JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2005; **40**: 307–316
Published online 31 January 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jms.785

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Measurement of caffeine and five of the major metabolites in urine by high-performance liquid chromatography/tandem mass spectrometry

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Received 9 September 2004; Accepted 2 November 2004

Analysis of caffeine and its metabolites is of interest with respect to caffeine exposure, for kinetic and metabolism studies and for opportunistic *in vivo* estimation of drug metabolizing enzyme activity in humans and animals. For the latter, analysis is usually done by high-performance liquid chromatography (HPLC) with UV detection. However, this method is close to the detection limit for certain of the metabolites and requires very long chromatography, 30–60 min. We have developed a fast method for the quantification of caffeine and its metabolites 1-methylxanthine, 1-methyluric acid, 1,7-dimethyluric acid, 5-acetylamino-6-amino-3-methyluracil (AAMU) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU) by HPLC tandem mass spectrometry (MS/MS) in urine that requires only its dilution with buffer and centrifugation before injection into the HPLC/MS/MS system. The chromatography lasts 7 min and is followed by 4.5 min for re-equilibration of the HPLC column, giving a total analysis time of 11.5 min. The method provides a great sensitivity improvement with detection limits for all analytes $\leq$25 nM in real samples. Also, the analysis provides much improvement in capacity to ~125 samples per 24 h. Intra- and inter-day coefficients of variation of a single analysis are <6.5% for all the analytes. The inter-day coefficient of variation of duplicate analyses is <4.8% for all analytes. The method is automated, including automated integration, and it is fast, robust and suitable for large-scale investigations in humans and animals. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: Caffeine; metabolites; electrospray; urine

INTRODUCTION

Estimation of drug metabolizing enzyme activity in humans is a difficult task. One approach is the use of probe drugs metabolized by known enzyme systems. Studies with such drugs have provided much information about the regulation of human drug metabolism by sex, exercise, environmental factors, life-style factors such as smoking and interaction with other drugs[1,2] also in animals[3] and *in vitro*.[4] The use of well-known probe drugs poses a small but real risk and the opportunistic use of a probe drug, i.e. a drug or substance that is ingested on a regular basis, is preferable. Caffeine is such a substance, as it is ingested by a large part of the population, e.g. in tea or coffee, and it has been shown that it is eliminated by cytochrome P450 1A2 (CYP1A2), *N*-acetyltranferase 2 (NAT2) and xanthine oxidase (XO).[5–8]

CYP1A2 belongs to the cytochrome P450 superfamily[9] that represents a major mechanism by which foreign compounds are eliminated by humans, animals and other organisms. A particular class of foreign compounds is drugs. Cytochrome P450 is often assigned a double-edged sword feature[10]. CYP metabolism often reduces the biological activity, but the metabolism can also produce highly reactive species, e.g. activation of carcinogens. The case of the drug paracetamol (acetaminophen) is a prime example of metabolic activation as the basis of organ specific toxicity.[11]

For investigations in humans, and also experimental animals, it is a clear advantage if such enzyme activities can be estimated non-invasively with non-toxic compounds.

Abbreviations: 13U, 1,3-dimethyluric acid; 137U, 1,3,7-trimethyluric acid; 17U, 1,7-dimethyluric acid; 37U, 3,7-dimethyluric acid; 17U*, [2-^{13}C, 1,3-$^{15}N_2$]-1,7-dimethyluric acid; 1U, 1-methyluric acid; 3U, 3-methyluric acid; 7U, 7-methyluric acid; 1U*, [2-^{13}C, 1,3-$^{15}N_2$]-1-methyluric acid; 137X, caffeine; 137X*, [2-^{13}C, 1,3-$^{15}N_2$]caffeine; 1X, 1-methylxanthine; 13X, 1,3-dimethylxanthine; 17X, 1,7-dimethylxanthine; 3X, 3-methylxanthine; 37X, 3,7-dimethylxanthine; 7X, 7-methylxanthine; 1X*, [2-^{13}C, 1,3-$^{15}N_2$]-1-methylxanthine; AAMU, 5-acetamido-6-amino-3-methyluracil; AAMU*, [2-^{13}C, 1,3-$^{15}N_2$]-5-acetamido-6-amino-3-methyluracil; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; AFMU*, [2-^{13}C, 1,3-$^{15}N_2$]-5-acetylamino-6-formylamino-3-methyluracil; FWHM, full width at half-maximum; HPLC, high-performance liquid chromatography; $[M + H]^+$, protonated ion in the positive ion mode; $[M - H]^-$, deprotonated ion in the negative ion mode; MS/MS, tandem mass spectrometry; NAT2, *N*-acetyltransferase 2; Q1, first quadrupole; Q3, third quadrupole; UV, ultraviolet; XO, xanthine oxidase.

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Contract/grant sponsor: Danish Research Council; Contract/grant number: 9501768 CPNC.

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Several methods based on high-performance liquid chromatography with UV detection (HPLC/UV) have previously been published for the analysis of caffeine and its metabolites with the aim of the non-invasive estimation of CYP1A2, NAT2 and xanthine oxidase activities *in vivo*, in both humans and animals. For our purpose, we found the present HPLC/UV[5–8,12] methods problematic owing to limitations on selectivity, specificity, sensitivity, reproducibility or resolution. Furthermore, several methods do not give the possibility of measuring the AAMU metabolite that is a breakdown product from AFMU on storage without acidification. When retention times are long, analysis time and thereby cost make it difficult to apply the method to large-scale epidemiological studies. This prompted us to develop a fast method based on HPLC coupled with tandem mass spectrometry (MS/MS) with a minimum requirement on sample preparation. Also, because the method includes the degradation product AAMU, it can be used to estimate CYP1A2 activity on samples that have not been stored in acid to stabilize the AFMU metabolite.

Four methods based on LC/MS have recently been published. The first[13] of these methods only measures 1-methyluric acid (1U), 1-methylxanthine (1X) and AAMU. The second method measures only xanthine derivatives.[14] The third method[15] does not include either AAMU or AFMU and a run lasts 50 min and prior solid-phase extraction is required. The fourth method[16] measures all the metabolites included in our method and some additional metabolites. The analysis time of this method is, however, fairly long, 37 min (plus an extra run), compared with 11.5 min. The analysis of the extra metabolites is not necessary to calculate the enzyme activities of CYP1A2, NAT2 phenotype and xanthine oxidase. Furthermore, the method presented here appears more sensitive and with the use of stable isotope-labelled internal standards the reliability is increased, and the peaks are very intense with complete baseline separation. All together this allows reliable auto-integration.

We conclude that the method presented here is rugged, fully automated and fast. It can be used on fresh and frozen urine samples, acidified or not, for the estimation of CYP1A2 activity, NAT2 phenotype and xanthine oxidase activity, non-invasively from opportunistic caffeine intake, even at very low doses. It requires minute amounts of urine, and is quantified by the isotope dilution method. The method is very robust and shows high performance in terms of sample throughput. In addition, it can be used on small experimental animals or even in cellular systems or purified enzyme systems because of the high sensitivity.

EXPERIMENTAL

Chemicals

Caffeine (137X), 1-methylxanthine (1X), 1-methyluric acid (1U) and 1,7-dimethyluric acid (17U) were purchased from Sigma (St. Louis, MO, USA). 5-Acetylamino-6-amino-3-methyluracil (AAMU) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU) were gifts from Morten Kall (Danish Veterinary and Food Administration). AFMU was also donated by René Fumeaux (Nestlé, Lausanne, Switzerland). [2-^{13}C, 1,3-$^{15}N_2$]caffeine (137X*) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). [2-^{13}C, 1,3-$^{15}N_2$]-1-methylxanthine (1X*), [2-^{13}C, 1,3-$^{15}N_2$]-1-methyluric acid (1U*), [2-^{13}C, 1,3-$^{15}N_2$]-1,7-dimethyluric acid (17U*), [2-^{13}C, 1,3-$^{15}N_2$]-5-acetamido-6-amino-3-methyluracil (AAMU*) and [2-^{13}C, 1,3-$^{15}N_2$]-5-acetylamino-6-formylamino-3-methyluracil (AFMU*) were synthesized by a 3 week caffeine-restricted human volunteer ingesting 200 mg of [2-^{13}C, 1,3-$^{15}N_2$]caffeine, collecting the urine and purifying the individual metabolites by HPLC.

The structures of caffeine and metabolites are given as a metabolic scheme in Fig. 1.

Apparatus

A CTC PAL autosampler (CTC Analytics, Zwingen, Switzerland), an HP 1100 binary HPLC pump, solvent cabinet, column oven and vacuum degasser (Hewlett-Packard, Palo Alto, CA, USA) were used. The HPLC system was fully controlled by the mass spectrometers: a Sciex API 365 or API 3000 triple-quadrupole mass spectrometer with a turboionspray source and controlled by Analyst software version 1.2 with service pack 3 (Sciex, Thornill, Canada). A fully automated software-controlled Valco two-position valve controlled by an EHMA microelectric actuator (Valco International, Schenkon, Switzerland) was used to divert the eluent fractions that contained the analytes into the mass spectrometer. The early eluting components were diverted to waste, thereby reducing contamination of the ion source.

The column used was a YMC-Pack C30 HPLC column (50 × 2.1 mm i.d., 3 μm film thickness) obtained from YMC Europe (Schermbeck/Weselerwald, Germany).

Eluent A was 0.5% acetic acid and eluent B was 100% acetonitrile. The flow-rate was 200 μl min^{-1} and the injection volume was 10 μl. The separation was performed by gradient elution from 0 to 1.5 min starting at 1.5% eluent B and ending at 18% B, then from 1.5 to 5 min gradient elution from 18% to 100% eluent B, and finished by isocratic elution from 5 to 7 min with 100% eluent B. The separation was performed at 60 °C. Between each run, the column was equilibrated with 100% eluent A for 4.5 min (flow-rate 1 ml min^{-1}). The eluate was diverted to waste from time 0 to 1 min and during the column re-equilibration.

In order to obtain maximum sensitivity, electrospray ionization was performed in the positive ion mode for 17U, 1X and caffeine, whereas it was performed in either the positive or negative ion mode for AAMU, AFMU and 1U. The ($[M + H]^+/[M - H]^-$) ions were selected by the first mass filter for all analytes. After collisional activation, the most abundant fragment ions were selected by the second mass filter. The MRM pairs used are shown in Table 1.

Nitrogen was used as nebulizer (15), curtain (8), heater (7.5 l min^{-1}) and collision gas (8) (the values for nebulizer, curtain and collision gas refer to the API 3000 settings). The electrospray probe temperature was 500 °C. The ionspray voltage was 4200 V. Individual tuning files were used for all the analytes to achieve maximum sensitivity. According to this, the potentials, collision gas pressure and the flow of the gases to the ion source were changed during each run. The respective potentials for AAMU, AFMU, 1U, 1X, 17U

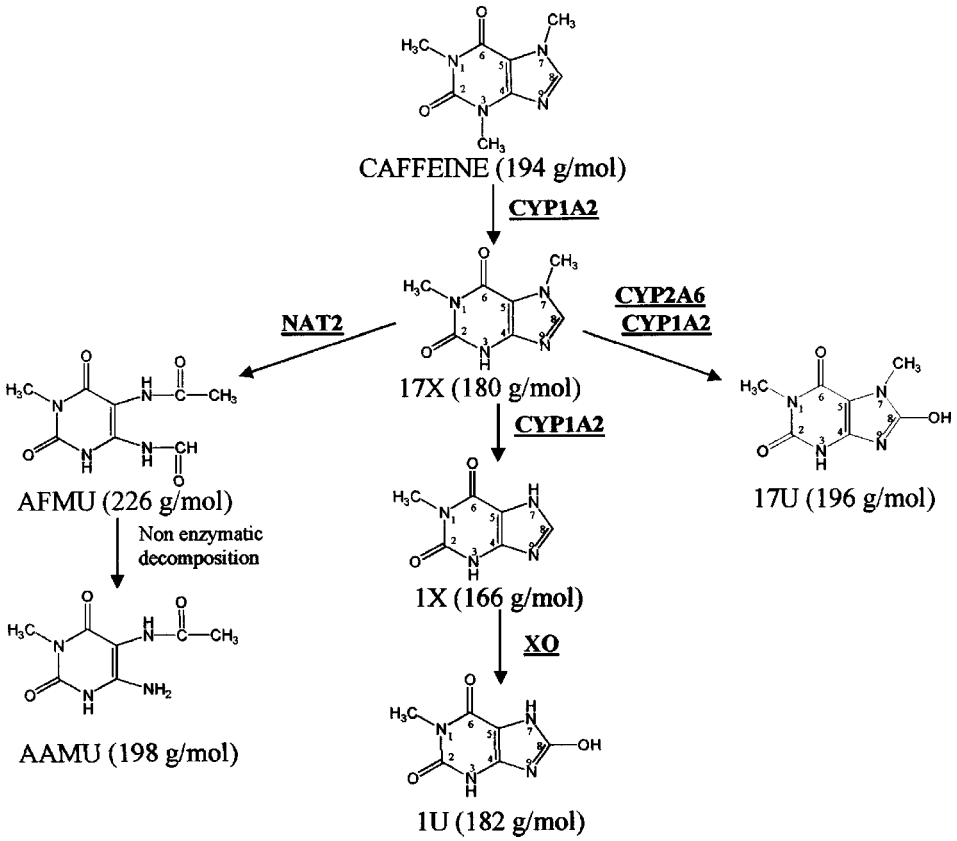

Figure 1. Schematic presentation of the metabolic fate of caffeine in humans.

Table 1. Acquisition parameters and detection limits

Compound	MRM pairs	Internal standard MRM pairs	Polarity	Detection limit (nM) (API 3000) (for standards)[a]	Detection limit (nM) (API 3000) (for real samples)[b]
AAMU	197/127	200/128	−		
AAMU	199/124	202/125	+	0.3	10
AFMU	225/197	228/200	−		
AFMU	227/157	230/160	+	0.5	10
1U	181/138	184/139	−		
1U	183/155	186/158	+	1.5	?
1X	167/110	170/111	+	0.1	?
17U	197/140	200/141	+	0.2	10
Caffeine (137X)	195/138	198/139	+	0.3	?

[a] The column gives the sensitivity measured directly on the standards.

[b] The column gives the sensitivity which can be obtained in urine samples (the sensitivity is actually 10 times better as the samples are diluted 10-fold before analysis). No values has been inserted for 1U, 1X and caffeine, as it was impossible to measure the values in real samples because of problems obtaining a true urine blank for these analytes. It is estimated that the detection limits for 1X and caffeine are better than 10 nM and the detection limit for 1U is roughly 25 nM.

and 137X are as follows: declustering potential, 38, 44, 73, 68, 57 and 46 V; focusing potential, 190, 215, 334, 308, 233 and 197 V; focusing lens 1, −4.7, −4.2, −4.7, −4.2, −10.8 and −10.6 V; prefilter, −8.8, −8.6, −11.4, −10.1, −18.2 and −14.4 V; focusing lens 2, −13.8, −14.0, −8.2, −11.6, −14.2 and −19.2 V; collision energy, 30.6, 26.5, 24.0, 28.1, 26.6 and 26.7 eV; and collision cell exit potential, 5.2, 7.5, 7.2, 4.7, 6.3 and 6.0 V. In order to remove contributions from other compounds, the 'high resolution mode' (peak width 0.5 u at 50% of peak height) was used in both the first quadrupole (Q1) and the third quadrupole (Q3) for all the analytes.

Preparation of internal standards

Heavy isotope-labelled internal standards of AFMU (AFMU*), 1U (1U*), 17U (17U*) and 1X (1X*) were biosynthesized by a human volunteer on a xanthine-restricted diet for 3 weeks (verified by analysis of urine by the method described here) ingesting 200 mg of [2-^{13}C, 1,3-$^{15}N_2$]caffeine dissolved in tap water, whereafter urine was collected for 24 h. After ingestion, the urine contained heavy isotope-labelled AFMU, 1U, 17U and 1X. The content of unlabelled AFMU, 17U and 1X in these standards was <3.6%. 1U*, however, was contaminated by 17.7% of unlabelled 1U.

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The labelled analytes (except AAMU*) were separated by injecting the urine samples on to a Luna C18 (2) HPLC column (250 × 10 mm i.d., 5 μm particle size) obtained from Phenomenex (Torrance, CA, USA) from which the relevant peaks were collected. The analytes were separated using the following gradient: eluent A, 0.5% acetic acid (pH 5.2); eluent B, acetonitrile; from 0 to 1 min, 100% eluent A; from 1 to 20 min, a linear gradient to 10% eluent B. The flow-rate was 5 ml min^{-1}. The analytes were detected using UV absorption at 280 nm. Stable isotope-labelled AAMU was prepared from the labelled AFMU by treatment with base.

The instrumentation used for purifying the internal standards was a Gilson (Middleton, WI, USA) ASPEC autosampler including a Model 401 diluter, two Gilson Model 306 pumps, a Gilson Model 811C dynamic mixer and a Gilson Model 805 manometric module. The UV detector was an HP 1100 diode-array detector (Hewlett-Packard).

The purity of the isolated compounds was verified by HPLC and MS/MS and their concentrations were roughly estimated by the method described here.

Preparation of standards

Stock solutions of 1U, 1X, 17U and caffeine were prepared by dissolving the analytes in Milli-Q-purified water. Small amounts of ammonium hydroxide can be added to help the dissolution of 1U. Stock solutions of AFMU were prepared by dissolving the analyte in 0.1% acetic acid to reduce its degradation. Stock solutions of AAMU were prepared by dissolving the analyte in 5 mM sodium hydroxide. Stock solutions were stored at −80 °C.

The concentration of the stock solution of each unlabelled analyte was determined by measuring the UV absorption using the extinction coefficients shown in Table 2.

Sample preparation

The frozen urine samples were thawed and mixed. During the mixing, a 10 μl sample was taken. The sample was diluted with 65 μl of 25 mM ammonium formate adjusted to pH 2.75 with formic acid and 25 μl of a mixture of the internal standards. After this, the sample was heated at 37 °C for 10 min during mixing to redissolve a precipitate that might contain analytes. Finally, the sample was centrifuged to remove residual particles before injection into the HPLC system.

Table 2. Extinction coefficients

Analyte	Wavelength (nm)	Extinction coefficient (M^{-1} cm^{-1})	Source Ref.
AAMU	266	14 700 (pH 11)	36
AFMU	207.5	17 400	37
1U	292.5	12 300 (pH >12)	38
	291	11 749 (pH 8)	39
17U	291	12 022 (pH 8)	39
1X	279	10 200	40
Caffeine	273	9900	41

RESULTS

The tandem mass spectra for the six analytes are shown in Figs 2 and 3. Some of the analytes were recorded in the positive ion mode and others in the negative ion mode. The choice was based on which gave the better sensitivity. The sensitivity for 1U, AAMU and AFMU is on average marginally better in the negative ion mode; owing to random variation in apparatus performance, however, sometimes better sensitivity can be obtained in the positive ion mode. The results were obtained in the MRM mode. In the first mass filter (Q1), the $[M + H]^+$/$[M - H]^-$ ions were selected in the positive ion mode/negative ion mode, respectively. A characteristic product ion was selected in the second mass filter (Q3).

As MS/MS detection is fairly specific, the need for extensive chromatography is reduced. It did turn out, however, that a resolution of 0.5 Da FWHM had to be used in both the first quadrupole Q1 and the third quadrupole Q3 for all the analytes. When this was introduced, only very few non-analyte peaks appeared in the MS/MS chromatograms of the urine samples and therefore the separation time could be reduced.

Retention of AAMU and AFMU on reversed-phase columns can be difficult. It was found that they were retained fairly well on a C30 phase, although the peaks were not as sharp as hoped. This is probably because the analytes cannot be fully retained and hence concentrated on the column. Several column temperatures between 1 and 60 °C were tried. AAMU and AFMU were retained longest at low temperatures, but the peaks were very broad. At increasing temperature, the peaks eluted earlier and the peaks were sharper; 60 °C was chosen as the best compromise. The other analytes were better retained and were gradient eluted as sharp peaks. The gradient was adjusted so that the peaks were sufficiently separated to allow the use of individual time windows and in addition the gradient was compressed in order to reduce the run time. Because of the use of gradient elution, the column had to be equilibrated before each new run. To speed up this part, a flow-rate of 1 ml min^{-1} was used during equilibration. As this flow-rate was too high for our ion source, an automatic switching valve was introduced between the outlet of the HPLC column and the mass spectrometer inlet. Also, the early eluting components from the urine samples (including the majority of the salts) were diverted to waste.

The detection limits for each of the analytes are shown in Table 1. If better detection limits are needed for real samples, it is possible to run the samples with less than 10-fold dilution.

Chromatograms of a standard and a urine sample are shown in Fig. 4.

Matrix suppression was calculated as the average signal suppression of the internal standards of which an equal amount has been added to each sample and standard. The values were AAMU 69%, AFMU 50%, 1U 12%, 1X 12%, 17U 56% and caffeine 20%.

The calibration curves for all six analytes are linear in the range 5 nM–50 μM with r-values ≥0.9994.

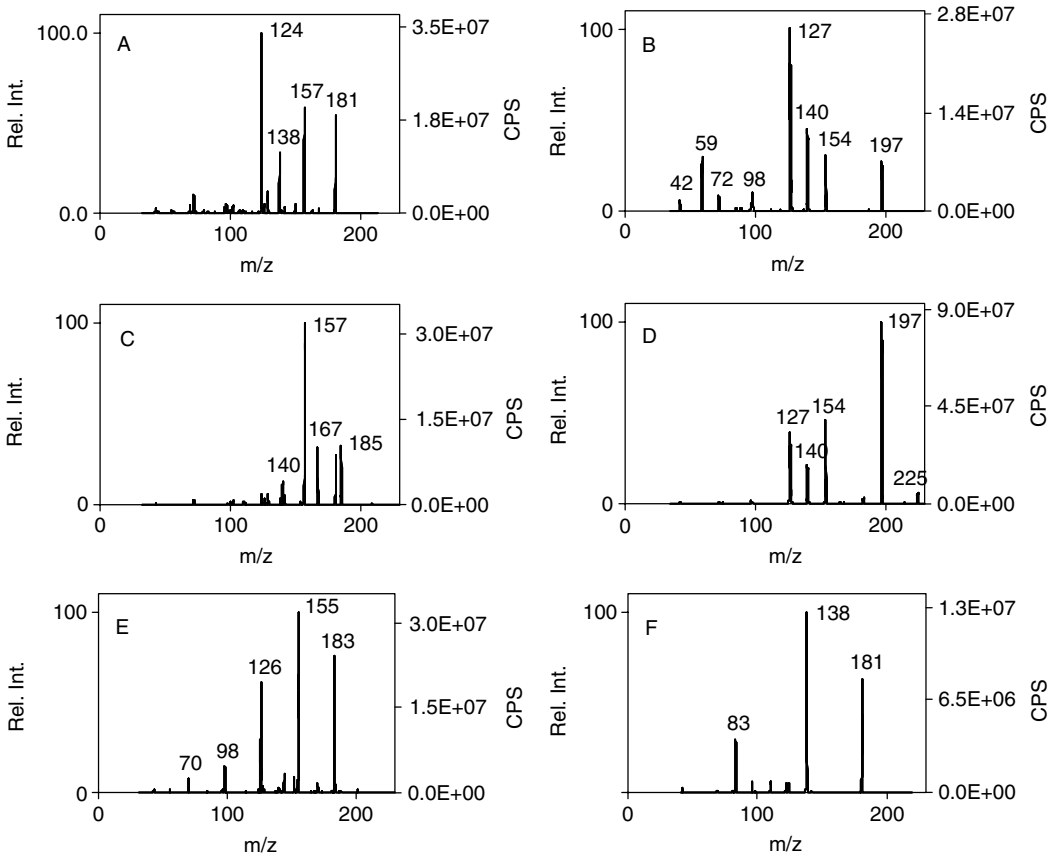

Figure 2. Product ion spectra of the three analytes which can be measured in either the positive or negative ion mode: (A) AAMU positive ion mode (precursor ion $m/z = 199$); (B) AAMU negative ion mode (precursor ion $m/z = 197$); (C) AFMU positive ion mode (precursor ion $m/z = 227$); (D) AFMU negative ion mode (precursor ion $m/z = 225$); (E) 1U positive ion mode (precursor ion $m/z = 183$); and (F) 1U negative ion mode (precursor ion $m/z = 181$). In the positive ion mode $[M + H]^+$ and in the negative ion mode $[M - H]^-$ were selected in the first mass analyzer before collisional activation.

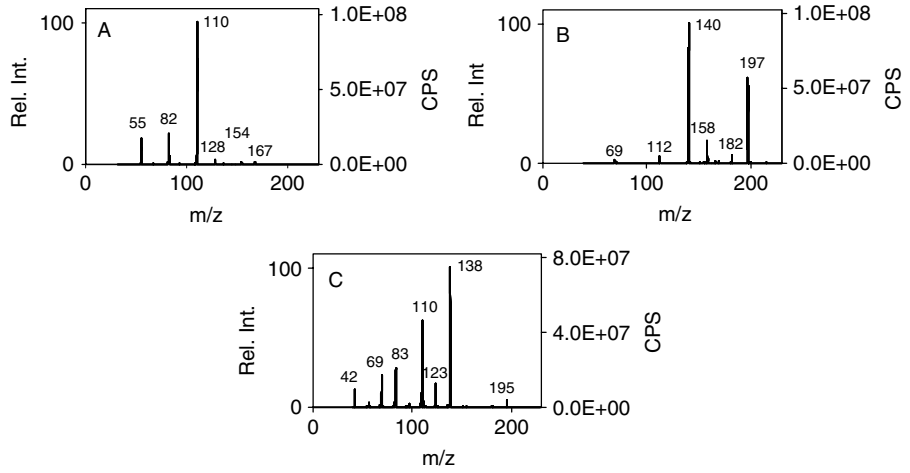

Figure 3. Product ion spectra of the three analytes which are always measured in the positive ion mode: (A) 1X positive ion mode (precursor ion $m/z = 167$); (B) 17U positive ion mode (precursor ion $m/z = 197$); and (C) caffeine positive ion mode (precursor ion $m/z = 195$). $[M + H]^+$ ions were selected in the first mass analyzer before collisional activation.

The intra- and inter-day coefficients of variation of single analyses are <6.5%. The inter-day coefficient of variation of duplicate analyses is <4.8% (see Table 3). Intra- and inter-day coefficients of variation were calculated by performing the analysis of 10 human urine samples four times, twice on two different days; on each day an analysis of the samples was performed as the first samples in the run and they were also performed as the last samples.

Injecting the same urine sample 233 times tested the ruggedness of the method and for every ~50 samples the concentration for all six analytes was calculated. The standard deviations were found to be 4.8, 3.5, 1.8, 5.8, 2.3

Table 3. Reproducibility[a]

Sample No.	Day 1-1 (μM)	Day 1-2 (μM)	Day 2-1 (μM)	Day 2-2 (μM)	Day 1 (% dev.)	Day 2 (% dev.)	Inter-day CV duplicate (%)	Inter-day CV single (%)
AAMU 1	16.4	16.2	13.8	15.1	0.9	6.4	8.5	12.2
2	14.1	11.7	11.3	11.3	13.2	0.0	9.4	15.6
3	2.8	3.1	2.5	2.4	5.5	2.3	13.6	9.6
4	67.5	65.6	53.4	62.0	2.0	10.5	10.1	16.5
5	17.4	19.5	20.9	21.2	8.0	1.0	9.3	12.9
6	1.2	0.9	1.2	0.9	19.2	16.9	4.3	5.3
7	116.0	98.6	97.0	98.0	11.5	0.7	6.8	12.6
8	12.9	11.3	11.4	11.3	9.4	0.6	4.5	8.7
9	13.7	12.6	12.2	13.4	5.9	6.6	1.9	8.2
10	17.3	16.1	15.3	15.0	5.1	1.4	6.9	8.7
					5.5	5.5	3.4	3.5
AFMU 1	3.8	4.2	3.3	3.7	8.5	7.8	10.1	9.7
2	13.4	11.7	10.6	10.3	9.6	2.0	12.9	16.5
3	0.2	0.3	0.2	0.2	20.2	5.1	3.1	10.3
4	24.9	27.5	20.7	24.3	7.0	11.3	10.7	13.0
5	1.1	1.4	1.5	1.6	19.1	1.8	13.7	22.8
6	0.3	0.3	0.4	0.3	5.8	13.2	9.3	12.7
7	39.9	37.5	34.5	34.9	4.4	0.8	7.7	10.3
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9	10.0	10.4	9.7	11.1	2.8	9.7	1.3	2.2
10	6.9	6.9	6.0	6.2	0.1	2.2	8.5	9.6
					7.0	4.8	4.7	6.5
1U 1	54.1	55.2	44.2	50.9	1.4	10.0	9.8	14.2
2	49.1	42.0	41.1	41.3	11.0	0.3	7.1	12.5
3	12.5	16.4	13.5	12.1	19.1	7.7	8.6	5.4
4	97.1	99.3	78.2	94.9	1.6	13.6	8.9	15.2
5	68.3	82.4	82.2	88.1	13.2	4.9	8.6	13.1
6	11.8	10.0	10.5	10.0	12.0	3.7	4.4	8.2
7	502.0	404.0	335.0	363.0	15.3	5.7	18.3	28.2
8	29.5	26.8	25.1	25.9	6.8	2.2	7.0	11.4
9	25.6	21.0	22.0	24.5	14.0	7.6	0.2	10.7
10	20.3	19.6	17.9	16.5	2.5	5.8	10.5	8.9

					6.3	3.8	4.6	6.2
1X 1	62.3	58.3	55.5	56.4	4.7	1.1	5.3	8.2
2	37.4	32.4	29.9	29.3	10.1	1.4	11.6	15.8
3	15.7	19.5	15.1	15.4	15.3	1.4	10.1	2.8
4	79.9	80.5	66.0	76.4	0.5	10.3	8.4	13.5
5	92.6	104.0	112.0	114.0	8.2	1.3	9.8	13.4
6	14.5	12.1	15.1	13.3	12.8	9.0	4.6	2.9
7	210.0	196.0	168.0	174.0	4.9	2.5	12.1	15.7
8	15.7	14.5	14.2	13.7	5.6	2.5	5.6	7.1
9	24.5	22.5	21.8	24.9	6.0	9.4	0.5	8.2
10	21.9	20.8	19.0	18.6	3.6	1.5	9.0	10.0
					4.5	3.9	3.6	4.8
17U 1	44.7	50.7	41.6	43.4	8.9	3.0	8.2	5.1
2	26.8	24.0	23.1	23.0	7.8	0.3	6.9	10.5
3	6.1	7.9	5.8	6.0	18.8	2.7	11.7	3.0
4	35.4	38.3	30.2	36.9	5.6	14.1	6.6	11.2
5	21.7	28.5	29.7	30.9	19.2	2.8	13.3	22.0
6	5.5	5.3	5.5	4.8	2.2	10.9	3.2	0.9
7	135.0	133.0	123.0	131.0	1.1	4.5	3.8	6.6
8	13.7	13.2	12.5	13.0	2.6	2.8	3.8	6.5
9	16.3	16.5	15.0	18.2	0.9	13.6	0.9	5.9
10	14.0	14.1	12.4	12.6	0.5	1.1	8.3	8.6
					7.1	5.2	3.9	5.8
Caffeine 1	47.8	52.6	42.2	45.4	6.8	5.2	9.6	8.8
2	21.9	18.6	17.7	17.2	11.5	2.0	10.5	15.0
3	6.8	8.6	6.8	7.0	16.9	2.9	7.4	0.1
4	18.5	19.1	15.2	17.3	2.3	9.1	10.3	13.8
5	14.3	17.8	18.4	19.5	15.4	4.1	11.7	17.7
6	8.6	7.8	9.7	7.8	6.7	14.9	4.9	8.7
7	23.8	22.3	20.9	21.2	4.6	1.0	6.4	9.2
8	43.5	40.0	40.6	39.7	5.9	1.6	2.8	4.9
9	21.8	22.4	21.7	30.4	1.9	23.6	11.6	0.3
10	23.9	22.7	20.7	20.9	3.6	0.7	8.0	10.1
					5.3	7.5	3.0	5.9

[a] Intra- and inter-day coefficients of variation was calculated by performing the analysis of 10 human urine samples four times: two times on two different days; on each day an analysis of the samples was performed as the first samples and they were also performed as the last samples in the run.

JMS

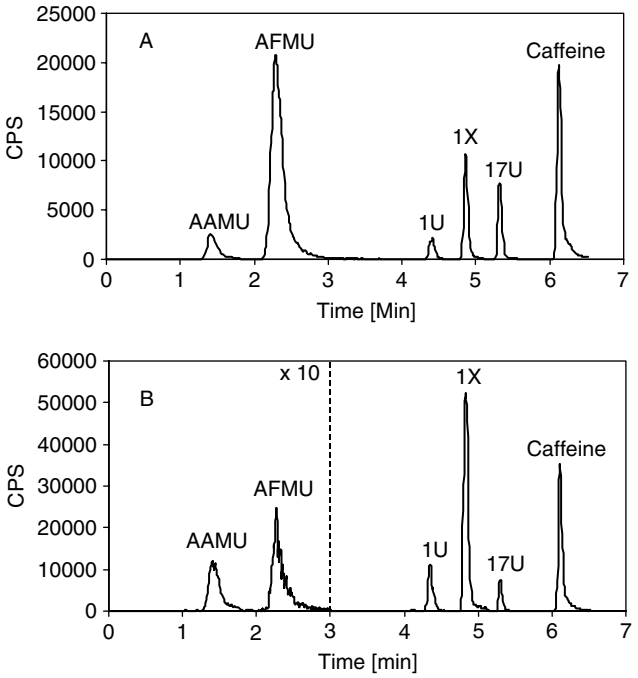

Figure 4. Chromatograms of (A) a 5 μM standard and (B) a 24 h human urine sample from a male volunteer. The sample is from an unpublished intervention trial with approval from the local ethical committee. AAMU and AFMU are recorded in the negative ion mode and 1U, 1X, 17U and caffeine in the positive ion mode.

and 1.5% for AAMU, AFMU, 1U, 1X, 17U and caffeine, respectively. During all 233 samples the retention times on the HPLC column were stable.

The accuracy and recovery of the method were tested by spiking a blank urine sample with standards corresponding to 20 μM of each of the six analytes in the sample. For each analyte the signal from the blank urine was subtracted from that of the spiked urine. The levels found were 19.4, 20.7, 18.4, 20.3, 19.8 and 19.6 μM for AAMU, AFMU, 1U, 1X, 17U and caffeine, respectively.

A study was undertaken in order to test the method for possible interferences from other caffeine metabolites. First a standard containing AAMU, AFMU, 1U, 1X, 17U and caffeine (10 μM each) was recorded. The chromatogram was overlaid by a chromatogram containing the caffeine metabolites not included in the method presented: 3-methylxanthine (3X), 7-methylxanthine (7X), 1,7-dimethylxanthine (17X), 1,3-dimethylxanthine (13X), 3,7-dimethylxanthine (37X), 3-methyluric acid (3U), 7-methyluric acid (7U), 1,3-dimethyluric acid (13U), 3,7-dimethyluric acid (37U) and 1,3,7-trimethyluric acid (137U) (20 μM each). The chromatogram is shown in Fig. 5. As can be seen, there is no interference from the metabolites not included in the method.

DISCUSSION

Enzyme activities can be evaluated from urinary caffeine metabolite ratios in several ways.[17] One way to calculate the ratio is to use (AFMU + 1U + 1X)/17U for CYP1A2,

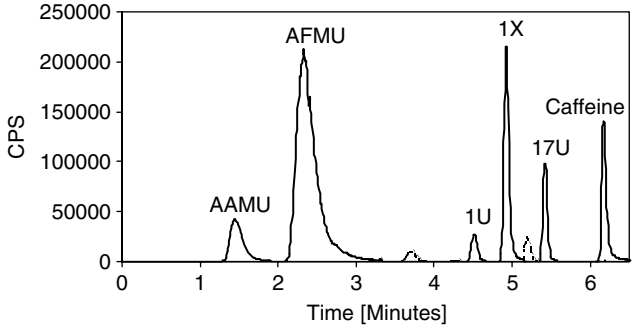

Figure 5. The total ion current (TIC) chromatogram of the MRM traces shows a standard (solid line) containing AAMU, AFMU, 1U, 1X, 17U and caffeine (10 μM each). The chromatogram is overlaid by a chromatogram containing caffeine metabolites not included in the method presented: 3X, 7X, 17X, 13X, 37X, 3U, 7U, 13U, 37U and 137U (20 μM each). As can be seen, there is no interference from the metabolites not included in the method.

AFMU/(17U + 1U + 1X) for *N*-acetyltransferase 2 (NAT2) and 1U/(1U + 1X) for xanthine oxidase (XO).[18–27] In stored urine samples AFMU may be partly converted to AAMU if samples are not acidified before storage to prevent this conversion. The sum of AAMU and AFMU may be used to represent the amount of AFMU that was originally present in the urine samples. Thus we found it mandatory that the method quantifies AAMU, AFMU, 1U, 1X and 17U to be able to calculate the enzyme activities of CYP1A2, NAT2 and XO.

Only few papers have been published on the measurement of caffeine and its metabolites by LC/MS.[13–16] In contrast, a number of methods has been developed based on HPLC with UV detection. These methods, however, often require time-consuming sample preparation such as liquid–liquid extraction,[20,28–31] solid-phase extraction[32] or the use of more than one chromatographic step.[33–35] The methods also often suffer with regard to selectivity, specificity, sensitivity and reproducibility or resolution. Our goal was to develop a method that was faster than the previously published methods and one that could be automated without extensive sample preparation.

During caffeine metabolism, several other metabolites are formed. Among these, 3-methylxanthine (3X) and 7-methylxanthine (7X) have the same molecular formula as 1X. Similarly, 1U and 17U have some isomers: 7-methyluric acid (7U), 3-methyluric acid (3U), 3,7-dimethyluric acid (37U) and 1,3-dimethyluric acid (13U). Isomers would be likely candidates as false positives. In addition, they will not appear in blank urines, as all metabolites of caffeine will be missing. Therefore, we had to investigate if 1X, 1U and 17U had the same retention times and transitions as their isomers. 3X and 7X do not show the same transition (m/z 167–110) as 1X and therefore will not interfere with the analysis. 37U also does not show the same transition as 17U (m/z 197–140), but 13U does show the same transition. With regard to 1U, both isomers show m/z 183–155 transitions in the positive ion mode, which is why it was necessary to verify that the retention times were different for the analytes and their isomers. A run including all the isomers mentioned above

plus 137U, 13X, 17X and 37X showed that none of them had the same retention times and transitions as the analytes (see Fig. 5).

It would be preferable if the stable isotope-labelled internal standards were added to urine at concentrations that correspond to those of the analytes. However, as some of the stable isotope-labelled internal standards contain some unlabelled metabolites, it is important that the amounts of added internal standard are low to reduce the impact of the impurities on the results. On the other hand, if the level of internal standard is too low then the contribution from the analytes' natural isotope distribution will add significantly to the internal standards' MRM trace. It would have been preferable to use internal standards that were more than three masses higher than the analytes, but we have been unable to obtain such standards. We usually choose a compromise where each final sample contains in the range 0.2–1.5 μM of each internal standard. As our samples are diluted 10-fold before injection, the amount of internal standard corresponds to a level between 2 and 15 μM if it had been added to the urine before dilution. Usually urine contains metabolites in the range 5–400 μM,[28] which is the range we can expect in an unknown sample. This means that the level of internal standards that we are using in our samples corresponds to the lower part of this range. We chose this low level of internal standard to reduce the impact of the unlabelled impurities on the results. If values below this range are expected, it is recommended to use smaller amounts of internal standards to reduce the impact of the non-labelled impurities. In some cases it might be necessary to extend the calibration curves to concentrations higher than 400 μM.

The detection limits for urine samples are somewhat lower than for standards. This is caused both by ion suppression and by the fact that the samples are diluted 10-fold before analysis. No values have been inserted in Table 1 for 1U, 1X and caffeine. The 'blank' urine which was obtained from a volunteer on a xanthine-restricted diet for 3 weeks still gave rise to considerable peaks for 1U, 1X and caffeine. After 3 weeks of abstaining from food know to contain caffeine and other xanthines, the urinary caffeine and metabolite excretion appeared to reach a stable level. Lower levels might be obtained if a defined synthetic diet is used before biosynthesis of the labelled internal standard; also, chemical synthesis is an option for synthesis of internal standards with higher purity; both methods, however, we have judged to be beyond the scope of the present paper. Because of the background level, it was impossible to measure the detection limits reliably for 1U, 1X and caffeine. It is estimated, however, that the detection limits for 1X and caffeine are better than 10 nM and that for 1U is roughly 25 nM. If better detection limits are needed, it is possible to analyse the samples with less dilution. The detection limits are, however, already more that 100 times lower than the levels normally found in urine, and considerably lower that those of the traditional UV detection methods.

The calibration curves were constructed on the basis of standards prepared in water. It would have been preferable to be able to prepare the standards in urine, but as mentioned above, it was not possible to obtain a 'blank' urine free from analytes. Because of this, it was estimated that more precise results could be obtained from aqueous standards. The aqueous standards will not show signal suppression as in the urine samples, but the internal standards should correct for this. Anyway, the signal suppression will vary from sample to sample as the urines have varying concentrations of salts, etc.

The analysis time with this method is 11.5 min compared with 40–60 min for the LC/MS/MS and UV detection-based methods. This represents an improvement that makes it possible to run larger scale projects. Also, the improvement in sensitivity is important, making it possible to measure the metabolites even after about 1 week on a xanthine-free diet. If a higher throughput is wanted, it should be possible to use two parallel HPLC columns connected to one mass spectrometer. While one column equilibrates, an analysis can be performed on the other column. This could cut down the efficient analysis time to 7 min but in order to do so an extra HPLC pump and an extra switching valve are required.

CONCLUSION

A fast, sensitive and fully automated method for measuring caffeine and five of its major metabolites has been developed. From the data obtained, it is possible to estimate the CYP1A2, NAT2 and XO activities. As the method is fast, it is suitable for large-scale investigations in humans and animals, and because of the high sensitivity, the method can be used on small experimental animals or even in cellular or purified enzyme systems.

Acknowledgement

This work was supported by the Danish Research Council (Grant 9501768 CPNC).

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