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Short Communication

Rapid method for the routine determination of caffeine and its metabolites by high-performance liquid chromatography

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Abstract

Caffeine is a popular compound for phenotyping individuals for CYP4501A2, xanthine oxidase (XO) and N-acetyltransferase (NAT) utilising urinary metabolites. The analysis is complex since at least thirteen metabolites are excreted by man. Past methods have been less than satisfactory in that either not all the metabolites have been resolved and/or extractions selective for particular groups of metabolites are required prior to chromatography. We report a method for the rapid analysis of caffeine and metabolites in urine that negates the requirement for an extraction step, and also a method for plasma analysis.

1. Introduction

Caffeine (Fig. 1) is widely available in dietary foodstuffs, particularly coffee, tea, cocoa and soft drinks – its wide consumption attributed at least in part to its stimulant properties [1]. It is extensively metabolised by a series of sequential and competing steps, the enzymes involved include CYP4501A2 (N-demethylation [2]), other CYP450 enzymes (ring hydroxylation [3]), N-acetyltransferase (NAT [4,5]) and xanthine oxidase (XO [3,5,6]). Interest in caffeine has been stimulated by its potential use as a metabolic probe in measuring enzyme polymorphisms both within and between populations. This is due not only to the variety of enzyme systems that can be mapped simultaneously, but to the fact that few therapeutic drugs utilise CYP4501A enzymes in their metabolism since CYP4501A oxidations are associated with the production of toxic free radical intermediates. Caffeine is consequently a popular substrate for measuring enzyme systems. Population studies necessitate the analysis of large numbers of samples requiring a reliable analytical method. Although there are several assays reported in the literature [2,3,6–11] none of these satisfactorily quantifies all the metabolites (listed in Table 1) due to factors such as poor recoveries [3], poor resolution [6], difficulties in reproducing the method without continual

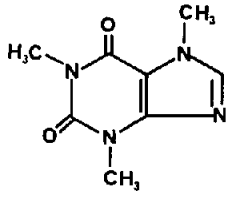

Fig. 1. Chemical structure of caffeine.

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Table 1
Caffeine metabolites and their retention times

No.[a]	Compound	Retention time (min)
Chromatography System 1		
1	5-Acetylamino-6-amino-3-methyluracil (AAMU)	4.32
2	3-Methyluric acid (3-MU)	6.19
3	7-Methyluric acid (7-MU)	8.26
4	1-Methyluric acid (1-MU)	9.66
5	7-Methylxanthine (7-MX)	10.05
6	3-Methylxanthine (3-MX)	10.96
7	3,7-Dimethyluric acid (3,7-DMU)	11.80
8	1-Methylxanthine (1-MX)	12.54
9	1,3-Dimethyluric acid (1,3-DMU)	15.06
10	3,7-Dimethylxanthine (3,7-DMX)	16.17
11	1,7-Dimethyluric acid (1,7-DMU)	17.38
12	4-Acetaminophenol (I.S.)(optional)	15.24
Chromatography System 2		
13	1,3,7-Trimethyluric acid (1,3,7-TMU)	3.11
14	1,7-Dimethylxanthine (1,7-DMX)	3.63
15	1,3-Dimethylxanthine (1,3-DMX)	4.03
16	1,3,7-Trimethylxanthine (1,3,7-TMX; caffeine)	7.03
17	β-Hydroxyethyltheophylline (I.S.)	4.76

[a] No. relates to peaks in Figs. 2 and 3.

minor adjustments [6], the lack of a method to measure total 5-acetylamino-6-formylamino-3-methyluracil (AFMU) [7] or caffeine simultaneously to the other metabolites [6,8]. Additionally, all the reported methods require one or more extraction procedures to analyse the metabolites and some require diode array analysis [6,11] or column switching [10]. We report HPLC analysis of caffeine and metabolites in urine without the need for prior extraction.

2. Experimental

2.1. Samples

Urine and plasma were collected from volunteers 2–6 h after a caffeine containing beverage and stored at −20°C prior to analysis. A brief medical history including a list of all medication taken in the last three weeks was recorded for each volunteer by a nursing sister.

2.2. Reagents and chemicals

Caffeine and all the metabolites in Table 1 except 1,7-DMU (Fluka, Glossop, Derbyshire, UK) and AFMU were purchased from Sigma (Poole, Dorset, UK). AFMU was the kind gift of Drs Arnaud and Philippossain (Nestlé, Vevey, Switzerland).

2.3. Procedure

To 0.1 ml of urine was added 0.1 ml of 1 *M* NaOH which was subsequently left at room temperature for 10 min to convert any AFMU to AAMU [12], after which 0.1 ml of 1 *M* HCl was added. The volume was adjusted to 1 ml with the starting mobile phase and the tube vortex-mixed. After centrifugation at 13 000 *g* for 2 min, the supernatant was used for HPLC. Addition of 4-acetaminophenol (50 mg/l; 0.2 ml) as an

internal standard prior to the addition of the mobile phase is optional. Plasma concentrations of caffeine-related compounds are low compared to those found in urine and an extraction procedure was employed to concentrate the samples. To 0.5 ml of plasma was added perchloric acid (sp. gr. 1.54; 0.1 ml) to denature the proteins, 0.2 ml of 1 *M* NaOH added to alkalise (pH >9) the plasma, converting AFMU to AAMU, and the plasma reacidified with 0.2 ml 1 *M* HCl. Internal standards (4-aminophenol and β-hydroxyethyltheophylline, both 50 mg/l; 0.2 ml each) and 6 ml of chloroform or chloroform–isopropranol (50:50, v/v) were added, and the tube agitated for 15 min on a rotating mixer. The tubes were centrifuged at 4000 *g* and the organic phase taken to dryness using a centrifugal evaporator (Genevac, Ipswich, UK). The residue was taken up in 0.1 ml of the mobile phase.

2.4. High-performance liquid chromatography

All the hardware used was supplied by Thermo Separation Products, Stone, Staffs, UK. Analysis was performed at ambient temperature using a Constametric CM4000 tertiary pump and programmable multiple solvent delivery system. Samples were automatically injected onto the column through a 20-μl loop from a rotating Promis I autosampler and the eluting products detected using a Spectromonitor 3100 variable wavelength detector set at 280 nm. The data was collected using a CI-10B integrator.

Separation of all but the 4 most lipid soluble compounds was achieved using a 25 cm × 4.5 mm I.D. Spherisorb ODS2 5 μm column (Phase Separations, Deeside, Clwyd, UK) by means of a gradient to cope with small adjustments in mobile phase as follows: 0–2 min, 2% acetoni-

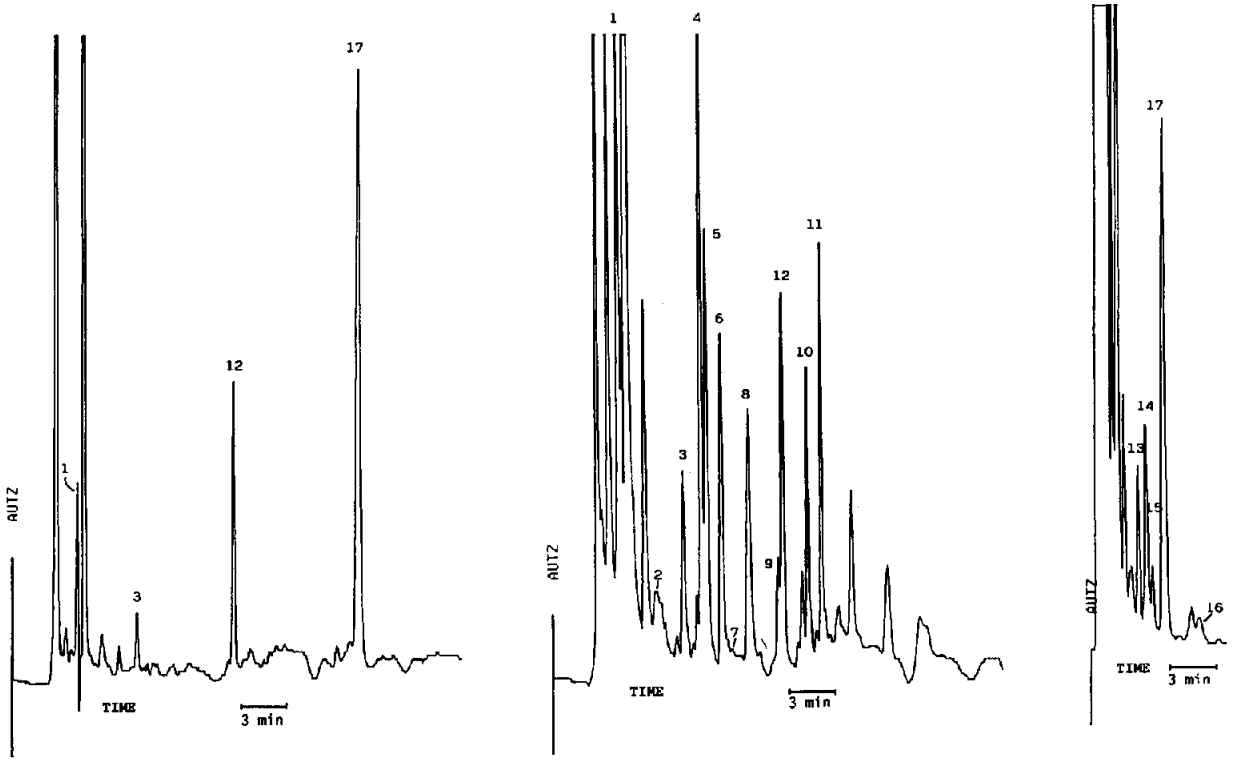

Fig. 2. (a) Chromatogram (System 1) for urine. Volunteer refrained from caffeine containing foodstuffs for 5 days. (b) Chromatogram (System 1) for urine collected 4 h after a caffeine containing beverage. (c) Chromatogram (System 2) for urine collected 4 h after a caffeine containing beverage. Peak identification is referenced in Table 1.

trile; 2.1–11 min, 4% acetonitrile; 11.1–25 min, 8% acetonitrile; all mobile phases were made up to 100% with acetic acid (0.5%) (System 1).

The remaining urinary compounds, caffeine, 1,3-DMX, 1,7-DMX, and 1,3,7-TMU were eluted using a 10 cm × 4.5 mm I.D. ChromSep column (Chrompack, London, UK) and run isocratically , the mobile phase consisting of 10 m*M* sodium acetate containing 8 ml of tetrahydrofuran per litre at 1 ml/min (System 2).

Plasma extracts were assayed using the isocratic system, the amount of the more polar metabolites being too low for detection.

3. Results and discussion

Representative chromatograms of caffeine and its major metabolites assayed in urine are presented in Fig. 2. Fig. 2a represents the chromatogram from a young volunteer who had refrained from xanthine containing products for 5 days. The chromatograms in Figs 2b and 2c are of urine from a volunteer 4 h after drinking a cup of instant coffee, the major metabolites being 1-MX, 1-MU, 1,7-DMU, 1,7-DMX, and AAMU. The two chromatography systems were necessary since the 4 least polar compounds could not be satisfactorily resolved in the first system due to peak broadening. Calibration curves using peak heights (or the peak-height ratio metabolite:4-acetaminophenol if internal standard was added) were linear over the range 0.5–100 mg/l and the coefficient of variation for all the metabolites and caffeine on an inter- and intra-day basis were equal or lower than 6% over the quoted range in each case. The limits of detection were between 0.5–2 ng on column (0.25–1 mg/l urine) depending on the metabolite.

Fig. 3 represents a plasma sample from a caffeine consuming volunteer. Unlike urine, not all the metabolites are identified in plasma, possibly due to their high rates of excretion. Only the more lipid soluble compounds (1,3,7-TMX, 1,3,7-TMU, 1,7-DMX, and 1,3-DMX) were routinely observed, their recovery after 100% chloroform extraction as described above being better than 93% in each case. Extraction

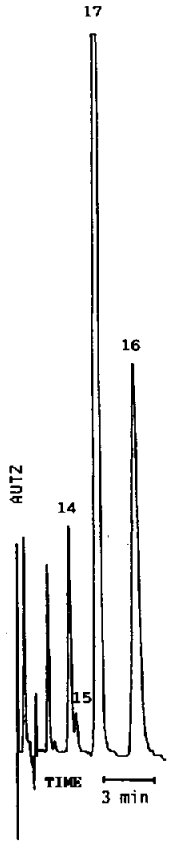

Fig. 3. Chromatogram (System 2) for plasma taken 4 h after caffeine containing beverage. Peak identification is referenced in Table 1.

of plasma using chloroform–isopropranol (50:50, v/v) did not recover other caffeine metabolites, suggesting that their excretion was rapid. The calibration curves using peak-height ratios of metabolite:internal standard, were linear over the range 1–100 mg/l with inter-day coefficient of variations being 4, 5, 5, and 6% respectively at 10 mg/l and 4, 3, 3, and 4% at 80 mg/l.

A number of HPLC methods have been published for the analysis of caffeine and metabolites. We found these inadequate for our purpose due to a lack of selectivity, sensitivity, reproducibility or resolution. Additionally, all the published methods involve at least one extraction procedure and none claim to assay all the metabolites of interest. The above methods negates the necessity for extraction in the case of

urine whilst retaining sensitivity, although plasma analysis requires an extraction step. These methods permit a rapid analysis of the metabolites required in measuring XO, NAT and P4501A enzymes suitable for use in population studies.

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