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HPLC method for rapid determination of acetylator phenotype by measuring urinary caffeine metabolites

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Abstract: A validated reversed-phase high-performance liquid chromatographic (RP-HPLC) method is developed for the selective and rapid determination of two major metabolites of caffeine, namely 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-methylxanthine (MX) from human urine. HPLC separation is achieved by means of a Supersphere-60 RP-Select B (4 μm) analytical column using a non-linear gradient elution programme of 70–95% solvent B (2.5% acetic acid–methanol, 60:40, v/v) in solvent A (water–acetonitrile, 80:20, v/v). A selective UV detection method is used for determination of AFMU, MX and internal standard with readings at 284, 268 and 248 nm, respectively. Urine samples are prepared for measurement by a simple chloroform–diethyl ether (80:20, v/v) extraction. The assay is validated with respect to linearity, sensitivity, accuracy, precision and system suitability. All validation parameters are found to be within the required limits. The limit of detection of AFMU and MX is found to be 50 ng/200 μl urine. Calibration curves show good linearity between 0.1 and 5 μg/200 μl urine concentration range for both metabolites. The assay is sufficiently sensitive and rapid (4.5 min chromatographic run) to be applied for routine monitoring of change in AFMU/MX molar ratio, indicating acetylation phenotype and change of caffeine metabolism in clinical cocktail studies.

Keywords: *Liquid chromatography; caffeine metabolites; urine; acetylation phenotype.*

Introduction

Caffeine (1,3,7-trimethylxanthine) is an important model compound for liver function [1–2]. *In vitro* and *in vivo* metabolism of caffeine in humans [3–10] and in newborns [11, 12] has been studied and more than 10 urinary metabolites isolated and determined by different HPLC methods [13–15]. AFMU (5-acetylamino-6-formylamino-3-methyluracil) and MX (1-methylxanthine) have been identified as the major urinary metabolites of caffeine [16–18]. The urinary molar ratio of AFMU/MX has been established as an index for the genetic polymorphism in acetylation capacity [7, 13, 17, 19].

Reversed- and normal-phase analytical columns have been used for the LC separation of AFMU and MX. A complete conversion of AFMU to AAMU (5-acetylamino-6-amino-3-methyluracil) is effected by treating urine samples with sodium hydroxide (pH 10) to overcome problems associated with the instability of AFMU during the preparation of urine samples [16, 18].

In the present study, a more sensitive and rapid HPLC method is developed for the selective determination of the caffeine metabolites AFMU and MX. This method is suitable for the routine monitoring of their molar ratio in urine, which indicates the individual acetylator phenotype status.

Experimental

Chemicals

All chemicals and solvents were of analytical grade. AFMU was obtained from Dr B.K. Tang (University of Toronto, Canada) [16]. MX and 4-acetamidophenol (internal standard) was obtained from Sigma (St Louis, MO, USA). Ammonium sulphate and diethyl ether were purchased from Merck (Darmstadt, Germany). Chloroform and acetic acid were purchased from Prolabo-Rhone-Poulenc (Paris, France) and May & Baker (Dagenham, UK), respectively. Acetonitrile and methanol were purchased from Rathburn Chemicals (Walkerburn, Scotland) and were of HPLC solvent grade. The water for HPLC was purified using a Milli-Q Purification System (Millipore, Bedford, MA, USA).

Standard solution

Stock solutions of 100 μg ml^{-1} AFMU, MX and internal standard were prepared in volu-

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metric flasks. AFMU was dissolved in 0.1% of acetic acid (pH 3.0) and was not found to degrade at this pH. MX was dissolved in distilled water and the internal standard solution was prepared in chloroform–diethyl ether (80:20, v/v) — the extraction solvent. The status of the HPLC-equipment was controlled daily by a standard mixture of AFMU, MX and internal standard prepared in 0.1% acetic acid. The stock solutions could be stored at 4°C for several months without deterioration.

Instrumentation and chromatographic conditions

A Merck–Hitachi HPLC System (Darmstadt, Germany) was used. The chromatographic system consisted of a LC-organizer with manual sample injector, (20 μl loop; Rheodyne), L-6200 type intelligent pump (gradient control), L-4250 type programmable variable wavelength UV–vis detector and D-6000 type electronic interface for computer connection. The HPLC system was coupled with an Ektaco EW-286 type IBM compatible computer (DR-Datatutkimus, Helsinki, Finland), and a Star-LC 10 Printer (Liverpool, UK). Solvent and detector programming, data collection and integration were accomplished on-line with D-6000 type HPLC manager software (Merck–Hitachi).

AFMU and MX were separated on a LiChroCART 250 × 4 mm (i.d.) Supersphere-60 RP-Select B (4 μm) (Merck) analytical column with a guard column LiChroCART 4 × 4 mm (i.d.) containing Lichrosorb RP-18 (5 μm) (Merck). The guard column was changed after about 100 analyses. A non-linear gradient elution programme was used. The mobile phase A consisted of water–acetonitrile (80:20, v/v) and the mobile phase B consisted of 2.5% acetic acid–methanol (60:40, v/v). Both were degassed before use. The initial mobile phase was 30% A and 70% B during 1 min. Thereafter the concentration of B was increased to 95% in 3 min and kept constant until 4.5 min. The analysis was performed at ambient temperature (22 ± 2°C) with a constant flow rate of 1 ml min^{-1}. The pressure was about 330 bar. Every day, before the biological sample analysis the reversed-phase column was equilibrated with the initial mobile phase for 1.5 h. The column eluate was monitored by a time programming UV-detection method. Each compound was detected on its maximum absorbance: AFMU, MX and internal standard at 284, 268 and 248 nm, respectively. The UV wavelengths were changed on-line at 2.85, 3.40 and at 4.50 min, respectively. Automatic baseline correction was used after each wavelength change.

Sample preparation

To 0.2 ml of urine in a glass tube were added 70 μl (7 μg) of internal standard (4-acetamidophenol) stock solution and 200 mg of ammonium sulphate for saturation and then the tube was vortexed (Vortex-Genie K-550-GE, Oriola Oy, Espoo, Finland) for 15 s. The metabolites were extracted with 6 ml of chloroform–diethyl ether (80:20, v/v) by vigorous shaking (EM-4, GWB KS 10, E. Büchler, Tübingen, Germany) for 10 min. Following centrifugation (Christ UJ3, Osterode, Germany) at 2400 rpm for 5 min, the organic phase was transferred to a conical test-tube and evaporated under pure nitrogen at 40°C. The dry residue was dissolved in 400 μl of 0.1% acetic acid and 20 μl of this solution was injected into the HPLC column.

Validation of the method

Calibration data were generated by spiking free urine samples with AFMU and MX to produce concentrations in the range 0.1–5 μg/200 μl. Calibration curves were constructed by plotting the ratios of peak areas (AFMU/IS; MS/IS) versus concentration and the best relationship was determined by a least-squares linear regression analysis. The slope of calibration curves were routinely controlled each day by analysing three concentrations of spiked urine.

Inter-day precision and accuracy were determined by using six urine samples spiked with metabolites in the concentration range (0.1–5 μg/200 μl). The samples were extracted and analysed on six different days ($n = 6$).

Intra-day reproducibility was assessed by using six samples spiked with AFMU and MX at two different concentrations (1 and 3.5 μg/200 μl). These samples were prepared and analysed on the same day.

The precision of the chromatographic system (system suitability) and the constancy of concentration ratio AFMU/MX were controlled by analysing six parallel urine samples of low and high concentration on the same day.

The precision and accuracy were characterized by relative standard deviation (RSD) and

by difference between nominal and found concentration, respectively, expressed as a percentage.

Results and Discussion

As a result of the present work a quicker and more sensitive method for the determination of the acetylator phenotypes has been developed that is suitable for the clinical cocktail study. The results of the analysis are expressed as the AFMU/MX molar ratio [4] which is an indicator of N-acetyltransferase activity [15]. Figure 1 shows the chromatograms of blank urine (I) and the two major caffeine metabolites (AFMU and MX) in human urine (II) after coffee drinking. The retention times of AFMU, MX and internal standard were 2.42, 2.97 and 3.54 min, respectively. The analysis time was 4.5 min, including about 1 min conditioning time after the elution of the internal standard. The peaks for AFMU, MX and internal standard were symmetrical. Figure 1 also demonstrates the gradient elution profile of mobile phase B. Other urinary caffeine metabolites and endogenous compounds did not interfere the measurement of AFMU and MX under the present conditions.

The calibration curves obtained with spiked human urine show good linearity within the range of 0.1–5 μg/200 μl AFMU and MX (Fig. 2). Linear regression analysis was used to determine the equations of the calibration lines (AFMU: $y = 0.04927x + 0.00401$; MX: $y = 0.08976x + 0.00618$). The limit of detection for both metabolites was 50 ng/200 μl urine or

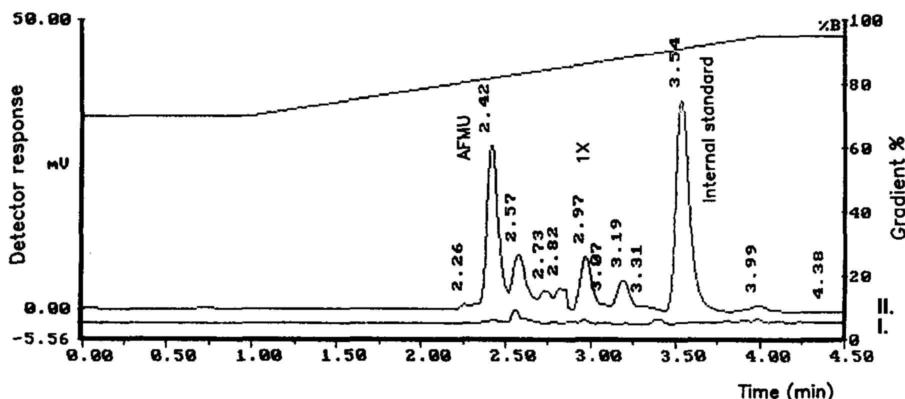

Figure 1
Representative gradient HPLC chromatogram of (I) blank urine extract, (II) volunteer's urine extract after the uncontrolled coffee drinking. See text for instrumentation and chromatographic conditions.

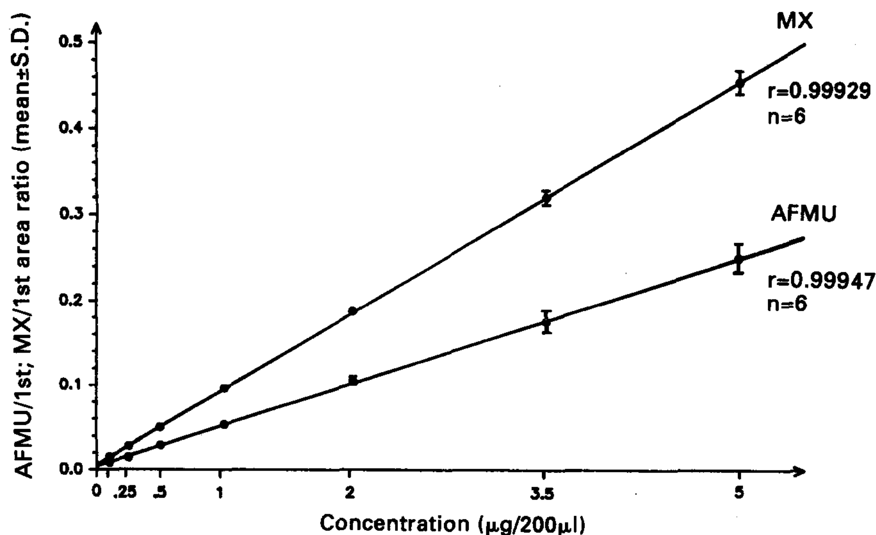

Figure 2
Calibration curves for AFMU and MX in human urine.

2.5 ng of injected substance (signal-to-noise ratio 3:1). The limit of determination of AFMU and MX was determined from the validation procedure and was found to be 100 ng/200 µl for both metabolites.

The stabilities of AFMU and MX in urine were investigated by storing (−20°C) spiked blank urine samples at different concentrations. For up to 3 months no significant degradation of AFMU and MX in urine or in stock solutions was observed.

The recoveries of AFMU and MX from spiked urine samples ranged between 84.7–102.6% and 96.0–100.8%, respectively.

Reproducibility of the assay was described by the all current validation parameters [21, 22]. The inter-day reproducibility data for the assay on six different days are presented in Table 1. The inter-day precision was characterized by relative standard deviation (RSD) and accuracy was generally less than 10%. The all intra-day reproducibility RSD values of AFMU and MX were less than 5% (Table 2). The analytical system reproducibility (system suitability) and the constancy of AFMU/MX ratio were found to correspond to RSD values of <5%. All validation parameters were within required limits [21, 22] (Table 3).

The method can be used to screen the acetylator phenotype status of patients after their habitual uncontrolled normal daily caffeine (coffee, tea, cola) consumption. In those rare cases where the patient is known to have taken the internal standard for therapeutic reasons an external standardization can be used. The subjects are classified according to their ratios of AFMU/MX: slow acetylators with molar ratio of <0.5 and rapid acetylators with molar ratio of >0.5 [15, 17, 20]. The change of AFMU/MX molar ratio could be

Table 1
Inter-day reproducibility of determination for AFMU and MX (n = 6)

	AFMU			MX		
Nominal conc. (µg/200 µl)	Found conc. (mean ± SD) (µg/200 µl)	RSD (%)	Accuracy (%)	Found conc. (mean ± SD) (µg/200 µl)	RSD (%)	Accuracy (%)
0.1	0.092 ± 0.005	5.43	8.0	0.100 ± 0.015	15.00	0.0
0.25	0.212 ± 0.037	17.35	15.2	0.241 ± 0.022	9.13	3.6
0.5	0.509 ± 0.023	4.52	1.8	0.491 ± 0.025	5.09	1.8
1.0	1.006 ± 0.024	2.39	0.6	1.006 ± 0.029	2.88	0.6
2.0	2.051 ± 0.024	1.17	2.6	2.015 ± 0.023	1.14	0.7
3.5	3.471 ± 0.105	3.03	0.8	3.496 ± 0.091	2.60	0.1
5.0	4.976 ± 0.062	1.25	0.5	4.995 ± 0.153	3.07	0.1

Table 2
Intra-day reproducibility of determination for AFMU and MX (n = 6)

	AFMU		MX	
Nominal conc. (µg/200 µl)	Found conc. (mean ± SD) (µg/200 µl)	RSD (%)	Found conc. (mean ± SD) (µg/200 µl)	RSD (%)
1.0	1.03 ± 0.046	4.46	0.95 ± 0.021	2.25
3.5	3.34 ± 0.123	3.68	3.43 ± 0.132	3.84

Table 3
Suitability of analytical system (n = 6)

	AFMU		MX		
Nominal conc. (µg/200 µl)	Found conc. (mean ± SD) (µg/200 µl)	RSD (%)	Found conc. (mean ± SD) (µg/200 µl)	RSD (%)	AFMU/MX ratio RSD (%)
0.5	0.49 ± 0.021	4.28	0.48 ± 0.012	2.55	4.45
3.5	3.49 ± 0.032	0.91	3.61 ± 0.079	2.19	2.53

used for the indication of caffeine metabolism changing after combined with different drug intake in clinical cocktail study [2, 23].

Conclusions

The described method appears to be suitable for determining the change of AFMU/MX molar ratio in human urine samples. It is more sensitive and quicker for the determination of the acetylator phenotype status than those previously reported. This effect was achieved by a new liquid-phase extraction procedure and a non-linear gradient elution with a programmed selective UV detection method.

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