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Short communication

Liquid chromatographic method for the simultaneous determination of caffeine and fourteen caffeine metabolites in urine

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Abstract

An HPLC method has been developed for the separation and the determination of caffeine and its metabolites in urine samples using a one extraction–analysis run and UV detection. The compounds were extracted by liquid–liquid extraction using chloroform–isopropylalcohol (85:15, v/v). Chromatographic separation was accomplished on an ODS analytical column with a mobile phase containing 0.05% acetic acid/methylalcohol (92.5:7.5, v/v). Compounds were monitored at 280 nm. The method was validated for the determination of AFMU, 1X, 1U, 17X and 17U caffeine metabolites required to assess the metabolic activity of the enzymes subject to in vivo caffeine testing. The validated assay was applied to urine samples from ten healthy volunteers. The method was proved to be suitable to assess simultaneously the enzymatic activity of cytochrome P450 CYP1A2 and CYP2A6, as well as *N*-acetyltransferase and xanthine oxidase.

Keywords: Caffeine

1. Introduction

Caffeine is commonly used as a probe drug to assess the metabolic activity of various isoforms of cytochrome P450 (CYP) and other drug metabolizing enzymes non-invasively and simply by measuring urinary elimination of its metabolites [1–3]. Caffeine metabolism is complex with significant differences between species as well as between individuals in different clinical situations [1,3–6]. In healthy human subjects, caffeine 3-*N*-demethylation (CYP1A2 dependent) producing paraxanthine (17X) is the dominant metabolic pathway (Table 1), (Fig. 1). Further 7-*N*-demethylation (CYPlA2 dependent) produces 1X. C-8-hydroxylation of 17X and 1X leads to the corresponding uric acids, 17U and 1U. These reactions depend on CYP2A6 and xanthine oxidase (XO), respectively. The acetylation of 17X producing AFMU is mediated by the polymorphic *N*-acetyltransferase (NAT2). A variety of metabolic indexes using urinary caffeine metabolites have been proposed to assess the metabolic activity of these enzymes [1–6]. The caffeine urinary metabolic ratios commonly used are (AFMU+1X +1U)/17U for CYP1A2, 17U/(AFMU+1X+1U+17X+17U) for

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Table 1
Abbreviations used

137X	1,3,7-Trimethylxanthine or caffeine
137U	1,3,7-Trimethyluric acid
37X	3,7-Dimethylxanthine or theobromine
17X	1,7-Dimethylxanthine or paraxanthine
13X	1,3-Dimethylxanthine or theophylline
37U	3,7-Dimethyluric acid
17U	1,7-Dimethyluric acid
13U	1,3-Dimethyluric acid
7X	7-Methylxanthine
3X	3-Methylxanthine
1X	1-Methylxanthine
7U	7-Methyluric acid
3U	3-Methyluric acid
1U	1-Methyluric acid
AAMU	5-Acetylamino-6-amino-3-methyluracil
AFMU	5-Acetylamino-6-formylamino-3-methyluracil
CYP	Cytochrome P450
I.S.	Internal standard
NAT2	Polymorphic *N*-acetyltransferase
XO	Xanthine oxidase

CYP2A6, AFMU/(AFMU+1X+1U) for NAT2, and 1U/(1X+1U) for XO.

Several drugs have been shown to be substrates for CYP1A2 [7]. Interest in CYPlA2 is also related to its implication in the mutagenic activation of heterocyclic amines [8]. CYP2A6 is involved in the metabolism of nicotine and coumarin [9,10]. NAT2 is a polymorphic enzyme with two nearly equal groups of ''slow'' acetylators and ''rapid'' acetylators in Caucasian populations [1–3]. NAT2 is responsible for the metabolism of many drugs relevant to human therapy such as dapsone, isoniazid, and amonafide [11,12]. Much of the interest in the acetylation polymorphism comes from the association of acetylator phenotypes with predisposition to develop drug-induced diseases including cancer [11]. XO is an important source of oxygen-derived free radicals [13]. XO is also implicated in the metabolism of drugs such as 6-mercaptopurine [14].

Reported analytical procedures for caffeine metabolites in urine have been essentially based on the methods of Grant et al. [1] and Tang et al. [15]. According to these procedures, two simultaneous extraction–HPLC analysis have to be carried out, one to measure xanthines and urates, and the other one to measure AFMU or its deformylated form, AAMU, after exposing the voided urine to alkaline pH. An assay allowing the simultaneous determination of AFMU, 1X, 1U, 17X and 17U, and using a single extraction–analysis run with isocratic and UV detection has been reported recently by Krul et al. [16]. However, in this study the resolution of the other caffeine metabolites, which are systematically co-extracted under the procedure described, was not investigated by the authors.

The present work reports a sensitive analytical High Performance Liquid Chromatographic (HPLC) method using a single extraction–analysis procedure and UV detection for caffeine and its 14 main metabolites. The assay was validated in urine and applied to the analysis of urine samples from ten healthy subjects.

2. Experimental

2.1. Chemicals

Caffeine and its metabolites (137U, 37X, 37U, 17X, 17U, 13X, 13U, 7X, 7U, 3X, 3U, 1X and 1U), *N*-acetyl-*p*-aminophenol and ammonium sulfate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). AFMU was generously donated by Nestle Research Center of Lausanne (Lausanne, Switzerland). HPLC-grade chloroform, glacial acetic acid, isopropylalcohol and methylalcohol were obtained from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Apparatus

The chromatographic HPLC system included a Spectra-Physics P200 pump (Spectra-Physics, San Jose, CA, USA), a Spectra-Physics SP8875 autosampler equipped with a 100-μl loop, a Rexchrom S5-100-ODS 5 μm (25 cm×4.6 mm I.D.) column (Regis, Chemical Co., IL, USA) with ODS kit Wide Pore 5 μm (1 cm×3 mm I.D.) Guard Column (Regis, Chemical Co., IL, USA), and a Spectra-Physics Spectra 100 variable wavelength detector (Thermo Separations, San Jose, CA, USA). The detector was operated at a wavelength of 280 nm. Solutes were eluted with the solvent A (0.05% acetic acid/methylalcohol, 92.5:7.5, v/v) according to the flow program described in Table 2. After the elution of the compounds needed for the enzyme assays, the column was flushed with solvent B containing

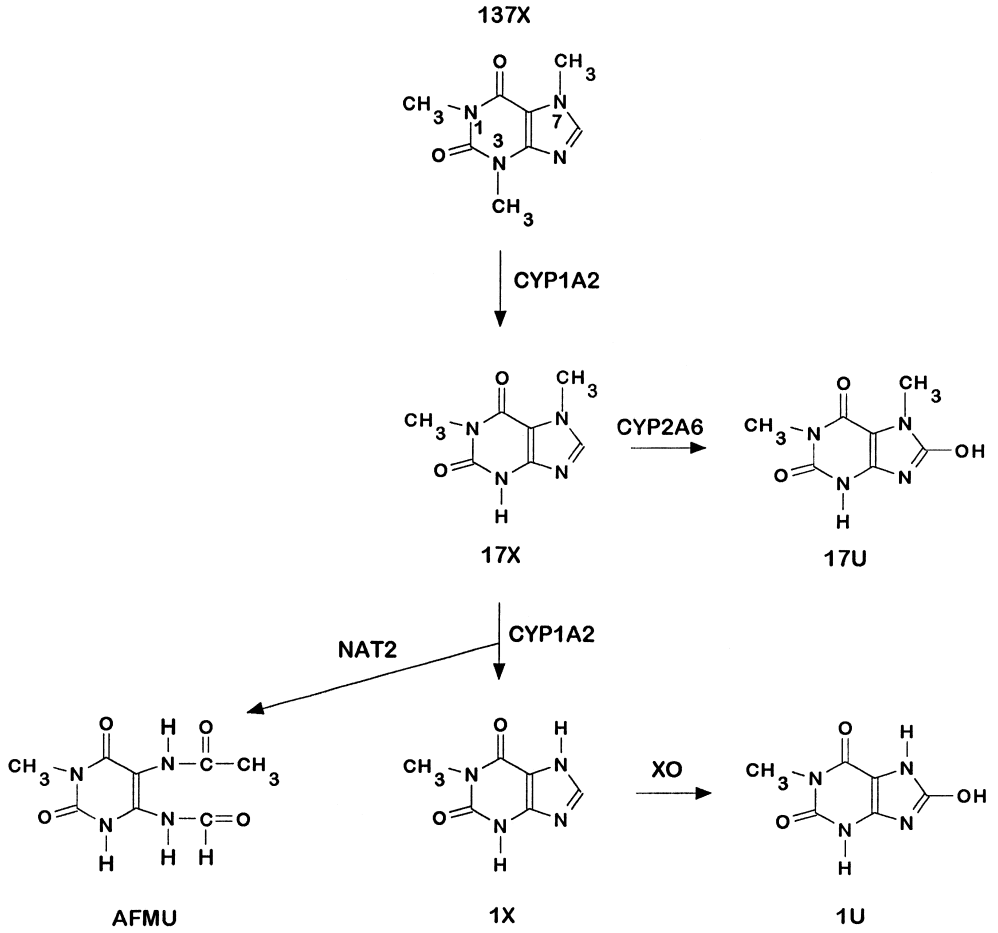

Fig. 1. Main pathways of caffeine metabolism in human healthy subjects and enzymes involved. Abbreviations are defined in Table 1.

Table 2
HPLC flow program used for the analysis of caffeine and its metabolites

Time (min)	Solvent A[a] (%)	Solvent B[b] (%)	Flow (ml/min)
0	100	0	0.5
10	100	0	0.5
11	100	0	1.0
45	100	0	1.0
46	0	100	1.0
60	0	100	1.0

[a] Solvent A: 0.05% acetic acid/methylalcohol (92.5:7.5, v/v).
[b] Solvent B: 0.05% acetic acid/methylalcohol (60:40, v/v).

increased methanol proportion (0.05% acetic acid/methylalcohol, 60:40, v/v) to remove the late-eluting compounds. Both solvents were filtered through a 0.45 μm nylon membrane (Millipore Filter Corporation, Bedford, MA, USA) and sonicated prior to use for at least 15 min. The column was re-equilibrated at initial conditions for 10 min before the next analysis.

2.3. Sample preparation

To a 100 μl of urine sample in 20 ml glass tube were added 800 μl of water, 120 mg of ammonium sulfate and 100 μl of internal standard (I.S., 30 mg/l *N*-acetyl-*p*-aminophenol in water). The sample was mixed with a hand vortex mixer, then extracted with

8 ml of chloroform/isopropylalcohol (85:15, v/v). Before centrifugation at 1000 *g* for 5 min, the sample was vigorously mixed for at least 1 min. The aqueous layer was removed by aspiration. The organic layer was transferred to a clean tube and evaporated to dryness using a speed-vac. The extract was reconstituted with 250 μl of elution solvent A, and 100 μl was injected directly into the HPLC system.

2.4. Standard solutions

A stock solution of caffeine and its metabolites, except AFMU, was prepared in distilled water at a concentration of 5 mg/l. AFMU stock solution was prepared in chloroform at a concentration of 5 mg/l. A stock solution of I.S. was prepared in distilled water at a concentration of 30 mg/l. All stock solutions were stored at 4°C. Our preliminary experiments showed that stock solutions are stable for at least one month under these conditions.

2.5. Method validation

For calibration curves and quality controls (QCs), samples were made by spiking caffeine-free urine with a known amount of stock solutions. Five concentrations in the range 0.5–20.0 μg/ml were used to construct the calibration curve for each compound. New standard curves were performed with each sample batch to minimize the analytical variability. Calibration curves were generated by plotting the ratio of the peak area of compound and I.S. against theoretical concentrations. Correlation coefficients and the equations describing the calibration curves were determined by linear regression analysis.

Three quality control samples were prepared. The concentration was equal to 1.0 μg/ml for the low quality control (LoQC), 4.0 μg/ml for the medium quality control (MeQC) and 17.5 μg/ml for the high quality control (HiQC). The intra and inter-day accuracy and precision of the method were performed from five and 15 replicates of each quality control, respectively.

The recovery of each compound was measured in triplicate by comparing peak-area of extracted quality control with the peak-area of the corresponding unextracted standards. The limit of quantification (LOQ) was set at the lowest concentration of the calibration curve. The limit of detection (LOD), defined as three times the signal-to-noise ratio, was determined by injecting diluted solutions of a mixture of caffeine and its metabolites.

2.6. Experimental subjects

Ten Caucasian healthy volunteers, university students and staff members who were regular caffeine consumers, provided a spot urine. The subjects were non-smokers, free from medication including oral contraceptives. There were seven men and three women, and the mean age was 26.6 years, with a range from 22 to 60 years.

Each subject was asked to collect a spot urine 5 h after drinking a regular cup of coffee and storing the sample in a refrigerator. On retrieval, the samples were acidified to pH 3–4 with concentrated HCl and stored at −20°C until analysis.

3. Results and discussion

The separation of caffeine and its main metabolites achieved using the experimental conditions is presented in Fig. 2. Blank urine extract (Fig. 3A) shows that no impurities are co-eluted with the compounds of interest. These results ensure that AFMU, 1X, 1U, 17X and 17U determination in urine is unaffected by other caffeine metabolites or by endogenous compounds during caffeine enzyme assays (Fig. 3B).

For each sample batch, the calibration curves of AFMU, 1X, 1U, 17X and 17U were linear over the range investigated (0.5–17.5 μg/ml). The standard curves were designed to address the expected range of concentrations encountered in regular caffeine consumers or after an administration of a standard dose (100–400 mg) of caffeine [1,6]. Correlation coefficients (r^2) were up to 0.998 for all the caffeine metabolites quantified. For each QC, the concentration was recalculated from the equation of the linear regression curve.

The results of intra-day ($n=5$) and inter-day ($n=15$) validation are summarized in Table 3. The method is reproducible with coefficient of variation

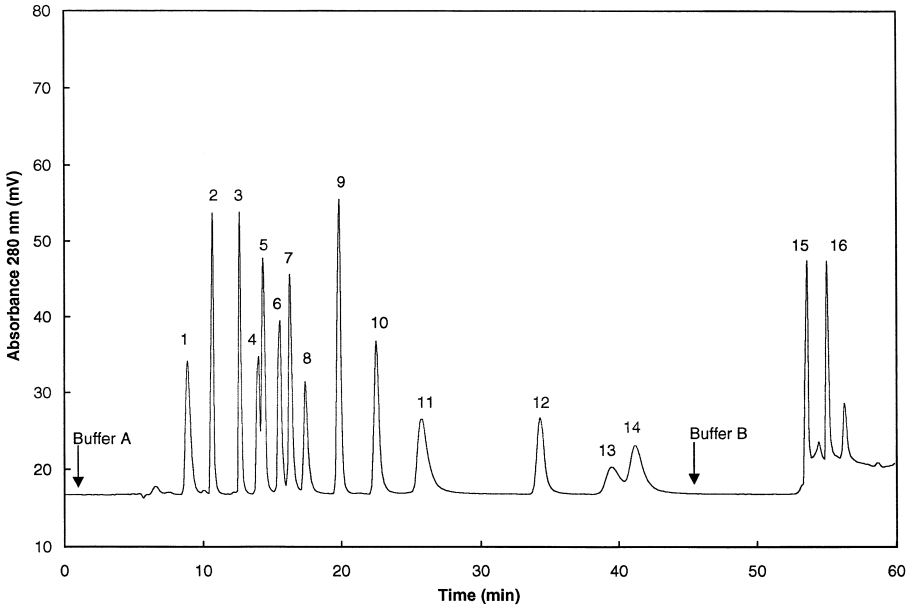

Fig. 2. HPLC profile of standard mixture of caffeine, 14 caffeine metabolites, and I.S.. 1=AFMU, 2=3U, 3=7U, 4=7X, 5=1U, 6=3X, 7=37U, 8=1X, 9=I.S., 10=13U, 11=37X, 12=17U, 13=17X, 14=13X, 15=137U, 16=137X. Abbreviations are defined in Table 1.

(C.V.) values between 1.23 and 7.89% for the MeQCs, and between 0.82 and 9.94% for the HiQCs. The C.V.s for the LoQCs were below 10% except for AFMU and 1U around 15% during inter-day validation. AFMU and 1U are the most important urinary caffeine metabolites in terms of molar fraction [1,6]. During a caffeine test, the lower concentrations of AFMU and 1U found usually in the urine of the subjects, even in slow acetylators, are in the range of MeQC. The standard accuracy values at all concentrations ranged from 87.60 to 114.00%, and from 92.64 to 109.07% during intra and inter-day validation, respectively.

The extraction efficiency of the assay for AFMU, 1X, 1U, 17X and 17U are presented in Table 3. The different QC concentrations gave rise to percent recoveries up to 77%, except for 1U which was about 50%. The moderate recovery of 1U did not affect the precision and the accuracy of 1U determination as shown in Table 3. The recovery of 1U can be increased however, by increasing the percentage of isopropylalcohol in the extraction solvent but in this case many impurities will be extracted with the caffeine metabolites (results not shown).

The minimum detectable concentration of each compound was about 1.0 ng/ml for 1X and 1U, and about 2.0 ng/ml for AFMU, 17X and 17U. The limit of quantification for each compound was determined to be 0.5 μg/ml. The sensitivity achieved by this method is sufficient to measure the caffeine metabolites present in urine during the caffeine test.

A standard dose of 100–400 mg of caffeine is commonly used during caffeine testing to optimize the precision of enzyme activity assessments [1–6]. Nevertheless, one to four cups of coffee on the test day have been shown to be sufficient for this purpose [3,17]. The timing of urine collection is relatively unimportant as long as there is a sufficient interval (4–5 h) between caffeine intake and urine collection. This procedure is ethically and practically convenient, especially for volunteer subjects and for epidemiological studies. Therefore, experimental subjects of this study were asked to collect their urine 5 h after drinking a regular cup of coffee. The validated method was applied to the urine collected for the determination of AFMU, 17X, 17U, 1X or 1U. The data summarized in Table 4 are in agreement with previously published results in healthy subjects using the classical two extraction–analysis procedures [1,2,6].

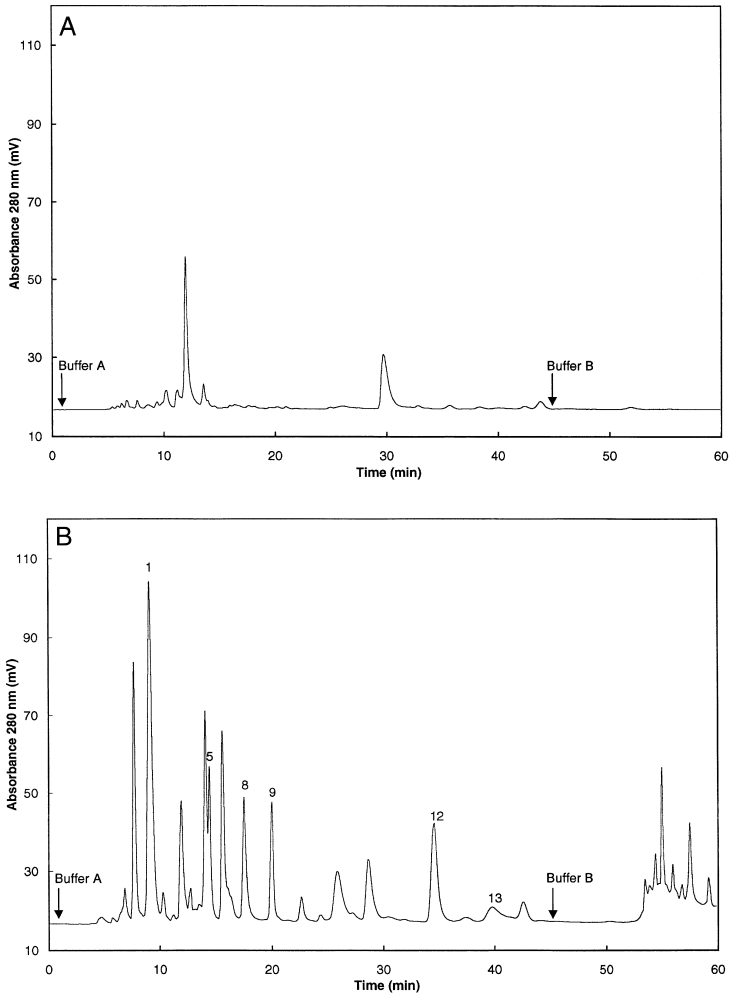

Fig. 3. HPLC chromatograms of urine extracts. A: blank urine. B: urine from an experimental subject collected 5 h after drinking a regular cup of coffee. The metabolites required for enzyme assays are indicated as: 1=AFMU, 5=1U, 8=1X, 9=I.S., 12=17U, 13=17X. Abbreviations are defined in Table 1.

Following an oral dose of caffeine, at least 14 metabolites are eliminated in urine [1,3,4]. These metabolites are systematically co-extracted with the chloroform–isopropylalcohol mixture commonly used for a liquid–liquid extraction. To avoid possible interferences, a relevant chromatographic method should be able to separate these caffeine metabolites even if they are not necessarily quantified. The present HPLC method allows the separation of caffeine and its 14 metabolites (Fig. 2).

In other respects, caffeine metabolism may vary considerably in different situations in comparison to healthy human subjects, e.g. under pathologic conditions, in neonates or in animal species [1,3,4,18]. Since caffeine metabolism pathways are interdependent, a relevant interpretation of the expression and the variation of the activity of a particular enzyme may require the simultaneous determination of the activity of the other enzymes implicated in the metabolism of caffeine. The possibility to measure simultaneously the activity of CYP1A2, CYP2A6, XO and NAT2 with the present method in one

Table 3
Results of intra- and inter-day validation spiked human urine[a]

Nominal concentration	LoQC					MeQC					HiQC				
(μg/ml)	AFMU 1.00	1U	1X	17U	17X	AFMU 4.00	1U	1X	17U	17X	AFMU 17.50	1U	1X	17U	17X
Intra-day validation															
n	5					5					5				
Measured mean concentration (μg/ml)	0.88	1.14	0.93	0.99	1.06	3.56	3.99	4.39	4.46	3.82	16.02	17.16	18.63	17.63	18.16
SD (μg/ml)	0.07	0.11	0.08	0.05	0.07	0.15	0.17	0.16	0.15	0.05	0.90	0.94	0.15	0.25	0.55
C.V. (%)	8.45	9.30	8.83	5.00	6.26	4.17	4.23	3.67	3.32	1.23	5.61	5.46	0.82	1.42	3.02
Accuracy (%)	87.60	114.00	93.00	99.00	106.20	89.00	99.70	109.70	111.55	95.50	91.54	98.06	106.43	100.77	103.79
Inter-day validation															
n	15					15					15				
Measured mean concentration (μg/ml)	0.99	1.06	0.99	1.09	1.12	3.77	4.15	4.24	4.18	3.89	16.21	17.28	17.90	17.73	17.59
SD (μg/ml)	0.18	0.15	0.10	0.09	0.10	0.30	0.24	0.17	0.23	0.11	1.61	1.22	0.58	0.40	0.85
C.V. (%)	18.57	14.09	9.71	8.67	8.76	7.89	5.88	4.04	5.61	2.72	9.94	7.06	3.21	2.27	4.83
Accuracy (%)	98.80	106.27	98.93	109.07	112.2	94.66	103.71	105.63	103.86	97.30	92.64	98.74	102.31	101.33	100.54
Recovery (%)	82	44	105	106	107	77	50	103	100	106	85	52	96	94	103

[a] Limit of detection is about 2.0 ng/ml for AFMU, 17X and 17U, and about 1.0 ng/ml for 1X and 1U.

Table 4
CYP1A2, CYP2A6, polymorphic *N*-acetyltransferase (NAT2) and xanthine oxidase (XO) enzyme activities as determined by the present HPLC method in ten healthy volunteers[a]

Subject	CYP1A2	CYP2A6	NAT2	XO
1	7.52	0.11	0.66	0.61
2	6.24	0.13	0.69	0.53
3	5.03	0.15	0.18[b]	0.51
4	7.21	0.11	0.67	0.59
5	42.86	0.02	0.12[b]	0.94
6	5.51	0.13	0.71	0.56
7	7.14	0.09	0.21[b]	0.48
8	3.46	0.18	0.13[b]	0.38
9	4.50	0.17	0.67	0.53
10	11.14	0.08	0.35	0.61
Mean	10.06	0.12	0.44	0.57
SD	11.72	0.05	0.26	0.15

[a] Enzyme activities were assessed by the urinary molar ratio of (AFMU+1X +1U)/17U for CYP1A2, 17U/(AFMU+1X+1U+17X+17U) for CYP2A6, AFMU/(AFMU+1X+1U) for NAT2, and 1U/(1X+1U) for XO.

[b] Subjects who can be classified as slow acetylators [6]. An application of the present HPLC method to more subjects will allow an accurate determination of the antimode value and consequently, a clear classification of subjects.

extraction–analysis run should therefore contribute to simplifying and optimizing the caffeine drug metabolizing enzyme assays.

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