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Extractionless method for the determination of urinary caffeine metabolites using high-performance liquid chromatography coupled with tandem mass spectrometry

Heiko Schneider*, Lan Ma, Hansruedi Glatt

Deutsches Institut für Ernährungsforschung, Abteilung Ernährungstoxikologie, Arthur-Scheunert-Allee 114–116, D-14558 Potsdam-Rehbrücke, Germany

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Abstract

Caffeine is metabolised in humans primarily by cytochromes P450 1A2 and 2A6, xanthine dehydrogenase/oxidase, and *N*-acetyltransferase 2. The activities of these enzymes show a large variation due to genetic polymorphisms and/or induction by xenobiotics. Ratios of different caffeine metabolites in urine or other body fluids are frequently used to characterise the individual/actual activity of these enzymes. The common analytical method involves extensive sample preparation, followed by HPLC–UV. The presence of numerous other UV-absorbing chemicals in body fluids affects the sensitivity and selectivity of this method. We have developed an HPLC–electrospray-MS–MS method for the determination of 11 caffeine metabolites and two internal standards after a simple, extractionless preparation. Blank urine, obtained after 5 days on a methylxanthine-free diet, contained small amounts of some caffeine metabolites, but no other components producing any confounding signals. Eleven metabolites and internal standards were recovered at 90 to 110% after addition to the blank urine (0.1 to 2.5 μM in the final sample involving a 20-fold dilution of urine) in the 0.1–2.5 μM concentration range. Other metabolites, 5-acetylamino-6-amino-3-methyluracil (AAMU) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU), were detected with similar recovery and precision, but required higher concentrations (3 to 30 μM). AFMU was completely converted into AAMU by a short alkalisation of urine. The method was explored in six healthy individuals after consuming coffee (4 mg caffeine per kg body mass). These experiments demonstrated the simplicity, high sensitivity and selectivity of the method under conditions used for phenotyping.
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1. Introduction

Urinary metabolites of caffeine are frequently utilised to characterise the activities of several xenobiotic-metabolising enzymes in humans. Caffeine is chiefly metabolised by cytochrome P450 (CYP) 1A2, CYP2A6, *N*-acetyltransferase 2 (NAT2) and xanthine dehydrogenase/oxidase (XDH). Minor contributions to some metabolic steps result from the activities of CYP2E1 and 3A4.

A scheme of the biotransformation pathways of caffeine is presented in Fig. 1, which also shows the

*Corresponding author. Tel.: +49-33200-88387; fax: +49-33200-88426.
E-mail address: schneide@mail.dife.de (H. Schneider).

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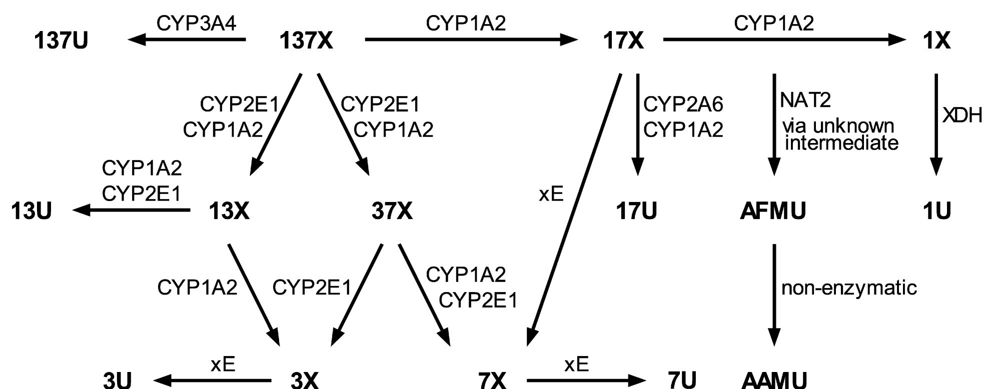

Fig. 1. Metabolic pathways of caffeine and the major enzyme forms involved. If more than one enzyme form is involved in a transformation step, that of greater significance is itemised at the top. The enzymes in some transformation steps have not yet been identified to the best of our knowledge (xE). The scheme is based on original and reviewed data [1,27–33]. The full names of the metabolites and enzymes are given in the first section of the Experimental section and in the Introduction, respectively.

abbreviations used for the metabolites (for full names see first paragraph of the Experimental section). Ratios of metabolites are normally used for phenotyping. Two different ratios have been employed as a measure of CYP1A2 activity: ([17X]+[17U])/[137X] (Ratio 1, R1) [1] and ([AAMU]+[AFMU]+[1X]+[1U])/[17U] (R2) [1,2]. The activity of CYP2A6 is represented by [17U]/[17X] (R3) [3,4] and that of XDH by [1U]/[1X] (R4) [1,5,6]. The activity of NAT2 is reflected in the ratio ([AAMU]+[AFMU])/([AAMU]+[AFMU]+[1X]+[1U]) [1,2,7,8].

Common procedures for the determination of caffeine metabolites involve HPLC with UV detection after liquid–liquid extraction of an acidified urine sample [5,6,9–11]. These procedures have some shortcomings in the extraction as well as the detection steps. Extraction is laborious and incomplete due to the heterogeneous physico-chemical properties of the metabolites. In particular, extraction by organic solvents is relatively poor for 1U, AFMU and AAMU [8]. This problem is aggravated by the spontaneous decomposition of AFMU to AAMU [2,12] and the poorer extraction of AAMU compared to AFMU. Although decomposition can be avoided by acidification of the urine sample [6,13], accurate quantification requires either separate, extraction-corrected determination of either metabolite, or their complete conversion into AAMU under basic conditions. Detection by UV is often insufficiently selective, as urine contains numerous UV-absorbing compounds which are not derived from caffeine but may overlay peaks of caffeine metabolites.

We have developed and validated a sensitive and selective HPLC method, coupled with tandem mass spectrometry (MS–MS), for the rapid detection of the major caffeine metabolites in human urine. It does not require any solid-phase or liquid–liquid extraction.

2. Experimental

2.1. Chemicals and standard solutions of reference compounds

1,3,7-Trimethyluric acid (137U), 1,3,7-trimethylxanthine (137X, caffeine), 1,3-dimethyluric acid (13U), 1,3-dimethylxanthine (13X, theophylline), 1,7-dimethyluric acid (17U), 1,7-dimethylxanthine (17X, paraxanthine), 1-methyluric acid (1U), 1-methylxanthine (1X), 3-methyluric acid (3U), 3-methylxanthine (3X), 7-methyluric acid (7U), 7-methylxanthine (7X), 3,7-dimethylxanthine (37X, theobromine), 3-propylxanthine (3PX, internal standard 1, I.S.1) and 7β-hydroxypropyltheophylline (IPX, internal standard 2, I.S.2) were obtained form Sigma (Deisenhofen, Germany). 5-Acetylamino-6-amino-3-methyluracil (AAMU) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU) were kindly provided by Drs. A. Kuhlow and E. Richter (Institute of Toxicology, University of Munich, Germany). All

solvents were of HPLC gradient grade and filtered through a 0.2 μm membrane filter.

In general, stock solutions of standards were prepared in methanol (1.00 mg/ml, 4.76–6.02 m*M*). If necessary, NaOH (10 *M*) was added to a suspension of the compound (1 mg) in methanol (500 μl) until it dissolved. The solution was neutralised with 1 or 10 *M* acetic acid and completed to 1000 μl with methanol. AFMU (0.29 mg/ml, 1.28 m*M*) and AAMU (0.24 mg/ml, 1.21 m*M*) were obtained as solutions in 0.01 *M* formic acid with 6% methanol (v/v) and stored at −80 °C.

Diluted solutions in methanol or blank urine were prepared daily. Blank urine was obtained from a volunteer after 5 days on a methylxanthine-free diet. Urine was then diluted with acetic acid and methanol, as described in the next section. For calibration, each metabolite was used in various concentrations and the resulting peak area was compared to that of a fixed amount of the respective internal standard.

2.2. Sample preparation

An aliquot of urine (50 or 250 μl) was diluted with an aqueous solution of acetic acid (0.05% v/v) and methanol (10% v/v) and 50 μl of an internal standard solution containing 5 μ*M* 3PX (I.S.1) and 4 μ*M* IPX (I.S.2) in the same solvent to give a total volume of 500 μl. Unless specified otherwise, AFMU was converted into AAMU by adding 50 μl NaOH (1 *M*). After 15 min, the solution was neutralised with acetic acid, and the volume was completed to 1000 μl by the addition of an aqueous solution of acetic acid (0.05% v/v) and methanol (10% v/v). Preceding experiments had demonstrated that this conversion is complete and results in 100% of AAMU (data not shown). The sample was then centrifuged at 8000 *g* for 10 min, before the supernatant was used for HPLC–MS–MS analysis.

2.3. Chromatographic system

A Waters 2960 HPLC system (Eschborn, Germany) was connected to a triple quadrupole mass spectrometer fitted with an electrospray source (Quattro II with Z-spray source, Micromass, Manchester, UK). Aliquots of 20 μl of urine sample (prepared as described in the preceding paragraph) or a solution of reference compounds in methanol were injected. Samples were separated on an Ultrasphere ODS column (250×4.6 mm, 5 μm; Beckman, Unterschleissheim, Germany). For elution, a gradient of 0.05% acetic acid (v/v) with 3% methanol (v/v) and 1.5% of 2-propanol (v/v) (solvent A), methanol (solvent B), and 0.05% acetic acid (v/v) (solvent C) was used. The gradient started with 47% A, 6% B and 47% C. This ratio was maintained for 8 min. Then, it was changed within 12 min to 90% A and 10% B, and then, within 8 min, to 55% A and 45% B. This ratio was maintained for 2 min before returning to the initial conditions within 2 min. These elution conditions were maintained for 5 min before the next injection. The flow-rate was 0.6 ml/min and the column temperature was 30 °C. Samples were maintained at 4 °C in an autosampler.

2.4. MS–MS analysis

The methods for detecting methylxanthines by electrospray-MS–MS were worked out with authentic standards, dissolved in methanol and injected into the HPLC system. Two HPLC runs were necessary for each sample, as some metabolites could only be detected in the negative ionisation mode with sufficient sensitivity, whereas other metabolites required the positive mode. The multiple reaction monitoring (MRM) mode was utilised with a cone voltage of 25 V (3X, 7X, 3U and 7U) or 30 V (other compounds). Parent and daughter ions (Table 1) were selected using intensity and selectivity as the criteria.

2.5. Studies in humans

Healthy, non-smoking volunteers (two males and four females, 23 to 34 years old) refrained from the consumption of methylxanthine-containing drinks, foods and drugs for at least 48 h before drinking coffee and during the observation period. They took a cup of instant coffee containing a defined amount of caffeine (4 mg per kg body mass). "Blank urine" was taken shortly before coffee consumption. The bladder was completely voided after 4 h. Then the total urine between hour 4 and hour 5 was collected ("challenge urine"). Samples were frozen immediately at −40 °C. For a good urine flow, subjects drunk 200 ml of water per hour after coffee con-

Table 1
Conditions used for the detection of caffeine metabolites by LC–MS–MS

Compound	Ionisation mode	Parent ion (m/z)	Daughter ion (m/z)	Collision energy (eV)	Retention time (min)	Limit of detection[a] (μM)
AFMU	Negative	224.7	196.7	15	4.7–4.8	1
AAMU	Negative	196.7	126.7	22	5.7–5.9	1
3U	Negative	180.6	137.6	14	7.6–7.8	n.d.[b]
1U	Negative	181.0	83.0	25	9.3–9.7	0.05
7U	Negative	180.6	165.6	14	10.0–10.3	n.d.
7X	Negative	164.4	121.8	14	10.7–10.9	n.d.
1X	Negative	164.4	107.7	20	12.0–12.5	0.05
3X	Negative	164.6	121.6	14	12.9–13.1	n.d.
13U	Negative	194.5	179.5	22	15.0–15.7	0.1
17U	Negative	194.5	179.5	22	20.5–21.0	0.05
137U	Negative	208.7	136.7	22	22.6–22.8	0.05
3PX (I.S.1)	Negative	192.6	149.6	22	23.7–23.9	0.05
37X	Positive	180.8	107.5	25	15.9–16.6	0.05
17X	Positive	180.6	123.5	20	21.2–21.6	0.05
13X	Positive	180.6	123.5	20	21.9–22.2	0.05
137X	Positive	194.4	137.6	22	24.1–24.3	0.05
IPX (I.S.2)	Positive	238.6	180.6	20	24.3–24.5	0.05

[a] With a 20 μl injection volume.
[b] n.d., not determined.

sumption until the collection of challenge urine. One volunteer (subject 1) refrained from methylxanthine-containing foods for 5 days. The urine collected from this person was used as blank urine for spiking.

3. Results

3.1. Establishment of conditions for the detection of caffeine metabolites by HPLC–MS–MS

Mass spectra were recorded for all reference compounds in the positive and negative electrospray ionisation modes. In general, the m/z giving the strongest signal was selected as the parent ion for the second fragmentation. From the second fragmentation, a daughter ion showing a strong signal was chosen for the MRM analysis. Different collision and cone energy levels were tested for the optimisation of detection. The optimised parameters and the retention times in HPLC are shown in Table 1. It was not possible to detect all metabolites in the same ionisation mode with sufficient sensitivity. Therefore, two chromatograms were recorded, one in the positive ionisation mode, the other in the negative ionisation mode.

All compounds were chromatographically separated and/or selectively detected in the MRM modes used. There were no interferences among the standard compounds, as they gave the same signals when chromatographed either individually or as a mixture of all 17 compounds listed in Table 1. Using an injection volume of 20 μl, the intensity of the signal increased linearly with the concentration of the compound, at least in the following ranges: 1U (0.25 to 15 μM), 13U (0.125 to 7.5 μM), 137U (0.05 to 12.5 μM), AAMU and AFMU (1.25 to 100 μM) and all other compounds of Table 1 (0.05 to 25 μM) (correlation coefficient $r^2 > 0.99$). 3X, 7X, 3U and 7U are very minor metabolites of caffeine [1] and have not been used in phenotyping. It was important to show that they do not co-elute with their isomers (1X and 1U) that are prominent caffeine metabolites. The analysis of 3X, 7X, 3U and 7U was not further validated, although small amounts of all these metabolites were regularly detected in the urine of volunteers after drinking coffee (data not shown).

3.2. Validation of the method using spiked urine

Blank urine, obtained from volunteer 1 after 5 days on a methylxanthine-free diet, was spiked with

varying concentrations of caffeine metabolites. This blank urine did not contain any components with the retention time and mass-spectrometric properties of AAMU, AFMU, 13U, 137U, 137X or the internal standards employed. However, it embodied substances that showed the same retention times and were detected by the same MRM mode as authentic 1U, 1X, 17U, 37X and 17X (Fig. 2A and B). The full daughter-ion spectra of these components of urine were similar to those of the standards, indicating that they really represented these methylxanthine metabolites rather than other confounding chemicals. Their levels, particularly in blank urine after 48 h on a methylxanthine-free diet, were highly variable and sometimes even exceeded those found in challenge urine from the same subject. Representative chromatograms from spiked urine samples are presented in Fig. 2C and D. In this experiment the spiked urine was not treated with NaOH in order to detect AAMU and AFMU separately. The signal in blank urine (if present for a given metabolite) was subtracted from the signal observed with spiked urine. The net value was then compared with the signal produced per concentration unit, when the same metabolite was injected in methanol rather than spiked urine. The intra-day and inter-day accuracy were between 91.5 and 109.7% at the three highest spike concentrations (approximately 0.25 to 2.5 μ*M* in the injected sample, involving a 20-fold dilution compared to the spiked urine sample) (Table 2). At the lowest spike concentration level (approximately 0.125 μ*M* in the 20-fold diluted samples), the recoveries were lower for most metabolites determined in the negative ionisation mode. The range was between 71.0 and 104.2%. Intra- and inter-day precision (standard deviation expressed as a percentage of the mean, RSD) was from 1.3 to 10.3% (depending on the metabolite and its concentration, Table 2). The limit of quantification was lower than 0.05 μ*M* (1 pmol/injection) for most metabolites. However, it was nearly 0.1 μ*M* (2 pmol/injection) for 13U, and substantially higher (1 μ*M*, 20 pmol/injection) for AAMU and AFMU. A four-fold, rather than a 20-fold, dilution of spiked urine samples gave similar recovery rates, whereas lower dilution factors led to a decrease in the recovery (data not shown).

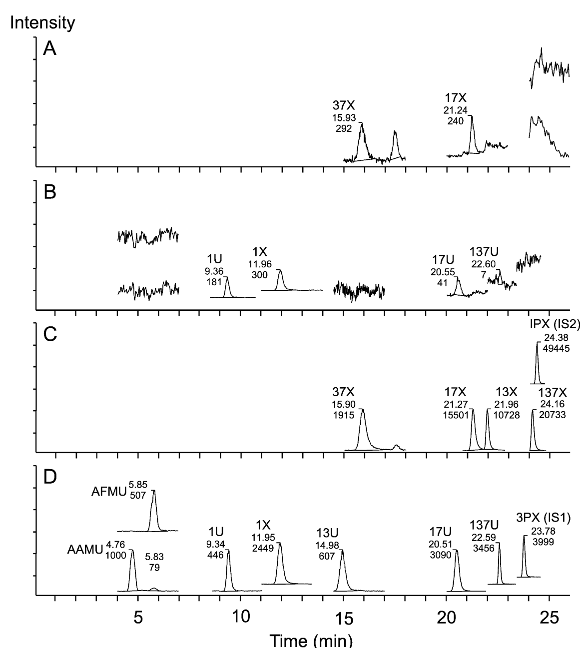

Fig. 2. HPLC–MS–MS chromatograms of a blank urine (obtained after 5 days on a methylxanthine-free diet) (A, B) and of the same urine spiked with 12.5 to 15 μ*M* of 1U, 1X, 13U, 17U, 137U, 3PX (I.S.1), 37X, 17X, 13X, 137X and IPX (I.S.2), 250 μ*M* AAMU and 300 μ*M* AFMU (C, D). The samples were prepared as described in the Experimental section but with omission of the NaOH treatment. Spiked and unspiked urines were diluted 20-fold during the sample preparation. Each xanthine derivative was recorded in the respective MRM mode (as indicated in Table 1) using positive (A, C) or negative (B, D) electrospray ionisation. The chromatograms in the various MRM modes are only presented around the retention time where corresponding metabolites eluted. Peaks are labelled with the short name of the compound, the retention time and the absolute area under the peak.

3.3. Urinary levels of methylxanthines before and after consumption of coffee

Fig. 3 shows chromatograms of urinary caffeine metabolites found in a healthy subject shortly before caffeine consumption (after 2 days on a methylxanthine-free diet) and 4 h afterwards. Whereas the signals of the internal standards were unchanged, the levels of all metabolites were increased after consumption of coffee. The increase was only 1.6-fold for 37X (a metabolite not used for calculating metabolic ratios), and 4.0 to 10.5 for AAMU and AFMU, 13U, 1X and 1U; it exceeded >20-fold for 17X, 13X, 137X, 17U and 137U. The result is representative, in as much as caffeine metabolites

Table 2
Intra-day and inter-day accuracy (recovery) and precision (standard deviation as percentage of the mean, RSD) of the determination of caffeine metabolites and internal standards in spiked urine

Compound	Concentration (μM)	Intra-day comparison		Inter-day comparison	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
AAMU+ AFMU[a]	25+30	102.7	4.9	93.2	5.6
	12.5+15	102.9	5.4	94.6	10.0
	6.25+7.5	104.2	4.0	94.0	8.1
	3.13+3.75	95.2	9.0	90.7	7.1
1U	3.0	100.8	1.5	98.5	7.1
	0.75	101.4	3.8	99.5	4.1
	0.3	95.7	2.4	95.8	7.0
	0.05	71.0	3.0	85.6	6.4
1X	3.0	103.6	1.6	101.4	3.0
	0.75	105.3	1.5	100.6	4.1
	0.3	96.9	1.5	93.7	7.0
	0.15	84.2	2.7	90.7	6.8
13U	2.5	106.6	2.8	101.0	4.2
	0.625	102.9	3.3	98.3	4.9
	0.25	99.6	1.5	99.5	7.1
	0.125	83.9	3.6	88.7	7.1
17U	2.5	96.5	1.3	99.9	2.9
	0.625	107.3	2.1	103.8	6.0
	0.25	100.1	1.7	96.7	5.1
	0.125	82.1	2.8	90.8	4.1
137U	2.5	107.6	3.2	100.1	2.8
	0.625	109.7	2.2	102.9	5.5
	0.25	98.0	2.2	99.5	2.6
	0.125	86.8	4.1	94.4	3.9
3PX (I.S.1)	2.5	101.4	3.2	100.4	2.3
	0.625	104.4	1.9	101.3	3.8
	0.25	97.9	1.4	97.9	3.9
	0.125	83.3	2.7	90.8	4.3
37X	2.5	105.5	2.2	98.6	5.1
	0.625	108.3	1.7	93.2	2.7
	0.25	103.4	2.9	101.1	6.0
	0.125	101.6	3.8	93.5	8.8
17X	2.5	99.8	2.2	98.6	3.5
	0.625	102.8	1.8	98.5	4.5
	0.25	96.0	1.5	96.2	2.0
	0.125	101.1	4.7	97.1	4.9
13X	2.5	97.7	2.5	99.5	3.6
	0.625	101.8	1.5	96.5	5.1
	0.25	93.5	2.0	91.5	3.4
	0.125	98.4	4.0	94.3	4.4
137X	2.5	97.6	3.2	97.2	3.6
	0.625	100.8	2.8	99.4	5.9
	0.25	101.7	2.2	93.5	8.7
	0.125	102.1	2.8	89.9	10.3
IPX (I.S.2)	2.5	100.1	2.4	97.9	2.6
	0.625	104.4	2.3	100.6	4.5
	0.25	106.0	3.2	100.8	6.4
	0.125	104.2	2.7	98.1	5.3

Urine was spiked concurrently with all methylxanthines (four series using different concentration levels), treated with base and analysed. The signal of the corresponding metabolite in blank urine was subtracted from that of the spiked urine. The resulting net signal was then compared to the signal produced by the same compound injected in methanol (linear range). For the intra-day comparison, six urine samples were used with each spike–concentration level. For the inter-day comparison, three urine samples were used on each of 3 days with each condition. The concentrations given here refer to the amount of standard in the 20-fold diluted sample after the preparation procedure.

[a] Detected as AAMU.

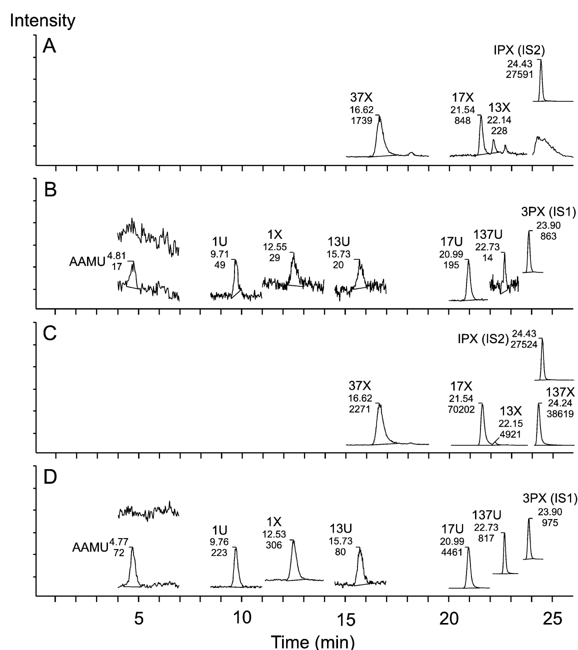

Fig. 3. Detection of caffeine metabolites in human urine before (A, B) and after (C, D) consumption of coffee using HPLC–MS–MS. The volunteer (subject 1-2 of Tables 3 and 4) refrained from the consumption of a methylxanthine-containing diet, drinks and drugs. Then, blank urine was collected and coffee (4 mg per kg body mass) was consumed. Urine was collected again in the 4 to 5 h interval after coffee drinking. Urines were diluted 20-fold during the sample preparation. Each xanthine derivative was recorded in the respective MRM mode (as indicated in Table 1) using positive (A, C) or negative (B, D) electrospray ionisation. The chromatograms in the various MRM modes are only presented around the retention time where corresponding metabolites eluted. Peaks are labelled with the short name of the compound, the retention time and the absolute area under the peak.

were detected in the blank urine of all six subjects investigated and some of these metabolites were strongly elevated after consumption of coffee. Table 3 shows the levels of urinary caffeine metabolites observed in these subjects. One subject performed the whole experiment on three separate occasions within 4 weeks (1-1, 1-2 and 1-3 in Table 3). Levels and patterns of the metabolites were similar between experiments 1 and 2. In experiment 3 the concentrations of most metabolites were approximately four times higher than in the initial experiments; these differences were inversely correlated with the volume of urine sampled (175, 240 and 45 ml in experiments 1, 2 and 3, respectively). However, the concentrations of 17X and 13X were only increased two-fold between the initial and last experiments, and that of 137X (caffeine) was virtually unchanged. Thus the absolute amounts of these metabolites excreted were lower in the last experiment than previously.

Each urine sample was analysed three times on separate days. In this trial, the inter-day variation of determination of some metabolites (in particular, AAMU + AFMU and 1U) was larger than previously observed with the spiked urine. The reasons have not been elucidated. The fact that the different metabolites were present at strongly varying levels in the caffeine challenge trial, but added at equal levels (except AAMU and AFMU) to the spiked samples, may have played a role. This variation was not a serious problem, as it was much smaller than the differences occurring between different urines. For example, the level of acetylated metabolites (AAMU + AFMU) was more than 100-fold higher in urine 1-3 than in urine 3.

3.4. Metabolic ratios

As mentioned in Section 3.3, some methylxanthine metabolites were still present in urine at substantial levels after 2 days on a methylxanthine-free diet, when the challenge with caffeine was performed. It is probable that methylxanthine metabolites from previous exposures continued to be present 4 h later (perhaps at a somewhat lower level), when urine was sampled again for studying the metabolites resulting from the caffeine challenge. We have determined the metabolic ratios introduced by other authors (Table 4). In agreement with previous studies we have not made any corrections for metabolites present in blank urine. With either metabolic ratio for phenotyping CYP1A2, subjects 1 and 6 showed higher activities of this enzyme than the other four subjects investigated. This was true with all three experiments conducted in subject 1, although both ratios were higher in the last experiment than in the initial experiments with this subject. R3 (reflecting CYP2A6 activity) varied 5.5-fold among the subjects investigated. R4 (indicative of XDH) showed the lowest variation among the investigated subjects and was virtually identical in all three trials with subject 1. NAT2 activity (indicated by R5) was high in

Table 3
Urinary caffeine metabolites in six subjects after ingestion of coffee (4 mg caffeine per kg body mass)

Metabolite	Urinary concentration (μM)							
	1-1	1-2	1-3	2	3	4	5	6
AAMU + AFMU[a]	107±22	125±31	425±78	39±10	3.2±2.0	2.5±0.8	11±3	60±18
1U	77±12	96±15	399±40	26±8	13±4	10±2	23±7	102±20
1X	31±3	39±1	164±6	12±3	17±2	14±1	20±2	44±5
13U	8.7±0.9	11±2	43±4	2.6±0.1	2.1±0.3	7.2±0.8	2.1±0.9	7.3±0.6
17U	23±1	26±4	77±13	11±1	7.2±0.3	14±1	12±1	30±1
137U	2.8±0.5	3.6±0.9	14±4	0.9±0.1	1.2±0.1	4.6±0.8	1.2±0.1	2.1±0.3
37X	14±1	13±1	55±1	3.2±0.2	9.1±0.7	4.4±0.2	5.8±0.5	5.3±0.1
17X	32±3	43±5	77±8	17±1	37±5	16±2	36±4	27±3
13X	1.6±0.2	1.8±0.2	3.5±0.6	0.7±0.2	1.3±0.2	1.9±0.2	1.3±0.2	1.0±0.2
137X	16±1	17±1	18±2	17±1	22±2	27±2	25±2	15±1
Volume of urine (ml)	175	240	45	450	370	150	280	160

Urine was collected in the 4–5 h interval after consumption of coffee. Urines were diluted five- to 20-fold during the preparation. Subject 1 was analysed on three separate occasions (columns 1-1, 1-2 and 1-3). Subjects 1 and 2 were males. Subjects 3 through 6 were females. Each sample was analysed three times on different days. Values are means±SD of these measurements. Values of blank urine were not subtracted.

[a] Detected as AAMU (after conversion of AFMU to AAMU by base treatment).

subjects 1 and 2, intermediate in subject 6, and low in subjects 3, 4 and 5. These ranges and frequency distributions of the various metabolic ratios may not be representative, as the number of subjects studied was very small.

4. Discussion

We have developed a simple procedure for the concurrent determination of 15 caffeine metabolites and two internal standards. It was validated for the quantification of 11 urinary metabolites commonly used for phenotyping of various xenobiotic-metabolising enzymes. The new procedure has several strengths compared to the established methods combining HPLC with UV detection. (i) It is fast. Samples can be processed within 15 min. The total HPLC run time is 60 min (including one run in the positive ionisation mode and one run in the negative ionisation mode). (ii) It does not require any extraction step, and it does not lead to loss of substance through any other mechanisms. (iii) The high selectivity of mass spectrometry in the MRM mode allows the separate detection of some metabolites (e.g. caffeine and the internal standards) even if their elution times do not differ significantly. Other metabolites that generated similar primary mass spectra and daughter ion fragmentation patterns (e.g. 13U and 17U, or 13X and 17X) could readily be sepa-

Table 4
Urinary caffeine metabolic ratios in six subjects (columns headed 1-1 to 6) after ingestion of coffee

Ratio, enzyme phenotyped	1-1	1-2	1-3	2	3	4	5	6
R1, CYP1A2	3.4±0.2	4.1±0.1	8.8±1.3	1.7±0.1	2.0±0.1	1.1±0.1	1.9±0.1	3.9±0.2
R2, CYP1A2	9.3±1.0	10.1±1.0	13.0±1.0	6.9±0.5	4.6±0.5	1.9±0.1	4.4±0.7	7.0±1.4
R3, CYP2A6	0.72±0.10	0.63±0.16	1.01±0.26	0.66±0.07	0.20±0.02	0.85±0.08	0.34±0.05	1.15±0.12
R4, XDH	2.5±0.3	2.5±0.4	2.4±0.2	2.2±0.2	1.5±0.3	0.7±0.2	1.2±0.2	2.8±0.2
R5, NAT2	0.50±0.07	0.48±0.06	0.43±0.05	0.51±0.12	0.10±0.07	0.09±0.03	0.20±0.03	0.28±0.04

Subject 1 was analysed on three separate occasions (columns 1-1, 1-2 and 1-3). Subjects 1 and 2 were males. Subjects 3 through 6 were females. The ratios were calculated from the raw data of Table 3, without subtracting the levels of metabolites in blank urine, using the formulas given in the Introduction. Values are means±SD of three measurements in the same urine sample.

rated by HPLC. The high selectivity of the detection method was also manifested by the lack of confounding signals by any other components of urine. Such signals are a significant problem with the conventional HPLC–UV method despite the usage of an extraction step. The high selectivity of the new method led to a high sensitivity. For example, various caffeine metabolites could be detected in ''blank urine'', obtained after 2 to 5 days on a methylxanthine-free diet.

The HPLC–MS–MS method has the following, minor shortcomings. (i) We did not succeed in detecting all metabolites with sufficient sensitivity using a single ionisation mode. Therefore, two runs were required for each sample, one with negative ionisation and one with positive ionisation. (ii) The intensity of the mass spectrometric signals was linear to the sample concentration only within a limited range ($r^2 > 0.99$ within an at least 25-fold concentration range). Ion suppression was observed at high metabolite concentrations, particularly in undiluted urine (data not shown). Reduced relative intensities were also observed at low concentrations of some metabolites when studied in spiked urine but not when directly injected in methanol (Section 3.2); the mechanism involved in this effect was not elucidated. If different metabolites are present in urine in very different concentrations, the accuracy and precision of the determination may be improved by conducting several HPLC runs using different dilutions of the urine sample. However, practical exploration of the method in caffeine-challenged subjects showed that one run was usually sufficient if an error of a few percent could be tolerated (data not shown).

To our knowledge this is the first description of an extractionless HPLC–MS method for the determination of almost all caffeine metabolites. Baud-Camus et al. [8] recently published an extractionless HPLC method combined with simple mass spectrometry; this method was only validated for AAMU, 1X and 1U. Therefore, it can be utilised for phenotyping NAT2 and XDH, but not for phenotyping CYP1A2 and CYP2A6. Kanazawa et al. [14] developed a HPLC–MS method for the determination of theophylline and eight of its metabolites (which are also formed from caffeine). As with our method, two HPLC runs (in the positive and the negative ionisation modes) were required. Since simple mass spectrometry was used, extraction was required in the sample preparation. Furthermore, this method has been validated for plasma samples but not yet for urine samples. In several other studies, caffeine, related compounds and/or individual metabolites have been determined in food, body fluids or in vitro incubations using GC–MS or HPLC–MS [15–18].

Ratios of the urinary concentrations of certain caffeine metabolites are often used as measures for the activities of various xenobiotic-metabolising enzymes. HPLC–UV has been used in most of these studies. The results of the present study indicate that HPLC–MS–MS is a very promising alternative. This method may give particularly reliable results as it does not require an extraction and is virtually free from signals from other urinary components. However, it revealed the presence of certain caffeine metabolites even in blank urine obtained after 2 to 5 days on a methylxanthine-''free'' diet. We suspect that some caffeine metabolites may persist at low levels in the organism for a relatively long time and that caffeine is present in small amounts in various food items without being declared. As long as high challenging doses of caffeine are used (as in the case of this HPLC–MS–MS study as well as in the published HPLC–UV studies) these traces of metabolites in blank urine may not create a serious problem in the determination of metabolic ratios. The values obtained for these ratios in the present study are only discussed briefly, as the number of subjects was very small.

The values for the arithmetic mean $\bar{x}$ or the median $\tilde{x}$ of R1 $\{=([17X]+[17U])/[137X]\}$, a measure of CYP1A2 activity$\}$ were lower in the present study ($\bar{x}=3.4$, $\tilde{x}=2.7$) than in various previous studies using HPLC–UV (e.g. $\bar{x}=5.0$ [13], $\bar{x}=10.6$ [19], $\bar{x}=10.8$, $\tilde{x}=9.8$ [6], $\bar{x}=8.8$ [20]). R2 $\{=([AAMU]+[AFMU]+[1X]+[1U])/[17U]\}$ is another measure of CYP1A2 activity. Its values in the present study ($\bar{x}=7.1$, $\tilde{x}=6.9$) were similar to figures determined using HPLC–UV (e.g. $\bar{x}=5.6$ [21], $\bar{x}=6.2$ [13]). We observed a correlation between the values for R1 and R2. Among others, the third values for R1 and R2 of subject 1 were clearly higher than the corresponding figures from the initial trials. McQuilkin et al. [22] observed strong within-

subject variation of CYP1A2 over a period of 3 weeks, whereas NAT2 activity was much less variable. Therefore, lifestyle, in addition to genetic factors [23], appears to have a substantial influence on CYP1A2 activity. However, in the present study, the variation in R1 and R2 within subject 1 was also associated with a change in urinary flow. It will be necessary to study the possible influence of urinary flow on metabolic ratios. The values for the mean $\bar{x}$ and median $\tilde{x}$ of R3 (=[17U]/[17X], a measure of CYP2A6 activity) were much smaller in the present study ($\bar{x}=0.69$, $\tilde{x}=0.69$) than in various previous studies using HPLC–UV (e.g. $\bar{x}=1.5$ [24], $\bar{x}=1.6$ [25], $\bar{x}=2.0$, $\tilde{x}=1.6$ [4]). Our values for R4 (=[1U]/[1X], a measure of XDH activity) were higher ($\bar{x}=2.0$, $\tilde{x}=2.3$) than those previously described using HPLC–UV (e.g. $\bar{x}=1.3$, $\tilde{x}=1.2$ [6], $\bar{x}=1.2$ [24], $\bar{x}=1.3$ [25], $\tilde{x}=0.95$ [5]). R5 {= ([AAMU] + [AFMU]) / ([AAMU] + [AFMU] + [1X] + [1U])} is used as a measure of NAT2 activity. Baud-Camus et al. [8], who also used an extractionless HPLC–MS method, defined slow and fast acetylators as subjects with R5 below and above 0.22, respectively. Three of six test subjects belong to each of these groups in the present study, a result which is consistent with a frequency of nearly 50% slow acetylators in the Caucasian population [26].

In conclusion, some metabolic ratios determined by HPLC–MS–MS may differ somewhat from those determined using HPLC–UV, but the differences appear to be moderate. Moreover, some of the apparent differences may not be due to the detection method, but result from other differences between the experimental protocols used (such as dose of caffeine and time interval for urine sampling).

The HPLC–MS–MS method developed requires more expensive instrumentation than the conventional HPLC–UV method. Otherwise, it appears to be superior, as the sample preparation is simpler and faster, and the detection is much more specific. A large number of caffeine metabolites can be determined concurrently.

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