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Extractionless method for the simultaneous high-performance liquid chromatographic determination of urinary caffeine metabolites for *N*-acetyltransferase 2, cytochrome P450 1A2 and xanthine oxidase activity assessment

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Abstract

Urinary metabolic ratios of caffeine are used in humans to assess the enzymatic activities of cytochrome P450 isoenzyme 1A2 (CYP1A2), xanthine oxidase (XO) and for phenotyping individuals for the bimodal *N*-acetyltransferase 2 (NAT2), all of them involved in the activation or detoxification of various xenobiotic compounds. Most reported analytical procedures for the measurement of the urinary metabolites of caffeine include a liquid–liquid extraction of urine samples prior to their analysis by reversed-phase HPLC. At neutral to basic pH however, 5-acetylamino-6-formylamino-3-methyluracil (AFMU), a metabolite of caffeine, spontaneously decomposes to 5-acetylamino-6-amino-3-methyluracil (AAMU). Since AAMU is not extracted in most organic solvents, the extent of AFMU decomposition cannot be precisely assessed. Although the decomposition reaction can be minimized by immediate acidification of the urine, accurate results can only be obtained when both AAMU and AFMU are monitored, or alternatively, if AAMU is measured after complete transformation of AFMU into AAMU in basic conditions. We report a liquid chromatographic method for the simultaneous quantitative analysis of the five urinary metabolites of caffeine used for the CYP1A2, XO and NAT2 phenotyping studies: AAMU, AFMU, 1-methylxanthine, 1-methyluric acid and 1,7-dimethyluric acid. These metabolites are satisfactory separated from all other known caffeine metabolites as well as endogenous urinary constituents. Sample treatment does not require any liquid–liquid extraction procedure. Urine samples are diluted and centrifuged before being injected (10 μl) onto a YMC-Pack Polyamine II (250×4.6 mm) column. A step-wise gradient elution program is applied using acetonitrile–0.75% (v/v) formic acid: (91:9) at 0 min→(75:25) at 25 min→(65:35) at 35 min→(65:35) at 45 min, followed by a re-equilibration step to the initial solvent composition. The flow-rate is 1.0 ml/min and the separations are monitored by UV absorbance at 260 and 280 nm. The procedure described here represents a substantial improvement over previous methods: a single analysis and a minimal urine sample treatment enables the simultaneous quantitation of five caffeine metabolites, notably AFMU and AAMU, used for the determination of CYP450 1A2, XO and NAT2 enzyme activity. Importantly enough, phenotyping individuals for the bimodal NAT2 is made possible without the uncertainty associated with the deformylation of AFMU, which is likely to happen at all steps prior to the analysis, during sample storage and even in the bladder of the subjects.

Keywords: Caffeine; *N*-Acetyltransferase; Cytochrome P450; Xanthine oxidase; Enzymes

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1. Introduction

Calculation of urinary metabolic ratios of caffeine (1,3,7-trimethylxanthine) is commonly used for phenotyping subjects for the bimodal *N*-acetyltransferase 2 (NAT2), and to assess the activity of cytochrome P450 isoenzyme 1A2 (CYP1A2) and xanthine oxidase (XO). These enzymatic systems are involved in the activation or detoxification of various xenobiotic compounds, including carcinogens [1,2], which may in turn influence their activity (induction and/or inhibition). Investigating these enzymes is of clinical relevance, since enzyme deficiencies can lead to unusually high plasma concentrations of several drugs due to impaired metabolism, to an increased incidence of side-effects, or conversely, to insufficient therapeutic effect. In environmental, epidemiological and toxicological studies, the determination of the metabolic activity of subjects belonging to a given population (i.e., patients, workers, etc.) may help to evaluate their risk of developing specific exposure-related diseases [3,4].

Fig. 1 shows the major metabolic pathway of caffeine in healthy human subjects which depends

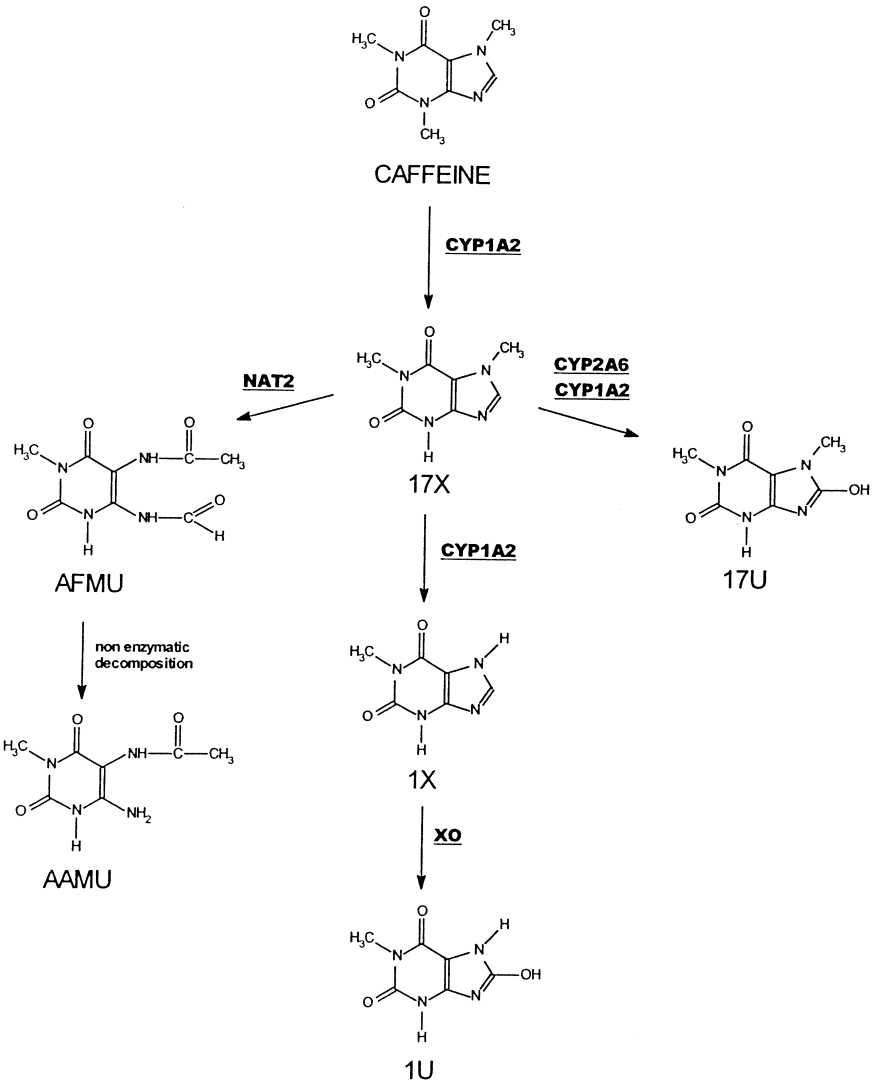

Fig. 1. Main pathways of caffeine metabolism in humans (for abbreviations see Table 1 and text).

notably on CYP1A2, XO and NAT2 enzymatic systems. In phenotyping studies, the enzyme activities are usually expressed as the urinary molar ratios of the caffeine metabolites (listed in Table 1) with the (AFMU+1X+1U)/17U, 1U/(1X+1U) and AFMU/(AFMU+1U+1X) ratio reflecting CYP1A2, XO and NAT2 activity [5–7], respectively. Different metabolite ratios have also been proposed [8–10], notably for CYP1A2 and NAT2 phenotyping, whereby the (AAMU+1X+1U)/17U and AAMU/(AAMU+1U+1X) ratios are used for the calculation, after the basic conversion of AFMU into AAMU. Moreover CYP1A2 activity can also be determined using the paraxanthine/caffeine (17X/137X) ratio albeit in other biological fluids such as plasma and saliva [11].

Most analytical procedures reported for the measurement of the urinary metabolites of caffeine are based on the early work of Grant et al. [12] which include a liquid–liquid extraction of urine samples prior to their analysis by reversed-phase high-performance liquid chromatography (HPLC). At neutral to basic pH however, 5-acetylamino-6-formylamino-3-methyluracil (AFMU), an acetylated caffeine metabolite, spontaneously loses a formyl group and decomposes into 5-acetylamino-6-amino-3-methyluracil (AAMU) (Fig. 1) which is not extracted in most organic solvents, precluding any precise direct assessment of the extent of AFMU decomposition. Although the decomposition reaction can be minimized by acidifying the urine immediately after voiding, complete control and assurance of accurate results is only provided when both AAMU and AFMU are monitored, or alternatively if AAMU is measured after the complete transformation of AFMU in basic conditions. The deformylation decomposition reaction is indeed likely to happen throughout analysis, during the extraction procedure, sample storage, urine collection, and possibly even in the bladder of the subjects [13,14].

The published procedures reporting the direct quantitation of AAMU require an additional separate analysis, either by HPLC on various chromatographic packings [13,15,16], by micellar electrokinetic capillary chromatography (MECC) [17], or by enzyme-linked immunosorbent assay (ELISA) [18]. The only method describing the simultaneous determination of AAMU together with other caffeine metabolites uses a direct injection of urine samples on a C_{18} reversed-phase column. The reported chromatographic profiles, however, clearly show interferences with endogenous compounds [19].

An important contribution using a normal-phase chromatography has been previously reported by Rodopoulos and Norman [15] but was applied to the assay of AAMU and AFMU only. We therefore propose an improvement of this method enabling the determination of AAMU and AFMU together with three additional metabolites 1X, 1U, and 17U in a single HPLC run with minimal sample preparation. It

Table 1
Caffeine and its metabolites with their respective retention times (chromatographic conditions in the text)

No.	Compound	Abbreviation	Retention time (min)
1	Caffeine	137X	4.1
2	1,3-Dimethylxanthine	13X	5.1
3	1,7-Dimethylxanthine	17X	5.3
4	3,7-Dimethylxanthine	37X	5.4
5	1,3,7-Trimethyluric acid	137U	7.1
6	1-Methylxanthine	1X	8.0
7	3-Methylxanthine	3X	8.5
8	7-Methylxanthine	7X	9.1
9	5-Acetylamino-6-formylamino-3-methyluracil	AFMU	12.2
10	1,3-Dimethyluric acid	13U	12.9
11	3,7-Dimethyluric acid	37U	16.3
12	1,7-Dimethyluric acid	17U	18.8
13	5-Acetylamino-6-amino-3-methyluracil	AAMU	21.2
14	3-Methyluric acid	3U	24.1
15	1-Methyluric acid	1U	26.4
16	7-Methyluric acid	7U	31.7

must be stressed that the proposed sample preparation procedure requires no liquid–liquid extraction step, enhancing phenotyping precision and accuracy by circumventing the poor stability of AFMU and the limited, if any, organic extraction of AAMU from urine.

2. Experimental

2.1. Chemicals

Caffeine (137X), 1,3-dimethylxanthine (13X), 3,7-dimethylxanthine (37X), 1-methyluric acid (1U), 7-methyluric acid (7U), 1,3-dimethyluric acid (13U), 1,7-dimethyluric acid (17U), 3,7-dimethyluric acid (37U), 1,9-dimethyluric acid (19U), 3-methylxanthine (3X), 7-methylxanthine (7X), 1,3,7-trimethyluric acid (137U) were purchased from Fluka (Buchs, Switzerland), 1-methylxanthine (1X) from Aldrich (Buchs, Switzerland) and 1,7-dimethylxanthine (17X), 3-methyluric acid (3U) from Sigma (Buchs, Switzerland). HPLC-grade acetonitrile was obtained from Romil (Cambridge, UK). AAMU and AFMU were kind gifts of the Nestlé Research Center (Vers-chez-les-Blanc, Switzerland). All other chemicals were purchased from Fluka.

2.2. Stock solutions, standard calibration urine samples

The stock solutions of caffeine and its metabolites were prepared separately in ultrapure water (caffeine, 137U, 13X, 17X, 13U), in 2.5 m*M* NaOH solution (for 37X, 17U, 37U, 3U) and in 5.0 m*M* NaOH solution (for 1X, 3X, 7X, 1U, 7U, AAMU).

For the considered NAT2, XO and CYP1A2 phenotyping studies, only 1X, AAMU, AFMU, 1U and 17U had to be quantitated in urine. Therefore, standard urine samples were prepared by mixing the appropriate volumes of solutions of 1X, AAMU, 1U and 17U, adjusted to pH 3.0 with 25% HCl, before being diluted with a blank urine previously acidified to the same pH, to obtain a standard urine containing these metabolites at a concentration of 250 μ*M*. Subsequent dilutions were prepared with a blank urine – acidified to pH 3.0 with 25% HCl – obtained from one healthy volunteer after a 3-day xanthine-free diet. All standard calibration solutions were stored as 150 μl aliquots at −20°C before their analysis.

A stock solution of AFMU was prepared at a concentration of 375 μ*M* in dimethylformamide (DMF)–ethyl acetate (30:70), a solvent mixture in which it had been found stable [20]. The solution was kept separately at −20°C to prevent its possible decomposition in the standard calibration samples. AFMU solution was added ex tempore to the calibration standard solutions just prior to the HPLC analysis.

The solution of the internal standard (19U) was prepared in acetonitrile–water (90:10) at a concentration of 320 μ*M*.

2.3. Urine sample collection

Healthy volunteers were given a 2.5 mg/kg dose of caffeine as a cup of coffee (Nescafé Gold) after a 1-day xanthine-free diet. Spot urine samples were collected 8 h after the caffeine intake. One portion of the urine samples was immediately acidified at pH 3 with 25% HCl (40–190 μl to 20 ml urine) to prevent at best the decomposition of AFMU to AAMU [7] before being analyzed.

2.4. Procedure

For comparative studies, the samples were processed according to two different procedures performed in parallel: (A) for the simultaneous assay of AFMU and AAMU in the calibration solutions and in the acidified urine samples and (B) for the analysis in urine samples of AAMU only, after complete transformation of AFMU into AAMU in basic conditions.

2.4.1. Sample preparation A

To 150 μl acidified urine sample, 70 μl water, 50 μl internal standard (19U) solution, 100 μl DMF–ethyl acetate (30:70) and 800 μl acetonitrile were added. The mixture was vortex-mixed for 30 s, centrifuged for 10 min at 10 000 *g* and directly injected (10 μl) onto the HPLC column.

2.4.2. Sample preparation B

To 150 μl unacidified urine sample, 30 μl 0.5 *M* NaOH solution was added to convert AFMU into AAMU. The mixture was allowed to stand at room temperature for 30 min before successive addition of 40 μl 0.5 *M* HCl solution, 50 μl internal standard (19U) solution, 100 μl DMF–ethyl acetate (30:70) and 800 μl acetonitrile. The mixture was vortex-mixed for 30 s, centrifuged for 10 min at 10 000 *g* and directly injected (10 μl) onto the HPLC column.

2.5. Chromatographic system and conditions

The chromatographic system consisted of a Merck–Hitachi L-6200A pump, a Merck–Hitachi AS-2000A autosampler (Merck, Darmstadt, Germany) and a LINEAR 206 PHD spectrophotometric UV–Vis detector (Thermo Separation Products, San Jose, CA, USA). The software ChromQuest version 2.1 (1998; Thermo Separation Products) was used to pilot the HPLC instrument and to process the data throughout the method validation and sample analysis. Baselines were visually inspected and manually adjusted using peak start and end features of the ChromQuest software.

2.5.1. First approach: chromatography using a Nucleosil 100-5 μm NH_2 packing

In a first series of experiments, separations of caffeine and its 15 metabolites (listed in Table 1) were performed on a ChromCart Nucleosil 100-5 μm NH_2 (250×4 mm I.D.) column (Macherey-Nagel, Düren, Germany) equipped with a guard column (8×4 mm I.D.) filled with the same packing material. Among the numerous gradient elution programs tested, the following elution conditions gave a baseline separation (data not shown) for caffeine and its 15 metabolites. Step-wise gradient elution was applied using acetonitrile–0.6% (v/v) acetic acid: (98:2) at 0 min→(85:15) at 45 min→(60:40) at 70 min at a flow-rate of 1.0 ml/min. For controlling at best the retention times and peak areas of analytes, the column had however to be washed for 15 min with 0.6% (v/v) acetic acid between each analysis, followed by a reequilibration step at the initial mobile phase composition. Moreover, prior to its first use, the new amino column had to be carefully conditioned by washing the stationary phase successively with tetrahydrofuran, acetonitrile (20 min of each at 1 ml/min) and 1.0% (v/v) acetic acid (30 min at 1 ml/min). The column was equilibrated thereafter with 0.6% (v/v) acetic acid (30 min at 1 ml/min) followed by the initial composition of the mobile phase gradient (see above).

Though efficient and giving a good separation of all 16 compounds, the chromatography on the Nucleosil 100-5 μm NH_2 packing was very long (up to 100 min, re-equilibration included) and obviously tedious, requiring numerous time consuming conditioning of the column. Moreover, the compounds tended to elute earlier with time (e.g., 2–3 min for the more retained substances) and the column resolution diminished after several series of analyses.

In addition, the signals of 1-methyluric acid and 1,7-dimethyluric acid in calibration samples were found to progressively decrease over time after multiple injections, without being explained by sample stability problems, as verified in subsequent studies. This poor reproducibility might be due to the instability of the conventional aminopropyl packing, especially noticable under gradient elution conditions. The complexity of the matrices of urine samples however does not allow to deliver the mobile phase isocratically.

In summary, this amino type stationary phase showed, as expected, less ruggedness compared to any standard octadecyl phase, but the variations in retention times on this normal phase, although minimized by regular column conditioning, precluded any straightforward peak identification in a routine analysis of large number of samples.

For all these reasons, another amino type packing material had to be considered for use on a semi-routine basis.

2.5.2. Final optimized method with a Polyamine II column

Optimal separations were finally achieved using the Polyamine II packing recently made commercially available, resulting in major improvements, notably on the precision (reproducibility of the retention time (typically RSD<1.7%) and peak areas and ruggedness of the analytical method (stability of the stationary phase packing when used on a regular basis), with concomitant analysis time reduction. Compared to conventional amino phases with pri-

mary *n*-propylamino ligands, the manufacturer claims that Polyamine II, a novel polymer coated amino phase, preserves the long-term retention characteristics and selectivity and gives the possibility of using gradient elution programs. The functionality of the stationary phase is achieved by a covalently bonded polymer layer containing secondary and tertiary amino groups not forming Schiff bases or other stable condensation products. In addition the secondary and tertiary amino groups of the polymer layer are to a large extent resistant to hydrolysis and oxidation.

The separations were therefore performed on a YMC-Pack Polyamine II (250×4.6 mm, 5 μm, 120A) column (GROM Analytik, Germany) equipped with a guard column (10×4 mm) filled with the same packing material. This change enabled more than 100 injections without any noticeable decrease in the performance of the HPLC column.

A step-wise gradient elution program was applied using acetonitrile–0.75% (v/v) formic acid: (91:9) at 0 min→(75:25) at 25 min→(65:35) at 35 min→(65:35) at 45 min→(91:9) at 50 min→(91:9) at 62 min. The last two steps correspond to the re-equilibration of the column with the initial solvent composition. The flow-rate was 1.0 ml/min. An aliquot of 10 μl of the sample was injected onto the HPLC column. The chromatographic separations was simultaneously monitored at UV 260 and 280 nm. The highest absorbance signal (i.e., at 260 nm for 1X and AAMU, and at 280 nm for AFMU, 17U and 1U) was chosen for the quantitative analyses. Chromatographic separations were carried out at room temperature. This last method was validated and applied throughout the phenotyping studies.

3. Results and discussion

This HPLC method provides a relatively simple procedure to quantify simultaneously AAMU, AFMU, 1X, 17U and 1U in a single run, without interferences from caffeine nor any of its known metabolites. The chromatographic profile of caffeine and 15 known metabolites is shown in Fig. 2a. Their identity and respective retention times are listed in Table 1. Baseline separation is achieved for all but the three dimethylxanthines – albeit not quantitated for our studies of the enzymatic activities – which are eluted together at 5.1–5.4 min as three non-resolved peaks. Representative chromatograms of the analyzed urine samples are presented in Figs. 2–4.

Fig. 2b shows the chromatogram of a blank urine sample from a healthy volunteer who was on a xanthine-free diet for 3 days. The major peak eluted at the end of the run (40.5 min) – found in all urine samples – was identified as uric acid by co-injecting the authentic substance. The chromatographic profile of a urine sample spiked with each of the analytes considered in this phenotyping study is shown in Fig. 2c. The chromatograms of a spot urine sample from a female healthy volunteer collected 8 h after caffeine intake, without and with the basic conversion of AFMU into AAMU, respectively, are presented in Fig. 3a and b. After the basic treatment, the AAMU/(AAMU+1U+1X) metabolic ratio measured in this sample is 0.51. Based on the 0.34 antimode value proposed by Tang et al. [9] this result corresponds to a fast acetylation NAT2 phenotype. By contrast, Fig. 4a and b show the chromatographic profiles of a urine sample collected from an individual with a slow acetylation phenotype AAMU/(AAMU+1U+1X)=0.28, after AFMU conversion. In these two urine samples, the calculated (AAMU+1X+1U)/17U and 1U/(1U+1X) ratios, reflecting the CYP1A2 and XO activity, respectively, were 6.29 and 0.54 in the fast acetylator subject while they were 3.83 and 0.51 in the slow acetylator subject. These results indicate a similar activity of XO in these two subjects, while the CYP1A2 enzymatic system, notoriously known to be highly variable and subject to induction or inhibition, shows a larger variation. Of note, these two subjects were both non-smokers, and their difference in CYP1A2 metabolism may depend upon other – non-determined – factors, outside the scope of the present analytical study.

Calibration curves using peak area of analytes vs. concentrations are linear over the 15–120 μ*M* range (r^2>0.997 for all metabolites). Spontaneous voidings were obtained from volunteers and the urinary flow-rate and urine volumes were not standardized. The concentrations of some analytes (notably AAMU in rapid acetylator individuals) were therefore found a posteriori in a few urine samples to lie outside the chosen range of the calibration curve, requiring in

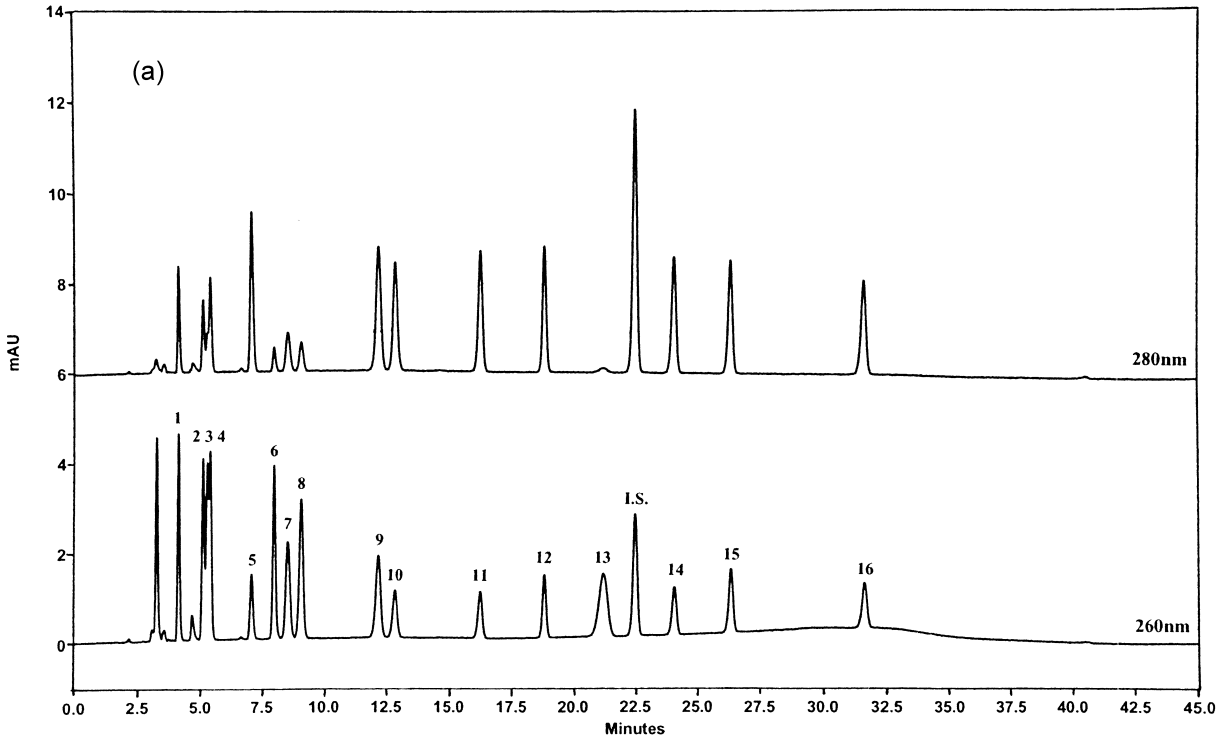

Fig. 2. (a) Chromatogram of a pure standard mixture of caffeine and its 15 metabolites (for peak identification see Table 1). (b) Chromatographic profile of a blank urine collected from one healthy individual after a 3-day xanthine-free diet. (c) Chromatogram of a control urine sample spiked with 1X, AFMU, 17U, AAMU and 1U at 75 μ*M* of each metabolite.

some cases higher urine dilution for achieving concentration within the established calibration range.

The intra-assay precision (expressed as RSD) of the method was evaluated by analyzing series ($n=5$) of blank urine samples spiked with each of the analytes at 20, 75 and 160 μ*M*, respectively (Table 2). The intra-assay RSDs are less than 4.2% for all metabolites. The deviations from nominal values (found minus nominal value over nominal value, expressed in %) range from −9.5 to +11.1% for all analytes.

These urine quality controls were also analyzed on three separate occasions. As shown in Table 2, the mean inter-assay precision were always lower than 9.4% and the accuracy ranged from −10.8 to +10.7% for all analytes.

The less polar xanthines are sufficiently retained on the amino-bonded stationary phase column, using a high percentage of acetonitrile in the mobile phase at the beginning of the gradient program. Polar metabolites – notably AAMU, 17U and 1U – are eluted later with a satisfactory separation from endogenous urinary components and other caffeine metabolites. This is in contrast with the C_{18} reversed-phase material which was also tested (data not shown), whereby AAMU is eluted very early, close to, or even simultaneously with the early eluting polar substances present in the urine, precluding the accurate measurement of this analyte, and thus the extent of the AFMU decomposition.

Even without any sample precleaning, a good selectivity was achieved with this Polyamine II phase column, with a gradient however of somewhat long duration.

Even though sample pretreatment was minimal (e.g., urine dilution, centrifugation and injection) and the injected volume precise, the internal standard method was nevertheless used throughout this method validation. The choice of a suitable internal

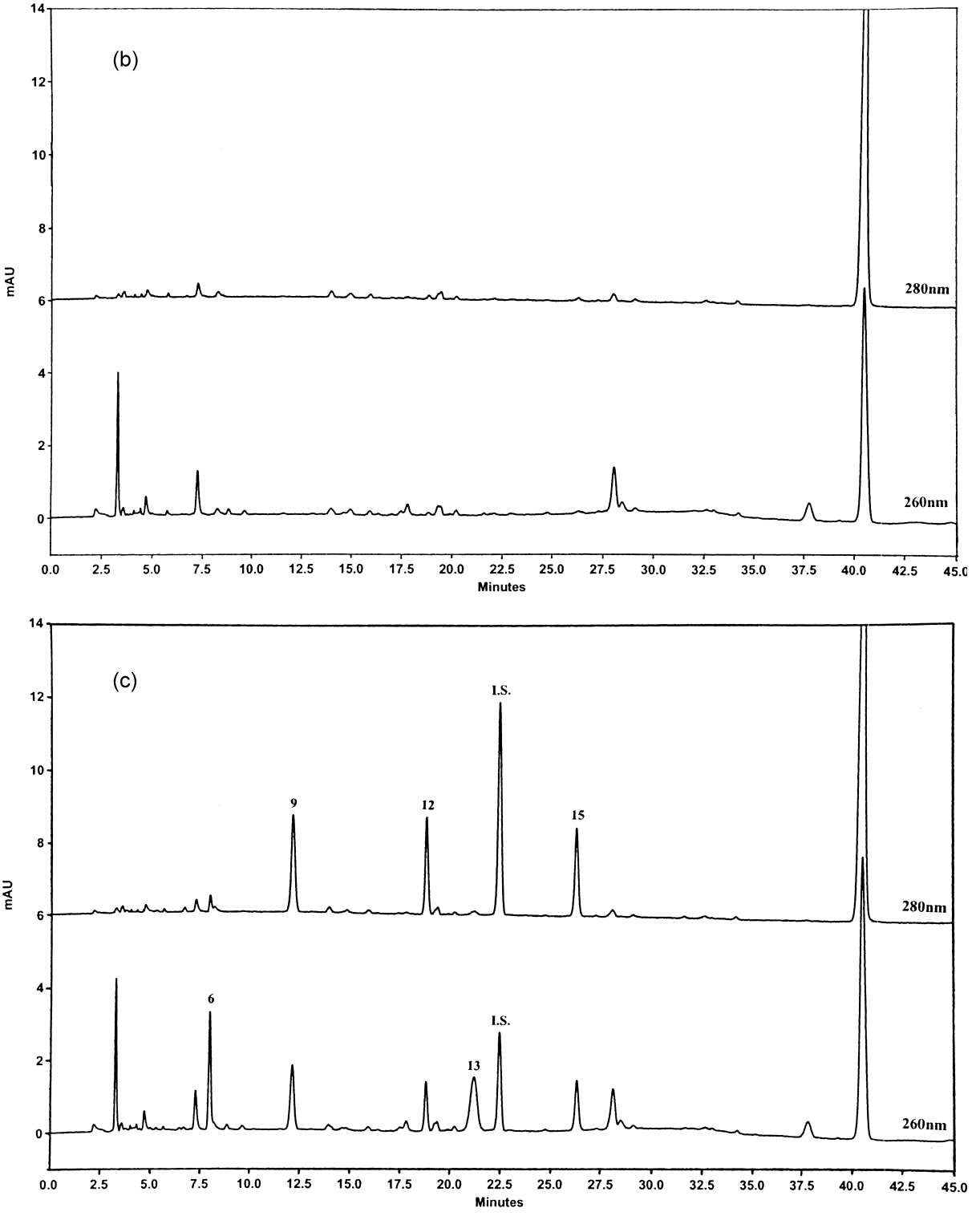

Fig. 2. (*continued*)

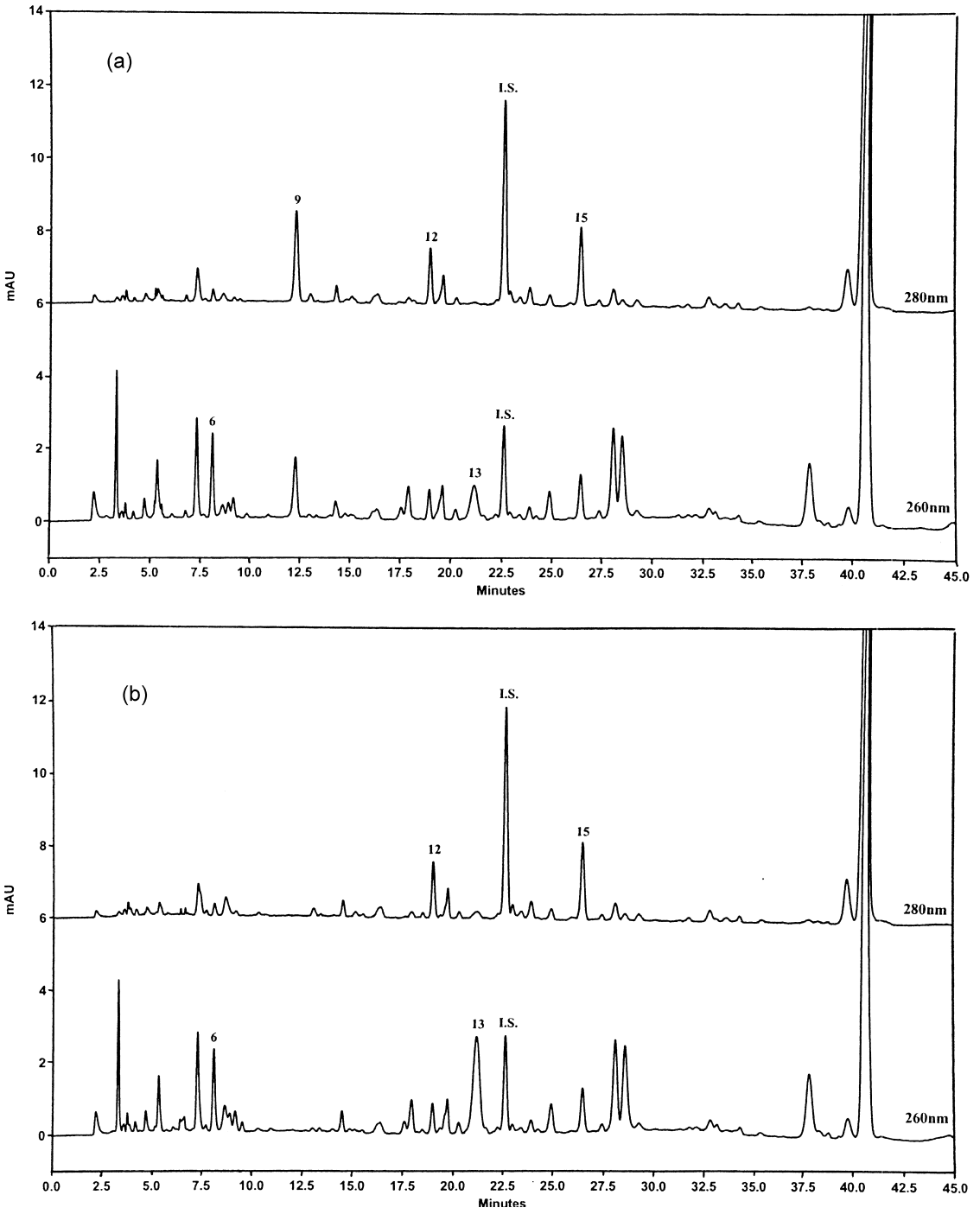

Fig. 3. Chromatogram of a urine sample of a rapid acetylator subject 8 h after caffeine intake before (a) and after (b) a treatment with 0.5 *M* NaOH solution to convert AFMU to AAMU. The basic treatment of the urine sample results in the complete disappearance of AFMU (peak 9) and in a corresponding increase of the concentration of AAMU (peak 13). Urinary levels of 1X, AFMU, 17U, AAMU and 1U in sample (a) were 62, 76, 43, 51 and 75 μ*M*, respectively. Of note, AAMU (peak 13) could already be detected in acidified sample (a) immediately analyzed (explanation in the text).

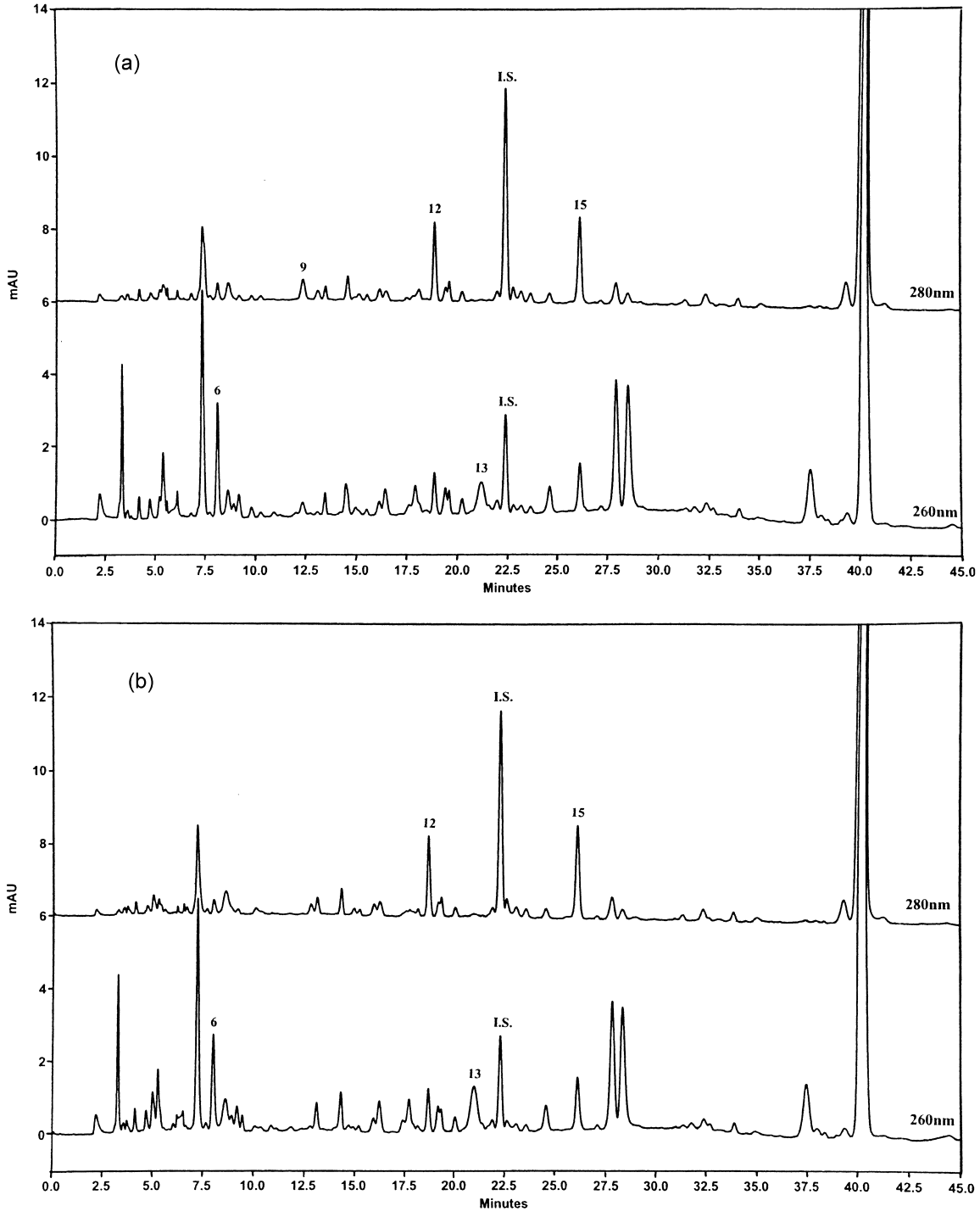

Fig. 4. Chromatographic profile of a urine sample of a slow acetylator subject 8 h after caffeine intake before (a) and after (b) a treatment with 0.5 *M* NaOH. Urinary levels of 1X, AFMU, 17U, AAMU and 1U in sample (a) were 80, 16, 60, 47 and 78 μ*M*, respectively. AAMU was already present in acidified sample analyzed immediately after voiding.

Table 2
Precision and accuracy of the measurements of five caffeine metabolites in urine

Nominal concentration	1-Methylxanthine			AFMU			1,7-Dimethyluric acid			AAMU			1-Methyluric acid		
(μ*M*)	Concentration found (μ*M*)	Precision, RSD (%)	Accuracy[a], deviation (%)	Concentration found (μ*M*)	Precision, RSD (%)	Accuracy[a], deviation (%)	Concentration found (μ*M*)	Precision, RSD (%)	Accuracy[a], deviation (%)	Concentration found (μ*M*)	Precision, RSD (%)	Accuracy[a], deviation (%)	Concentration found (μ*M*)	Precision, RSD (%)	Accuracy[a], deviation (%)
Intra-assay (n=5)															
20.0	18.3±0.6	3.5	−8.3	18.1±0.7	4.1	−9.5	21.4±0.5	2.4	7.1	20.2±0.2	1.1	0.8	22.2±0.9	4.2	11.1
75.0	74.4±1.0	1.3	−0.8	77.7±1.6	2.0	3.6	77.4±1.4	1.8	3.2	76.9±2.0	2.6	2.6	76.8±1.1	1.5	2.3
160.0	157.4±2.1	1.4	−1.7	166.1±2.4	1.5	3.8	157.6±2.0	1.2	−1.5	162.2±2.4	1.5	1.4	148.5±2.3	1.6	−7.2
Inter-assay (n=15)															
20.0	20.5±1.9	9.4	2.7	17.8±1.2	6.7	−10.8	21.0±0.7	3.1	4.8	21.5±1.5	6.9	7.3	22.1±0.8	3.5	10.7
75.0	75.8±1.7	2.3	1.0	75.4±3.7	4.9	0.6	76.9±1.9	2.4	2.5	76.7±1.5	1.9	2.3	77.0±1.3	1.7	2.7
160.0	155.5±3.6	2.3	−2.8	163.9±3.1	1.9	2.5	156.8±3.3	2.1	−2.0	160.0±3.4	2.1	0.0	149.7±4.4	2.9	−6.5

[a] Accuracy=(found value−nominal value/nominal value)·100.

standard (I.S.) was evaluated. 7-Methyluric acid (7U) has been proposed by another group [15] as a valid I.S., but may be questioned since 7U itself is a minor urinary metabolite of caffeine. In fact, we observed in almost all urines of subjects having taken caffeine a small peak at 32 min, at the retention time of 7U. By contrast, 1,9-dimethyluric acid (19U), a related compound which is not a caffeine metabolite, was found to be a suitable I.S. with the proposed gradient program. It is unlikely to be present in urine and was shown not to interfere with any other urinary components.

Importantly enough, the proposed method enabled one to demonstrate that, in spite of the immediate acidifying of urine samples followed by the HPLC analysis, AAMU could be detected in appreciable amount in urine (see Fig. 3a and Fig. 4a). During the method validation, it has been verified that AFMU is indeed stable during the duration of the HPLC analysis: it decomposes neither in the vials stored at room temperature in the autosampler rack, nor during its elution into the HPLC column. This indicates that the deformylation of AFMU into AAMU is likely to happen very early, in the bladder of the subjects, or even at an earlier stage. The influence of this finding on the NAT2 metabolic ratio values – and hence enzymatic activities determination – has yet to be established and is currently underway.

The duration of the analysis of the five considered urinary metabolites of caffeine is 45 min. Since each HPLC analysis must be followed by a 17-min re-equilibration step, this HPLC assay is therefore not rapid. This long duration is counterbalanced however by the highly simple and cheap sample preparation. Since the method can be fully automated, analyses of large number of samples can be easily carried out overnight.

The procedure described here represents a substantial improvement over previous methods: a single analysis and a minimal urine sample treatment enables the simultaneous quantitation of five caffeine metabolites, notably AFMU and AAMU, used for the determination of CYP450 1A2, XO and NAT2 enzyme activity. Importantly enough, phenotyping of individuals for the bimodal NAT2 is made possible without the uncertainty associated with the deformylation of AFMU, likely to happen at all steps prior to the analysis, during sample storage and even in the bladder of the subjects.

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