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Determination of *N*-acetylation phenotype using caffeine as a metabolic probe and high-performance liquid chromatography with either ultraviolet detection or electrospray mass spectrometry

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Abstract

A rapid, sensitive method using liquid chromatography–electrospray mass spectrometry (LC–ES-MS) was developed and evaluated for the simultaneous quantitative determination of caffeine metabolites 1U, 1X and AAMU in human urine. This method involved a simple dilution of urine samples. The chromatographic separation was achieved on a C_{18} reversed-phase column using a gradient of acetonitrile in 2 m*M*, pH 3.0 ammonium formate as mobile phase. After ionisation in an electrospray source, mass spectrometric detection was performed in the negative ion, selected ion monitoring mode. This method yielded acceptable accuracy and precision within the range 0.25–50 μg/ml. This analytical method was applied to investigate the *N*-acetylator phenotype of HIV-infected patients and compared with high-performance liquid chromatography with UV detection. Its specificity was better, which appeared to be absolutely necessary to prevent errors in metabolic ratios and phenotype interpretation.

Keywords: *N*-Acetylation phenotype; Caffeine; 1-Methylxanthine; 1-Methyluric acid; 5-Acetylamino-6-amino-3-methyluracil

1. Introduction

Acetylation phenotype is genetically determined by the expression of the polymorphic arylamine *N*-acetyltransferase-2 (NAT2) [1]. Two major phenotypes can be distinguished: fast and slow *N*-acetylators. The investigation of such a polymorphism is useful because of its association with adverse drug effects of amine-containing drugs and of predisposition to disease such as bladder cancer and systemic lupus erythematosus [1]. In particular, slow acetylator phenotype status has been associated with a greater risk of side effects when sulfonamides are used in human immunodeficiency virus (HIV)-infected patients [2]. The in vivo metabolic phenotype can be readily determined with the use of a probe such as caffeine. Urinary concentrations of metabolites after an oral dose of caffeine have been used as safe and useful indicators of NAT2 phenotype. Caffeine undergoes *N*-demethylation at three sites forming paraxanthine, theophylline and theobromine. Paraxanthine is further demethylated to a short-lived intermediate that is subsequently stabi-

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lised either as 5-acetylamino-6-formylamino-3-methyluracil (AFMU) – step catalysed by NAT2 – or as 1-methylxanthine (1X). The further hydroxylation of 1X to 1-methyluric acid (1U) is catalysed by xanthine oxidase. The NAT2 phenotype may be determined by measuring the AFMU/1X molar metabolic ratio in urine [3]. A complete conversion of AFMU to 5-acetylamino-6-amino-3-methyluracil (AAMU) with sodium hydroxide (pH 10) was often proposed to overcome problems associated with the instability of AFMU during sample storage or sample preparation [4,5]. In order to take into account the possible inter-individual variation in xanthine oxidase activity, the metabolic ratio of AAMU over the sum of AAMU, 1X and 1U has been used [6]. Many analytical methods have been used for the quantitation of these caffeine metabolites. These include high-performance liquid chromatography (HPLC) [4,7,8], capillary electrophoresis (CE) [9,10], or enzyme-linked immunosorbent assay [11]. A combined method with HPLC and CE was also described [12]. For sample preparation prior to the quantitation of these compounds in urine, either dilution [4] or liquid–liquid extraction [7,13,14] have been used. In our hands, these methods were not specific enough to measure the metabolic ratio AAMU/(AAMU+1U+1X) in urine of HIV-infected patients.

The purpose of our work was to develop an analytical method which could specially provide a better selectivity for AAMU and 1U. Finally we propose a new liquid chromatography–electrospray mass spectrometry (LC–ES-MS) method for AAMU, 1U and 1X measurement in urine.

2. Experimental

2.1. Reagent and chemicals

1X, 1U, dyphylline and tetrabutylammonium hydrogen sulfate (TBA) were purchased from Sigma (Saint Quentin Fallavier, France). AFMU was kindly supplied by Nestec, Nestle Research Centre (Lausanne, Switzerland). The chemical structures of the analytes are shown in Fig. 1. Water was delivered by a Milli-Q Water purification system (Waters, Saint Quentin en Yvelines, France). Glacial acetic acid, sodium acetate, ammonium formate, ammonia, sodium hydroxide and hydrochloric acid, all of analytical quality, were from Prolabo (Paris, France). Acetonitrile, ethyl acetate, propanol of HPLC grade quality were from J.T. Baker (Noisy-le-sec, France).

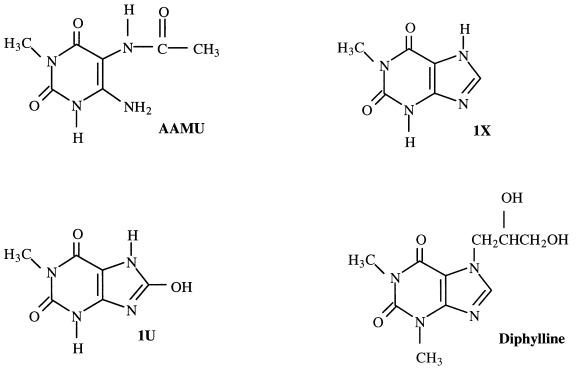

Fig. 1. Chemical structures of 1-methylxanthine (1X), 1-methyluric acid (1U), 5-acetylamino-6-amino-3-methyluracil (AAMU) and diphylline (I.S.).

2.2. Preparation of standards, calibration samples and quality control samples

Stock solutions of 1U, 1X in methanol and of dyphylline, internal standard (I.S.), in deionised water were prepared at 1 mg/ml in volumetric flasks. Sodium hydroxide (0.01 *M*) was added to improve the dissolution of 1U. AFMU was dissolved in 0.1% of acetic acid, to avoid its degradation. Diluted solutions of metabolites and I.S. were prepared daily from the stock solutions. Calibration data were generated by spiking drug-free urine samples with AAMU, 1X and 1U over a concentration range from 1 to 50 μg/ml for HPLC–UV and 0.25 to 50 μg/ml for LC–ES-MS. The calibration curves were obtained by linear regression analysis applied to the concentration versus peak area of metabolites for HPLC–UV and versus peak area ratio of each metabolites to I.S. for LC–ES-MS. For quality control samples, aliquots of convenient working solutions of 1X, 1U and AAMU in deionised water, were added to 5 ml of metabolite-free urine in a volumetric flask, to yield final concentrations of 1, 10 and 50 μg/ml of each compound. These samples were stored at −20°C. Only material frozen for less than 6 months was used.

2.3. HPLC–UV method, apparatus and operating conditions

2.3.1. Sample treatment

For 1U and 1X a solid-phase extraction was used. First, the column (Bond Elut 100 mg; Varian, Harbor City, CA, USA) was conditioned with 1 ml methanol and 1 ml of 0.1 *M* pH 3.8 sodium acetate–acetic acid (1:9). A 100-μl urine sample was diluted with 1 ml acetate buffer and transferred onto the column. The column was washed with 500 μl acetate buffer and finally, the analytes were eluted with 300 μl methanol. The eluate was evaporated to dryness under nitrogen and the residue was dissolved in 1 ml mobile phase. 50 μl was injected onto the HPLC column.

AFMU was converted into AAMU by adding 100 μl of 0.1 *M* NaOH to 100 μl urine. After 10 min of incubation, 100 μl of 0.1 *M* HCl was added. The volume was adjusted to 1 ml with water and 50 μl was injected onto the HPLC column.

2.3.2. Chromatographic conditions

For 1U and 1X, the column used was a Hypersil ODS (5 μm particle size, 250×4.6 mm I.D.) (Hypersil, Les Ulis, France) preceded by a guard column. The mobile phase consisted of 10 m*M* sodium acetate and 5 m*M* TBA, adjusted to pH 5.4 with NaOH. Immediately after the elution of 1U, a linear gradient elution was used to elute other caffeine metabolites and endogenous compounds prior the next injection, by adding acetonitrile up to 50% in the mobile phase. Between each run, the original mobile phase was used for 10 min conditioning.

For AAMU, the column used was a μBondapack NH_2 (5 μm particle size, 250×4.6 mm I.D.) (Beckman, Gagny, France) preceded by a guard column. The mobile phase consisted of a mixture of acetonitrile–0.5% glacial acetic acid (95:5, v/v).

In both cases, chromatography was performed at ambient temperature using a 1 ml/min flow-rate.

The eluent was monitored at 273 nm for 1U and 1X and 254 nm for AAMU, with a detector sensitivity of 0.01 a.u.f.s.

2.3.3. Chromatographic apparatus

The chromatographic apparatus was comprised of a Shimadzu LC-6A (Touzart et Matignon, Courtaboeuf, France) solvent delivery pump, a Waters 715 ultraWISP autosampler injector (Waters, Saint-Quentin en Yvelines, France), a Shimadzu SPD-6A variable-wavelength UV detector and a Shimadzu C-R6A reporting integrator (Touzart et Matignon).

2.4. LC–ES-MS analysis

2.4.1. Sample preparation

To 50 μl urine samples were added 100 μl of a 10 mg/l I.S. solution and 50 μl deionised water. After 30 s vortex-mixing and 5 min centrifugation at 10 000 *g*, 3 μl was injected into the LC–ES-MS system.

The conversion of AFMU to AAMU with sodium hydroxide was not possible, due to the necessary neutralisation with hydrochloric acid which lead to the precipitation of sodium chloride in the mass spectrometer. Therefore, this conversion was induced overnight with 0.2 *M* ammonia, and neutralisation was performed with acetic acid.

2.4.2. Chromatographic conditions

The HPLC system consisted of two Perkin-Elmer Series 200 LC micropumps connected to a Series 200 autosampler (Perkin-Elmer, Norwalk, CT, USA). The eluate was passed through a MIC 15 CP, C_{18}, 3.5 μm column (150×1 mm I.D.; LC Packings, Marseille, France). Two solvents were used: (A) 2 m*M* ammonium formate, pH 3 and (B) acetonitrile. The gradient elution profile was: 0–2 min, 100% A (isocratic); 2–11 min, from 0 to 50% B in A (linear gradient); 11–12 min, A–B (50:50). The mobile phase was delivered at a flow-rate of 50 μl/min.

2.4.3. Mass spectrometric conditions

LC–ES-MS analysis was performed using a Sciex API 100 triple quadrupole mass spectrometer (PE-Sciex, Toronto, Canada). This instrument is equipped with an ionspray (pneumatically-assisted electrospray) interface and a MacIntosh data system. The mass spectrometer was operated in the negative ion mode. Full mass spectra were obtained by acquiring data between 100 and 1000 u, then quantitation was performed in the selected ion monitoring mode, using the $[M+H]^+$ pseudo-molecular ion of each compound for quantitation and, when possible, one or two fragments as confirmation ion(s). The follow-

ing conditions were used in ES-MS negative ion experiments: ionisation voltage 5500 V, fragmentation (orifice) voltage optimised for each ion selected (Table 2).

2.5. Validation studies

Within-day precision and accuracy was studied at concentration levels used for calibration by analysis on the same day of a single injection of six samples spiked with the three metabolites for each level. For the between-day precision and accuracy study and for studying the linearity of the method, sets of calibration samples were prepared in advance and analysed each day for 6 days. Accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of the measured concentration (C_{mes}) as follow: $[(C_{mes}-C_{nom})/C_{nom}]\cdot 100$. The precision relative standard deviation (RSD) was calculated from the measured concentration [standard deviation (SD)/C_{mes}]·100.

The limit of detection was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3:1. The limit of quantitation (LOQ) was determined as the lowest amount of the analyte which could be determined in a sample with a precision and accuracy better than 20%.

Extraction recovery was determined by comparing the peak area of drug-free urine spiked with known amounts of metabolites versus peak area of the same concentrations prepared in purified water and injected directly on the column. Six replicate analyses were carried out at each concentration.

2.6. Application of the method to the determination of the acetylation phenotype in HIV-infected patients

The subjects were instructed to refrain from consumption of products containing methyl xanthine such as coffee, tea, cola and chocolate for 18 h before the test and for the duration of the test. The subjects drank two cups of coffee or very strong tea. The dose of caffeine was between 80 and 140 mg. Urine samples were collected during the first 6 h following the ingestion of caffeine. Aliquots of urine samples were immediately stored at −20°C until analysis.

3. Results

3.1. HPLC–UV

Fig. 2 shows typical chromatograms obtained from human urine samples. The retention times for 1X and 1U were, respectively, 9 min and 12.9 min on the Hypersil ODS column and that of AAMU was 10.6 min using the μBondapack NH_2 column.

The recovery of 1U and 1X from spiked urine samples ranged from 80.1 to 88.8% and 93.1 to 99.1%, respectively.

A linear relationship was obtained for 1U, 1X and AAMU, covering the concentration range 1–50 mg/l.

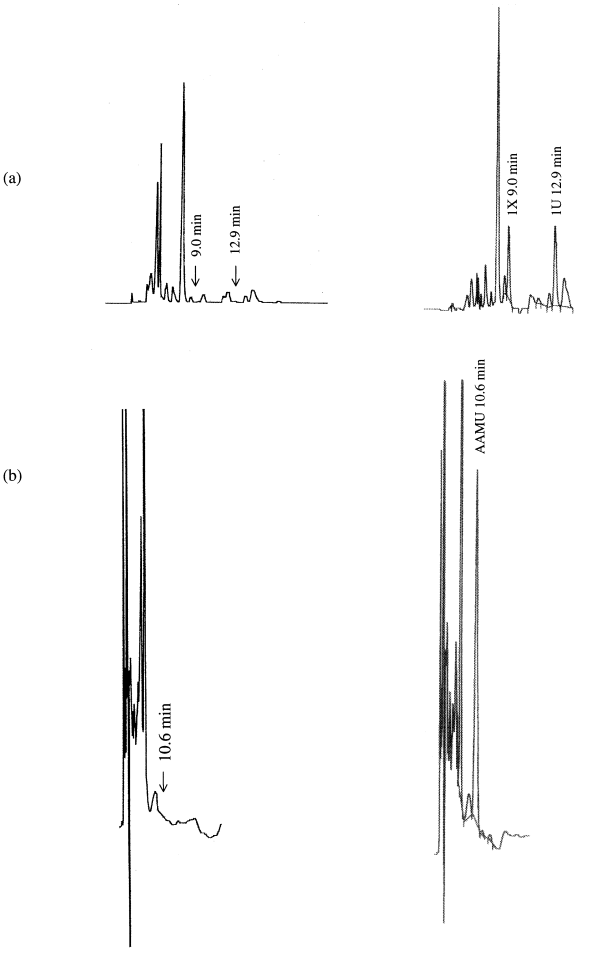

Fig. 2. HPLC–UV chromatograms of a blank urine (left) and a human urine sample after drinking two cups of strong coffee (right): (a) 1U, 1X and (b) AAMU.

Regression analysis gave squared correlation coefficients of $r^2=0.9999$, $r^2=0.9993$ and $r^2=0.9996$ for AAMU, 1U and 1X, respectively.

The intra-day and between-day precision and accuracy values are reported in Table 1. The RSDs for the three metabolites were equal or less than 15%, except for AAMU at the concentration of 1 μg/ml (RSD=16.8%).

The lowest concentration of the calibration graph was 1 μg/ml, which was therefore the practical lower limit of quantitation for each compound.

3.2. LC–ES-MS

Representative ion chromatograms of human urine samples assayed by LC–ES-MS are shown in Fig. 3. The substances were determined using their pseudo-molecular ion (MH+). Table 2 show retention times and selected quantitation and confirmation ions for the three metabolites and I.S. On the reconstructed ion chromatograms the signals from $(1U+H)^+$ at m/z 183.1 and $(1X+H)^+$ at m/z 167.1 were well separated from major endogenous compounds but also from other caffeine metabolites. Endogenous compounds interfered sometimes with the signal from $(AAMU+H)^+$ at m/z 199.1, but did not impede quantification. 1U and 3-methylxanthine (3X) had similar retention times but 3X ions were different from those of 1U.

The three caffeine metabolites showed a linear behaviour in the concentration range studied. Standard curves calculated using a $1/x$ weighted regression analysis showed squared correlation coefficients (r^2) of 0.999 or better.

The intra-day and between-day precision and accuracy, as reported in Table 3, were satisfactory.

The limit of quantitation could be set at 0.25 μg/ml for the three compounds.

3.3. Clinical applications

The suitability of the LC–ES-MS method for clinical samples was demonstrated by determination of the AAMU/(AAMU+1U+1X) molar ratio in urine samples of 43 healthy subjects (30 females, 13 males aged between 20 and 50 years). The subjects

Table 1
Within-day and between-day precision and accuracy obtained with HPLC–UV ($n=6$)

Compound		Concentration (μg/ml)					
		1	2.5	5	10	25	50
AAMU	Within-day						
	Precision RSD (%)	16.8			2.3		0.7
	Accuracy (%)	111.1			102.1		99.0
	Between-day						
	Precision RSD (%)	9.8	12.6	8.1	10.4	4.7	6.4
	Accuracy (%)	105.6	91.6	102.3	95.2	103.8	93.1
1X	Within-day						
	Precision RSD (%)	7.6			6.0		4.1
	Accuracy (%)	15.6			96.1		98.2
	Between-day						
	Precision RSD (%)	3	2.2	3.6	2.1	4.6	3.1
	Accuracy (%)	106.3	105.0	102.9	93.6	103.5	102.8
1U	Within-day						
	Precision RSD (%)	10.9			2.7		1.1
	Accuracy (%)	115.1			99.8		99.8
	Between-day						
	Precision RSD (%)	13.7	4.2	7.3	4.2	6.8	1.6
	Accuracy (%)	106.8	105.0	107.5	93.2	101.7	99.6

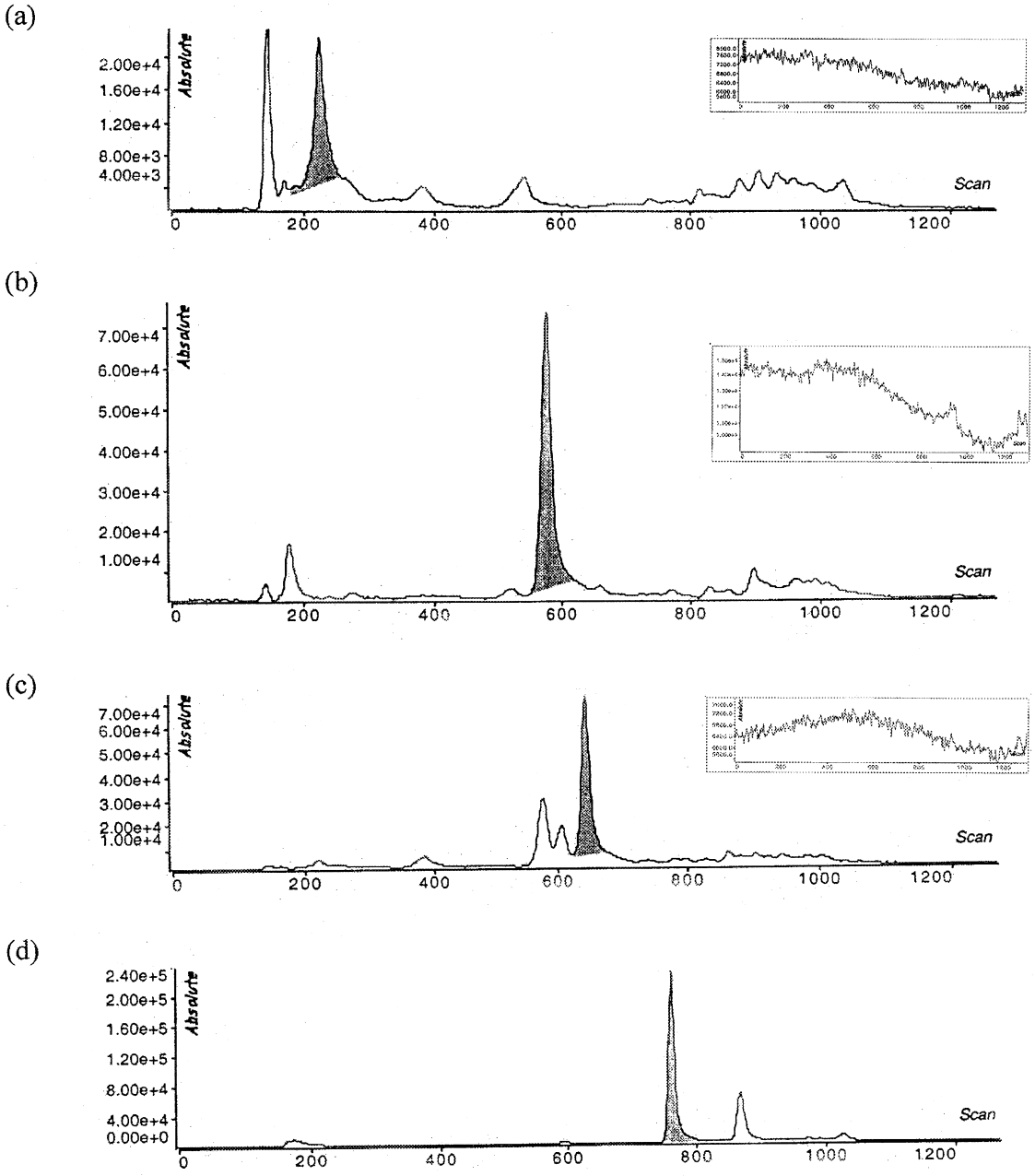

Fig. 3. LC–ES-MS ion chromatograms of a blank urine (inset) and a human urine sample after drinking two cups of strong coffee (a) AAMU; (b) 1U; (c) 1X; (d) dyphilline.

were classified according to the estimated value of this ratio (Fig. 4). Acetylator status was defined using probit plot [15]. When molar was lower than 0.22, the patient was considered as slow acetylator.

4. Discussion

The aim of our work was to develop a method to determine the acetylation phenotype in humans and

Table 2
Retention times and selected ions of AAMU, 1X, 1U and internal standard

	Retention time (min)	Selected ions[a] (a.m.u)	Orifice voltage (V)
AAMU	3.6	**199.1**	**10**
		157.1	45
1U	9.0	**183.1**	**15**
		–	
1X	10.1	**167.1**	**15**
		110.0	45
Dyphilline (I.S.)	11.9	**255.1**	**15**

[a] Quantifying ions in bold.

to apply this method to HIV-infected patients. We first used HPLC–UV methods. Many HPLC methods have been reported in the literature. Most of them used exclusion chromatography to determine AAMU (after converting AFMU to AAMU under basic conditions) and reversed-phase chromatography to measure other caffeine metabolites. Adequate sample purification prior to HPLC is needed for the determination of caffeine metabolites, since the analysis of human urine is affected by the presence of interferences. All our attempts failed to extract AAMU from urine samples with a good yield (>50%) either with liquid–liquid extraction using different solvents (chloroform–isopropanol mixtures, *tert*.-butyl-methyl-ether) or with SPE (with C_{18} or NH_2 phase). A simple dilution of urine (after treatment with NaOH and neutralisation by HCl) was finally used as previously described [4]. For 1U, previously described extraction methods [7,14,16] using acetate–isopropanol or chloroform–isopropanol

Table 3
Within-day and between-day precision and accuracy obtained with the LC–ES-MS assays (n=6)

Compound		Concentration (μg/ml)							
		0.25	0.5	1	2.5	5	10	25	50
AAMU	Within-day								
	Precision RSD (%)			12.2			7.8		1.8
	Accuracy (%)			93.2			88.0		93.3
	Between-day								
	Precision RSD (%)	8.8	16.7	9.9	3.8	5.4	5.3	4.5	5.5
	Accuracy (%)	101.7	84.6	107.1	100.7	103.1	102.2	103.2	96.5
1X	Within-day								
	Precision RSD (%)			4.7			8.9		9.6
	Accuracy (%)			83.2			88.8		82.7
	Between-day								
	Precision RSD (%)	17.25	8.3	7.5	6.3	11.7	8.2	6.4	7.6
	Accuracy (%)	92.0	101.4	105.5	100.5	104.9	105.5	94.8	91.3
1U	Within-day								
	Precision RSD (%)			9.1			8.0		9.8
	Accuracy (%)			92.8			97.2		99.7
	Between-day								
	Precision RSD (%)	7.2	9.5	12.8	11	9.7	6.0	12.3	5.3
	Accuracy (%)	101.1	101.1	100.6	105.3	100.5	96.7	101.9	93.9

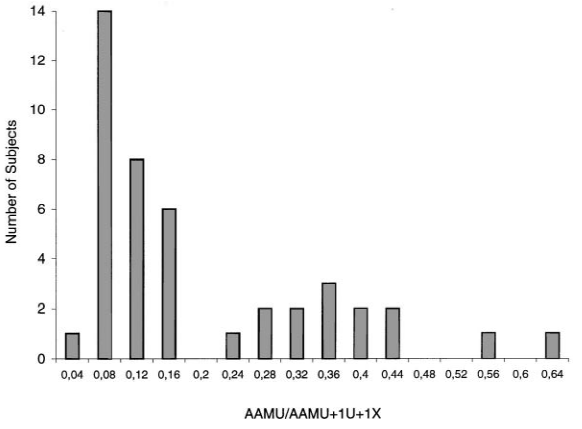

Fig. 4. Histogram showing the distribution of AAMU/(AAMU+ 1U+1X) ratio in 43 subjects, as determined by LC–ES-MS.

mixtures lead to recoveries not higher than 40%. We were not able to find a good compromise between satisfactory recovery and thorough sample cleaning using liquid–liquid extraction. We finally used SPE for 1U and 1X, which gave good recovery. AAMU and 1U were not retained or separated in the isocratic method previously described [7,8]. The separation on a C_{18} column [8] was not an interesting method in our hands: AAMU was not retained enough and endogenous polar compounds interfered. The use of two columns in tandem as previously described [7] or of a column switching technique with various columns (CN, C_{18}, SCX) did not yield suitable separation from interferences. For these reasons, we developed two different methods, for AAMU on one hand and for 1U and 1X on the other hand. A suitable retention of AAMU was allowed with a μBondapack NH_2 column. For 1U, the addition of TBA in the mobile phase ensured more retention on the Hypersil C_{18} column, especially when the pH was increased, and avoided coelution with interfering substances. In both HPLC methods, a mobile phase gradient was necessary to enable adequate retention and separation of AAMU, 1X and 1U and to ensure the elution of caffeine and other strongly retained compounds. Population studies necessitate the analysis of large numbers of samples, requiring a reliable analytical method. Although our assay satisfactorily quantified AAMU, 1U and 1X in healthy subject urine samples, it was not convenient for urine samples of HIV-infected patients, because they showed a lot of interfering substances eluting at the retention time of AAMU.

To solve these difficulties, we attempted to use CE according to Lloyd et al. [9] (data not shown). The main problem was also related to interfering peaks with all metabolites involved in the metabolic ratio.

The problem of selectivity was only solved using LC–ES-MS, which even need no prior extraction. To our knowledge, no LC–ES-MS method had been previously described for the acetylation phenotype determination based on caffeine metabolism. This method showed improved specificity and selectivity as compared with HPLC. To avoid the precipitation in the mass spectrometer of sodium chloride generated by successive addition of NaOH and HCl, the conversion of AFMU to AAMU was induced by incubation with ammonia overnight. However, in these conditions, 1U was unstable. Because AAMU may be already formed in the bladder or during manipulation or storage [5] we maintained two separate sample preparations, with and without pretreatment with ammonia, to determine AAMU in the first case and 1U and 1X in the second. The method yielded a reliable determination of AAMU, 1U and 1X down to 0.25 μg/ml in urine. These limits of quantification were lower than those obtained with HPLC methods with UV detection and lower than the urine concentrations expected for phenotype determination in our conditions.

5. Conclusion

A sensitive LC–ES-MS method for the determination of caffeine metabolites molar ratio is presented. This method was validated over the 1–50 μg/ml concentration range, showing a good selectivity, precision and accuracy. This method requires no sample extraction and is highly specific, as compared with the other methods. Finally, this method was suitable for the determination of the urinary molar ratio of caffeine metabolites in healthy volunteers and HIV-infected patients.

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