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Highly sensitive and rapid determination of theophylline, theobromine and caffeine in human plasma and urine by gradient capillary high-performance liquid chromatography–frit-fast atom bombardment mass spectrometry

Yoko Hieda*, Seiichi Kashimura, Kenji Hara, Mitsuyoshi Kageura

Department of Forensic Medicine, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-80, Japan

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Abstract

A sensitive and reliable analytical procedure has been established for the detection of theophylline (TH), theobromine (TB) and caffeine (CA) in human plasma and urine by gradient capillary high-performance liquid chromatography (HPLC)–frit-fast atom bombardment mass spectrometry (FAB-MS) (LC–frit-FAB-MS). Two capillary columns and a column-switching valve were used in this LC system to allow all of the sample injected to be introduced into the MS system. 7-Ethyltheophylline was used as the internal standard (I.S.). The xanthines in the specimen were extracted with an Extrelut column. The lowest detected amount was ca. 5 ng/ml using this method.

1. Introduction

Theophylline (TH) and theobromine (TB) (which are dimethylxanthine species), and caffeine (CA) (which is a trimethylxanthine species) are present in various kinds of foods and drinks we consume each day. In addition, TH is one of the most popular drugs for the treatment of bronchial asthma, being used in emergencies to expand the bronchia. TH, TB and CA are metabolites of the xanthine group [1]. These xanthines are usually analyzed by high-performance liquid chromatography (HPLC) [2-6]. From the perspective of legal medicine, the peaks detected by LC–UV have to be confirmed by mass spectrometry (MS) because other compounds might be detected at the same retention time. There have been two reports on the analysis of xanthines using LC–MS equipped with thermospray (TS) and atmospheric pressure chemical ionization (APCI) as the interface between LC and MS [7,8]. In the present study, we were able to develop a highly sensitive and reliable method using on-line gradient capillary HPLC–frit-fast atom bombardment (FAB)-MS

* Corresponding author.

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(LC–frit-FAB-MS) for the detection of TH, TB and CA in human plasma and urine.

2. Experimental

2.1. Reagents

TH, TB and CA were purchased from Research Biochemical International (Natic, MA, USA). 7-Ethyltheophylline used as the internal standard (I.S.) was synthesized by ethylation of TH with diethylsulfate (sublimation temperature: 143°C). The structures of these compounds are shown in Fig. 1. Water used in this study was passed through a Milli-Q purification system (Millipore, Milford, MA, USA) and then distilled. All other reagents used were of analytical grade.

2.2. Samples

The samples analyzed in this study were human plasma and urine obtained from healthy volunteers. These volunteers consumed a usual diet and did not take any drug containing the three xanthines.

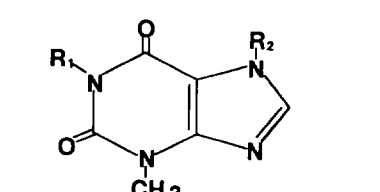
2.3. Sample preparations for LC–frit-FAB-MS

Urine (0.2–1.0 ml) spiked with I.S. (5 μg) was adjusted to pH 3 with acetic acid, and subsequently applied onto an Extrelut-1 glass column (Merck, Darmstadt, Germany) packed with resin (0.6 g). The resin was washed with dichloromethane, dried at 150°C for 6 h, and packed into a glass column. Fifteen minutes after application of the urine sample the column was eluted with dichloromethane (10 ml). The eluent was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 200 μl of distilled water and passed through a membrane filter (0.22 μm). The filtrate was then injected onto the LC–frit-FAB-MS system. The sample volume injected was 10–100 μl.

Plasma (0.2–1.0 ml) spiked with I.S. (5 μg) was diluted with water (0.1–0.5 ml) and deproteinized with 11.6 *M* perchloric acid (0.04–0.2 ml). The supernatant was adjusted to pH 3 with sodium hydroxide and/or ammonia and then applied onto an Extrelut-1 column. For 1.0 ml of supernatant, 1.0 g of resin was used. From here on, the procedures were the same as for the urine sample, except that 18 ml of dichloromethane was used to elute the 1.0 g of resin.

2.4. LC–frit-FAB-MS

A schematic diagram of the LC–frit-FAB-MS system is shown in Fig. 2. The capillary LC system consisted of an isocratic pump (Model 45, Waters, Milford, MA, USA), a gradient pump



R_1	R_2		M
H	CH_3	theobromine (TB)	180
CH_3	H	theophylline (TH)	180
CH_3	CH_3	caffeine (CA)	194
CH_3	C_2H_5	7-ethyltheophylline, IS	208

Fig. 1. Structures of xanthines used in this study.

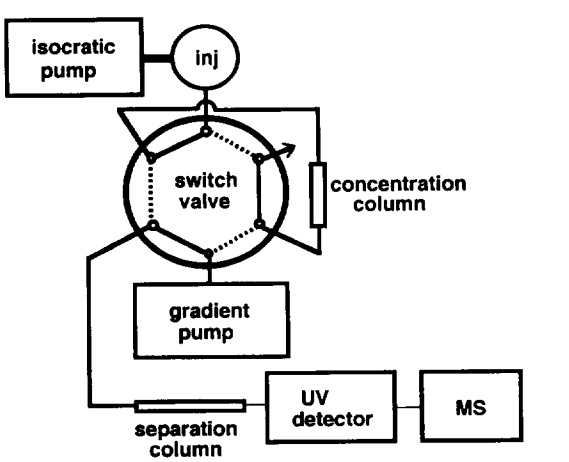


Fig. 2. Schematic diagram of the capillary HPLC–frit-FAB-MS system.

(Model M600, Waters), one injector (Model 9125, Rheodyne, Cotati, USA), one switching valve (Model N6W, Valco, Houston, TX, USA), two capillary columns, and one UV variable-wavelength detector (Model UV-8000, TOSO, Tokyo, Japan) set at 273 nm. The injector 9125 was equipped with a 500-μl sample loop. Develosil ODS-HG-15/30 (35 × 0.3 mm I.D., 15–30 μm) (Nomura Chemicals, Seto, Japan) and Develosil ODS-HG-5 (150 × 0.3 mm I.D., 5 μm) (Nomura Chemicals) were used as the pre-concentration column and separation column, respectively. A 10–100 μl aliquot of the sample was injected and trapped onto the precolumn for 1 min. The mobile phase used for trapping the injected sample was 17 *M* acetic acid–glycerol (Gly)–water (0.1:0.5:99.4, v/v) (flow-rate, 0.05 ml/min). After the valve was switched, the sample trapped on the precolumn was eluted to the separation column with a second mobile phase of 17 *M* acetic acid–Gly–methanol–water (0.5:0.5:10–99:89–0, v/v) (flow-rate, 4 μl/min). The gradient program for the methanol concentration in this second mobile phase is shown in Fig. 3. The total eluent from the separation column was introduced into the mass spectrometry without being divided. The UV signal was detected and calculated with an integrator (Model 741 data module, Waters).

The interface between the LC and MS system was a frit made of a porous stainless steel filter and fused-silica tubing (0.05 mm I.D.). A JMS-LX-2000 double-focusing MS instrument fitted with an FAB ion source was used (JEOL, Tokyo, Japan). Xenon (applied voltage, 3 keV in positive mode) was used to generate the fast atom beam. The mass spectrometer was scanned over a mass range of *m/z* 50–1000 for 5 s.

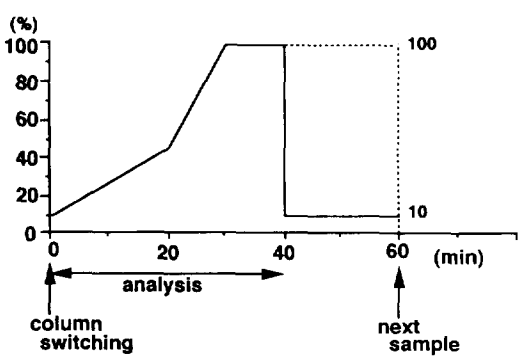


Fig. 3. Methanol gradient in the mobile phase used for separation.

3. Results and discussion

The xanthines trapped on the pre-concentration column began to elute when the mobile phase contained more than 10% of methanol. With a lower concentration of methanol in the mobile phase the xanthines took a long time to elute, and the late-eluting peaks had a bad shape and showed trailing. On the other hand, with a higher concentration of methanol in the mobile phase the xanthines eluted in a short time, with sharp peaks, but no separation was obtained. These problems could be solved by gradient analysis. A typical LC–UV chromatogram, mass chromatogram and mass spectra of the standard mix solution for the four xanthines are shown in Fig. 4. Each compound could be separated completely on both the LC and mass chromatograms. The quasi-molecular $[M+H]^+$ ion was observed as the base peak for each xanthine. The $[M+H+Gly]^+$ ion and some fragment ions, e.g. $[MH-CO-NCH3]^+$, $[MH-CO-NH]^+$ or $[MH-CO]^+$, were also observed; however their intensities were small. No response was found in the negative mode.

The peak-area ratio to I.S. (U.V. detection) was linear for TB, TH and CA over the range of 5–500 ng (on column), and linear for each compound in the concentration range from 10 ng/ml to at least 1 μg/ml for plasma and urine (correlation coefficients were 0.999, 1.000 and 0.999, respectively); however, the lower concentration could be reduced to ca. 5 ng/ml with an increase in the sample volume extracted or in the volume injected. The lowest detected amounts of the three standard solutions by LC–UV and mass chromatography in the scanning mode, at a signal-to-noise ratio of 5, were 3 and 10 ng (TB), 2 and 2 ng (TH), and 2 and 5 ng (CA), respectively. The lowest detectable amounts with mass spectrometry were 50 ng

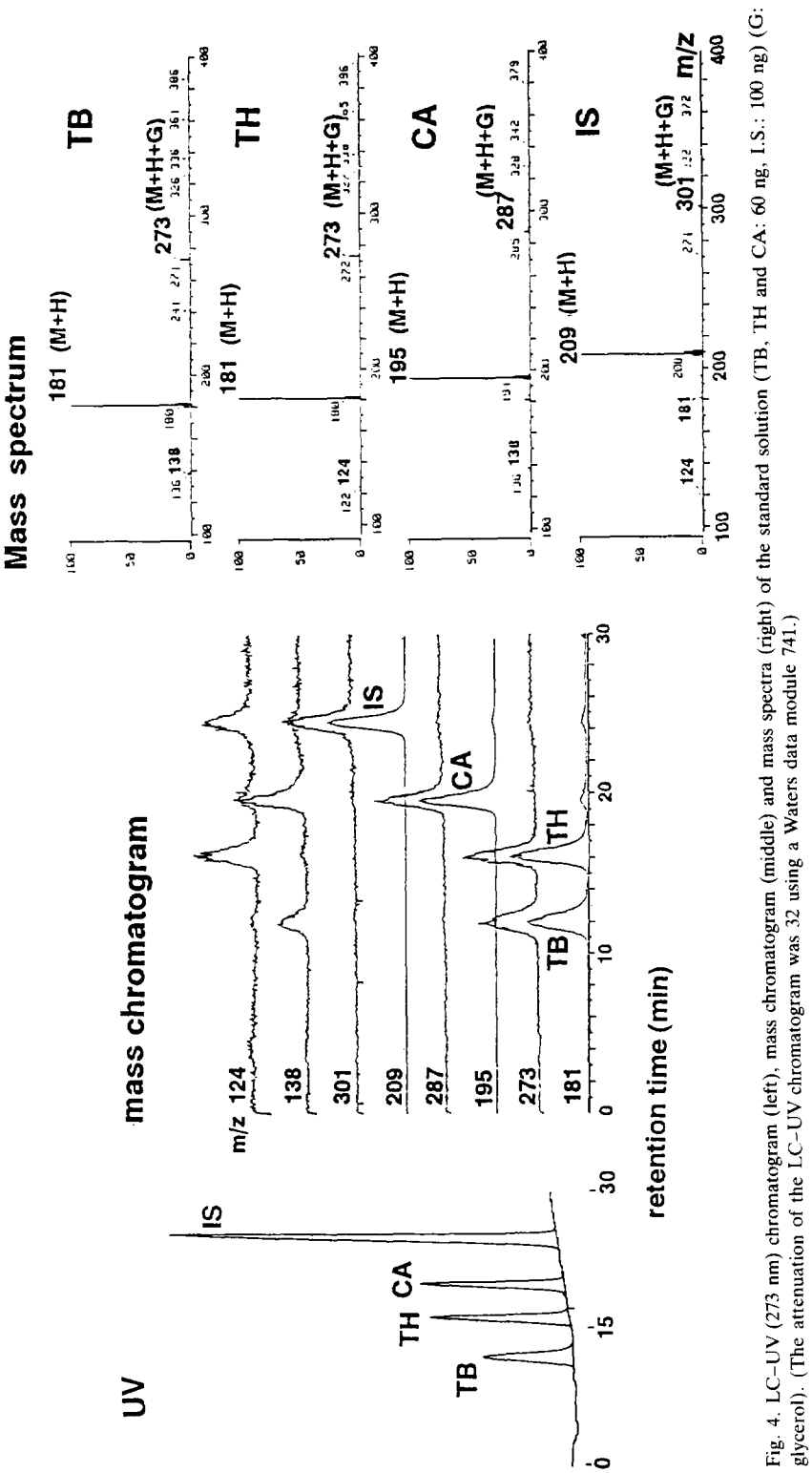


Fig. 4. LC–UV (273 nm) chromatogram (left), mass chromatogram (middle) and mass spectra (right) of the standard solution (TB, TH and CA: 60 ng, I.S.: 100 ng) (G: glycerol). (The attenuation of the LC–UV chromatogram was 32 using a Waters data module 741.)

Table 1
Recovery of TB, TH and CA in human plasma and urine

Specimen	Concentration (μg/ml)	Recovery (mean ± S.D.) (%)		
		TB	TH	CA
Plasma	0.1	94.5 ± 4.8	97.6 ± 5.6	83.8 ± 1.2
	0.5	90.1 ± 6.8	92.9 ± 7.3	82.2 ± 6.1
	1.0	94.6 ± 3.4	95.7 ± 2.4	82.2 ± 1.5
Urine	0.1	91.6 ± 2.8	93.2 ± 3.2	85.8 ± 1.7
	0.5	93.3 ± 5.5	93.2 ± 7.2	83.5 ± 4.9
	1.0	90.9 ± 2.2	94.6 ± 2.7	87.2 ± 3.0

For each concentration $n = 5$; theoretical recovery = 100%.

(TB), 10 ng (TH) and 10 ng (CA), respectively. The detection limit in the single-ion monitoring (SIM) mode was lower than in the scanning mode, suggesting that the SIM mode is better for quantitation by MS when the compound in the matrix is already known. For forensic medical analysis, however, the scanning mode is more suitable, since unknown compounds might be present in the sample which have the same retention times as those of the analytes of interest. Even in the scanning mode, the xanthines can be semi-quantified by comparing their base peak heights with the base peak height of the I.S.

Table 2
Precision and accuracy

Specimen	Spiked concentration (μg/ml)	Intra-day ($n = 5$)		Inter-day ($n = 5$)	
		Concentration (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration (mean ± S.D.) (μg/ml)	C.V. (%)
Plasma	0.1 TB	0.097 ± 0.0074	7.6	0.099 ± 0.0080	9.9
	TH	0.103 ± 0.0077	7.5	0.103 ± 0.0076	7.3
	CA	0.087 ± 0.0068	7.8	0.094 ± 0.0052	5.6
	0.5 TB	0.482 ± 0.035	7.4	0.504 ± 0.046	9.1
	TH	0.489 ± 0.033	6.8	0.504 ± 0.030	6.0
	CA	0.530 ± 0.051	9.6	0.486 ± 0.026	5.3
	1.0 TB	1.022 ± 0.040	3.8	1.017 ± 0.058	5.7
	TH	1.009 ± 0.026	2.6	1.011 ± 0.027	2.7
	CA	0.977 ± 0.047	4.8	0.960 ± 0.041	4.3
Urine	0.1 TB	0.095 ± 0.0054	5.6	0.103 ± 0.0051	5.0
	TH	0.096 ± 0.0054	5.6	0.094 ± 0.0049	5.2
	CA	0.102 ± 0.0062	6.1	0.098 ± 0.0072	7.3
	0.5 TB	0.536 ± 0.045	8.3	0.488 ± 0.044	9.0
	TH	0.505 ± 0.035	7.0	0.492 ± 0.043	8.7
	CA	0.499 ± 0.044	8.8	0.487 ± 0.031	6.5
	1.0 TB	1.000 ± 0.024	2.4	1.023 ± 0.051	5.0
	TH	0.974 ± 0.050	5.1	0.983 ± 0.027	2.7
	CA	1.003 ± 0.039	3.9	0.982 ± 0.038	3.9

The calculated recoveries of the xanthines were 82.2–97.6% over the concentration range tested (Table 1).

The reproducibility of the assay was checked by determining the intra- and inter-day variation (using three different concentrations) by adding the xanthines to blank plasma and urine. The coefficients of variation (C.V.) ranged from 2.4 to 9.6% for the intra-day assay and from 2.7 to 9.9% for the inter-day assay (Table 2).

These values are considered to be sensitive and reliable enough to measure levels of xanthines consumed in daily life. Fig. 5 shows the LC–UV and mass chromatograms of the extracts of plasma and urine provided by one volunteer.

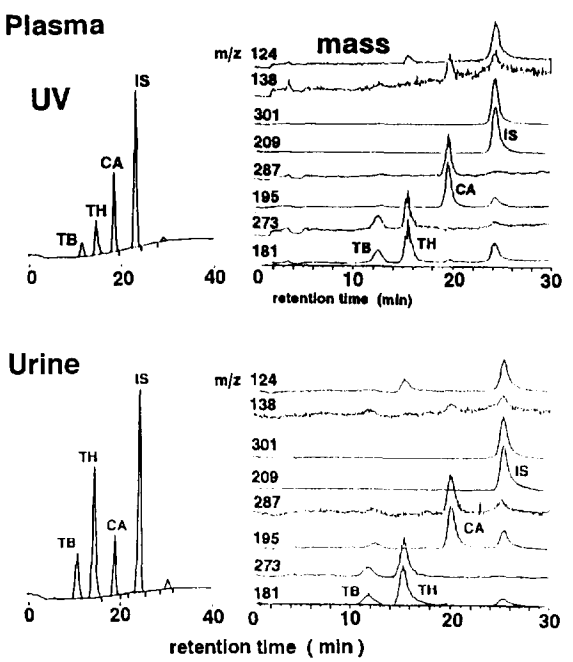


Fig. 5. LC–UV (273 nm) (left) and mass chromatogram (right) for extracts of human plasma and urine. The concentrations in plasma and urine were 0.63 and 1.5 μg/ml (TB), 0.88 and 2.8 μg/ml (TH), and 2.2 and 1.7 μg/ml (CA), respectively. (The attenuation of the LC–UV chromatogram was 32 using a Waters data module 741.)

The xanthines were detected in the plasma and urine of almost all subjects tested. TH, TB and CA were quantified by UV detection, and their identity was confirmed by monitoring the major ions and by obtaining the same mass spectra patterns as for the standard solution. The volunteers' plasma and urine ranged from 16.3–473 ng/ml and n.d.–3.0 μg/ml for TB, 7.0–990 ng/ml and n.d.–5.4 μg/ml for TH, and 61 ng/ml–2.2 μg/ml and n.d.–2.2 μg/ml for CA, respectively (n.d. = not detected).

4. Conclusions

In this study, a sensitive and reliable analytical method for TH, TB and CA in human plasma and urine was established using capillary gradient LC–frit-FAB-MS. The lowest detected amount was ca. 5 ng/ml.

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