metabolites may also contribute to a large extent, to the variability of CRMs [28].

OCS intake decreased significantly all ratios in non-smoking women (group D versus group C). On the contrary, smoking habit increased significantly all ratios in women taking OCS (group E versus group D). This result is in accordance with previous studies on the inhibitory effect of OCS on the CYP1A2 activity. In fact, it has been shown that the CMR (AFMU + 1X + 1U)/17U [6,29] decreased with OCS intake. Similar results have been obtained with the caffeine breath test [22]. The significant inductive effect of CYP1A2 activity in smoking women taking OCS was also in accordance with the previous observation using either the HPLC–UV detection or the breath test [15,22]. Smoking habit in women taking OCS resulted in an overall metabolic activity not significantly different from that measured in non-smoking women taking no OCS.

No significant difference was observed between smokers and non-smokers men. In a study done on 37 smokers and 70 non-smokers, Carrillo and Benitez [26] reported no significant difference for the CMR_3 and a significant difference for the CMR_1. Butler et al. [30] showed that CRM_2 increased significantly in Arkansas and Italian smokers compared to non-smokers but it was not affected in Italian smokers of black tobacco and Chinese smokers compared to Italian and Chinese non smokers. Consequently, these results show that not all of the calculated CMRs are able to point out the induction of CYP1A2 activity by smoking. Moreover, as previously mentioned [30], CMRs showed much larger inter-individual variability inside groups than differences between groups. Finally, it has been shown that inter-ethnic variation may have an influence on the clearance of dimethylxanthines [7]. Nevertheless, 3 h after caffeine intake, urine of smokers contained twice more 17X and 17U than urine of non-smokers, reflecting the rapid main first *N*-demethylation of caffeine through the induction of the CYP1A2 (Fig. 4, group B versus group A). A significant increase in all the calculated CMRs was observed when women taking OCS are smoking (Fig. 5, group E versus group D).

Table 2
Correlation matrix (Spearman correlations) between the different calculated caffeine metabolic ratios (CMRs) and between these ratios and caffeine breath test (CBT) previously measured [22]

	CMR_1	CMR_2	CMR_3	CMR_4	CBT
CMR_1	1.00				0.62**
CMR_2	0.90***	1.00			0.59**
CMR_3	0.81***	0.83***	1.00		0.64**
CMR_4	0.83***	0.81***	0.91***	1.00	0.80***

$CMR_1 = (7X + 37U + 37X)/137X$, $CMR_2 = (17X + 17U)/137X$, $CMR_3 = (3X + 13U + 13X)/137X$, $CMR_4 = (13X + 17X + 37X)/137X$. ** $P < 0.001$ and *** $P < 0.0001$, using the Student's *t*-test. For compound abbreviations, refer to Fig. 1.

As previously reported in other studies, no gender difference was observed of the CYP1A2 indexes (Fig. 5, group A versus group C) [26,31].

Table 2 presents correlation coefficients between the different CMRs and shows that all CMRs were strongly correlated with each other ($P < 0.0001$). These significant correlation coefficients are in accordance with the high participation of CYP1A2 in the three *N*-demethylation of caffeine and confirm that any of those CMRs are relevant markers of the CYP1A2 activity. Nevertheless, the different ratios do not have the same specificity and sensitivity. A study from Rostami-Hodjegan et al. [27] used computer simulations with values measured in vivo to examine the validity of the various CMRs. Among the ratios used in the present study, CMR_4 seemed to be the most sensitive to the CYP1A2 activity and all CMRs could be affected by the renal clearance of caffeine but also by changes in the CYP2A6 activity.

As all the subjects presented in this study were also part of a caffeine breath test study [22], we have calculated correlation coefficients between the CMRs and the results of the areas under curve of the breath test (Table 2). A very good concordance of the results performed with the two tests was observed ($P < 0.001$) and the inter-individual variations were of similar magnitude. However, it is interesting to note that caffeine breath test was able to significantly differentiate CYP1A2 activity between non-smoking and smoking men.

In conclusion, the present LC–MS method provides a new sensitive way for measurement of caffeine and its metabolites in urine. MS detection records higher selectivity and sensitivity than UV detection (50-fold). The increased sensitivity of MS detection allows urine collections for only a 3 h period with the possibility of relevant metabolic ratio calculations to assess the CYP1A2 activity. The use of labelled caffeine for the calculation of CMRs as indexes of CYP1A2 activity avoids taking into account the important background of endogenous caffeine metabolites, difficult to eliminate even after a specific diet. Comparisons between the different specific groups show that CMRs are sensitive markers of changes in CYP1A2 activity. However, the large inter-individual variations and the small number of subjects included in the present study limit the extent of the results interpretation. Further investigation should take into account the influence of race and gender and should compare CMRs to the clearances of caffeine and its metabolites.

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