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ORIGINAL ARTICLE

High-throughput determination of theophylline and caffeine in human serum by conventional liquid chromatography-mass spectrometry

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Abstract Automated high-performance liquid chromatography/mass spectrometry (HPLC-MS) with backflush column-switching was established for ultra-fast determination of theophylline and caffeine. A 400-μl portion of serum sample diluted with ultrapure water was injected and transferred to an Oasis HLB cartridge used as a precolumn for extraction. After switching the valves, the analytes trapped in the precolumn were eluted in the backflush mode and separated with a Chromolith Performance RP-18e column (C_{18}-bonded monolithic silica); the compounds in column effluents were then detected by atmospheric pressure chemical ionization (APCI)-MS. The present method successfully provided high-throughput determination of theophylline and caffeine within 2 min. Satisfactory linearity, reproducibility, and sensitivity could be obtained for analysis of therapeutic and toxic levels of both compounds. Because of the very simple procedure and high throughput using the conventional HPLC system, the present method seems to have high potential in the fields of forensic toxicology and emergency medicine.

Keywords Theophylline · Caffeine · LC-MS · Backflush column-switching · Monolithic silica column · High throughput

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Introduction

Theophylline and caffeine are natural alkaloids that are commonly ingested, and show various pharmacological effects in humans [1–3]. Caffeine is a constituent of coffee and other beverages [2] and is included in many medicines. Theophylline is used as a bronchodilator and is also formed as a metabolite of caffeine in humans [1,3]. Theophylline poisoning is occasionally encountered in the field of forensic toxicology, because of its relatively narrow safe range of therapeutic doses [1]. These alkaloids have been analyzed by various methods, such as gas chromatography (GC) [4], GC-mass spectrometry (MS) [5–12], and high-performance (HP) liquid chromatography (LC)-MS [13–16].

In the existing analytical methods for the drugs, sample pretreatments such as liquid–liquid extraction, solid-phase extraction, filtration or centrifugation were usually required to remove proteins and other impurities contained in biological matrices. The analytical methods including time-consuming multiple-step sample pretreat-

ments are unsuitable for rapid detection of poisonous materials in human specimens. To avoid such sample pretreatments, special HPLC columns, which enable direct injection of crude biological samples, were developed [17–29], and our group has applied them to compounds of forensic toxicological interest [17–23]. Furthermore, backflush column-switching systems using the special HPLC columns have become powerful analytical tools for simultaneous determinations of various drugs in biological samples [23–29].

In this study, we established an automated on-line method for ultra-fast determination of theophylline and caffeine in human serum by HPLC-MS with backflush column-switching and with a monolithic separation column. To our knowledge, our method presented in this study is the fastest MS determination technique for theophylline and caffeine in human serum using conventional HPLC instrumentation.

Materials and methods

Materials

Theophylline, caffeine, and 3-isobutyl-1-methylxanthine (internal standard, IS) were purchased from Wako (Osaka, Japan); 0.1% formic acid/acetonitrile, ultrapure water, and formic acid were of LC-MS grade also from Wako. Blank human serum was obtained from a healthy subject who had not ingested any food or drink containing theophylline and/or caffeine for 4 days. The target compounds in the blank serum were not detected by the present method. The blank serum was decanted and stored at −25°C until analysis.

Procedure

A mixture of theophylline, caffeine (10 µl, a known concentration), IS (10 µl, 500 µg/ml), and ultrapure water (4.0 ml) was added to a 50-µl volume of serum in a 4-ml vial, and was shaken for a few seconds. Each vial was then set in the rack of an autosampler (L-7200, Hitachi, Tokyo, Japan). The time required for this procedure was about 1 min.

HPLC conditions

The precolumn used for extraction from serum was an Oasis HLB cartridge (20 × 2.1 mm i.d., Waters, Milford, MA, USA). The analytical column used for chromatographic separation of the compounds was Chromolith Performance RP-18e (100 × 4.6 mm i.d., Merck, Darmstadt, Germany).

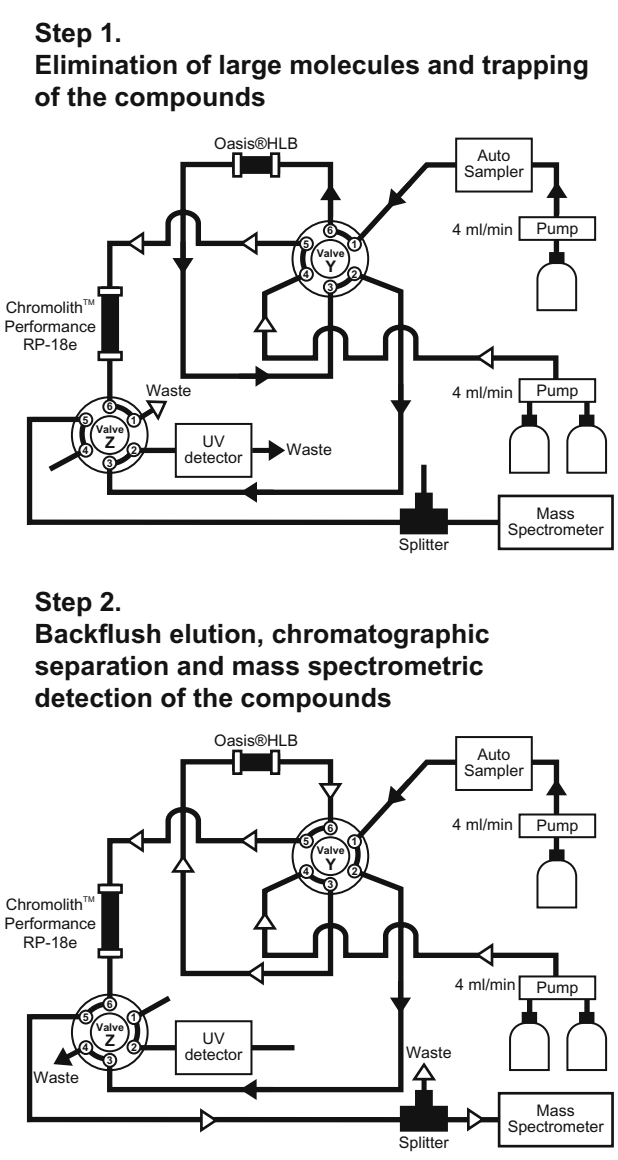

Fig. 1 Diagrams of a backflush column-switching system constructed in this study

Schematic diagrams of the backflush column-switching system equipped with two pumps constructed for this study are shown in Fig. 1. The HPLC-MS instrument used was an M-8000 LC/3DQMS with an atmospheric pressure chemical ionization (APCI) interface (Hitachi). Two L-7100 pumps, an L-7200 autosampler, an L-7300 column oven (all obtained from Hitachi) set at 40°C both for the precolumn and the separation column, two six-port switching valves (VICI Valco, Houston, TX, USA), and the MS system were arranged as shown in Fig. 1. The whole system was controlled by an M-8000 LC/3DQMS system manager (Hitachi).

In step 1 (Fig. 1), a large volume (400 µl) of the diluted serum sample was injected into a 500-µl sample-loop by the autosampler, and transferred to the precolumn (Oasis

HLB cartridge) with 0.1% formic acid/ultrapure water as a mobile phase set at a flow rate of 4.0 ml/min. The mobile phase was discarded by monitoring proteins with an ultraviolet (UV) detector (L-7420, Hitachi) at 280 nm.

In step 2, the valves Y and Z were switched 0.7 min after injection to connect the precolumn with the separation column (Chromolith Performance RP-18e) and to introduce the analytes into the mass spectrometer. The isocratic elution with a mobile phase (0.1% formic acid/acetonitrile : 0.1% formic acid/ultrapure water = 20 : 80) was performed for the analytes trapped in the precolumn and for their chromatographic separation on the column (Chromolith Performance RP-18e). During the elution of target compounds, the mobile phase was split (45 : 55 = volume introduced into mass spectrometer : waste volume) by a splitter just before introducing them into the mass spectrometer.

MS conditions

The mass calibration was performed by flow injection of reserpine as a mass marker. All MS detection was made in the positive mode. The MS conditions for APCI were: low mass cutoff, 47.8 amu; scan range, 50.0–300 amu; accumulation voltage, 0 V; accumulation time, 250 ms; the temperatures of the nebulizer, desolvator, aperture-1, and aperture-2 were 200°, 380°, 150°, and 120°C, respectively; the voltages of drift, focus, needle, and detector were 70 V, 30 V, 3.5 kV, and 400 V, respectively. Helium was used as buffer gas with an output pressure of 0.50 MPa and an ion source inlet pressure of 0.29 MPa.

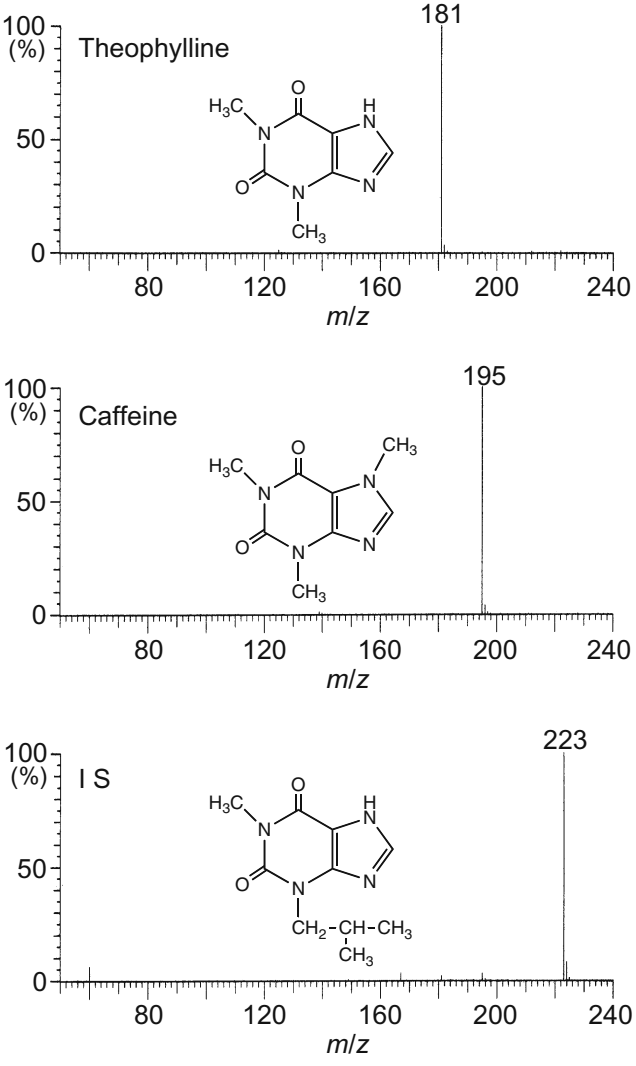

Fig. 2 Atmospheric pressure chemical ionization mass spectra for theophylline, caffeine, and 3-isobutyl-1-methylxanthine (internal standard, *IS*) and their chemical structures

Results

APCI mass spectra

The structures and mass spectra obtained by HPLC-MS with the APCI interface for theophylline, caffeine, and 3-isobutyl-1-methylxanthine used as IS are shown in Fig. 2. The APCI mass spectra gave protonated molecular ions constituting the base peaks for all compounds, and notable fragmentation peaks were not observed. The $[M + H]^+$ ions were used for quantitation of the drugs by mass chromatography.

Mass chromatograms

Figure 3 shows typical mass chromatograms by HPLC-MS obtained from blank human serum spiked with the three compounds including IS. The drugs could be selectively detected by monitoring $[M + H]^+$ ions, and all drug peaks appeared within 2 min.

Reliability of the method

The recoveries of theophylline and caffeine from sera at concentrations of 5, 10, 20, 30, 60, and 120 μg/ml are shown in Table 1. They were more than 75%.

The calibration equations and detection limits were investigated for theophylline and caffeine over the concentration range of 0.015–120 μg/ml using spiked sera. Both calibration curves for theophylline and caffeine showed good linearity in the range of 5–60 μg/ml. Their equations and correlation coefficients were: $y = 0.00262\,x - 0.00507$, $r^2 = 0.986$ for theophylline; $y = 0.00834\,x - 0.00117$, $r^2 = 0.994$ for caffeine. The limits of detection

(signal-to-noise ratio = 3) for theophylline and caffeine were 1.0 and 0.5 μg/ml, respectively.

Intraday and interday accuracy/precision for quantitation of the drugs was tested for human sera spiked with five concentrations of analytes (Table 2). The coefficients of variation (CVs) were not greater than 15.0%, and accuracy values were generally satisfactory.

Application of the method to an actual human sample

Figure 4 shows mass chromatograms for blank serum spiked with IS (left column) and a serum specimen collected 3 h after drinking coffee (right column); the serum concentration of caffeine was 5.1 μg/ml, but theophylline was not detected.

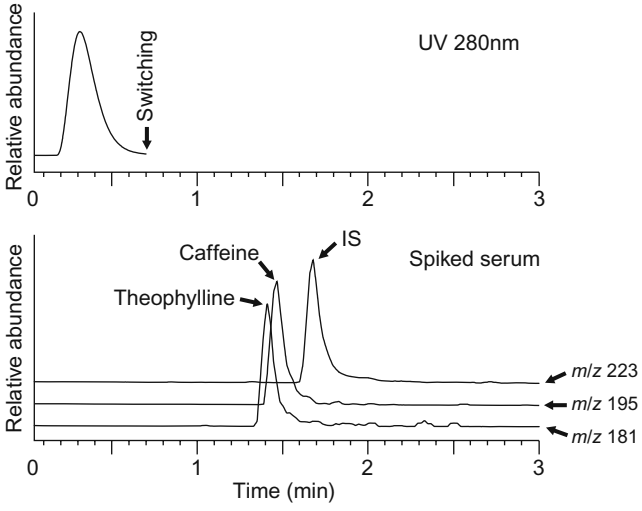

Fig. 3 Liquid chromatography-ultraviolet detection chromatogram at 280 nm for monitoring mainly proteins (*upper*), and mass chromatograms monitored at *m/z* 181, 195, and 223, showing peaks of theophylline, caffeine, and IS, respectively, for spiked human blank serum (*lower*)

Discussion

In this study, we established an automated on-line method for ultra-fast determination of theophylline and caffeine in human serum by HPLC-APCI-MS using a backflush column-switching system and special columns. The precolumn used for extraction, an Oasis HLB cartridge, was packed with the copolymer poly(divinylbenzen-*co*-*N*-vinylpyrrolidone), which exhibits both hydrophilic and lipophilic retention characteristics, and enabled direct injection of biological samples [20]. The large particles of sorbent material in this column enabled very high flow rates of the mobile phase [20]. The separation column (Chromolith Performance RP-18e) was packed with C_{18}-bonded monolithic silica and had a significantly high porosity. Both columns used in this study enabled very high flow rate (4 ml/min) of the mobile phase. Due to the characteristics of the two

Table 1 Recoveries of theophylline and caffeine spiked into human blank sera, measured by the present liquid chromatography-mass spectrometry method

Analyte	Concentration added (μg/ml)	Mean ± SD (%)[a]
Theophylline	5	93.5 ± 23.4
	10	116 ± 10.0
	20	75.4 ± 12.6
	30	79.2 ± 5.9
	60	84.5 ± 4.8
	120	97.2 ± 2.3
Caffeine	5	100 ± 12.5
	10	108 ± 4.4
	20	85.2 ± 5.5
	30	80.6 ± 4.6
	60	94.1 ± 8.4
	120	104 ± 5.3

SD, Standard deviation
[a] $n = 5$

Table 2 Intraday and interday accuracy and coefficients of variation (CV) for theophylline and caffeine measured by the present method

Analyte	Concentration added (μg/ml)	Intraday ($n = 5$)			Interday ($n = 5$)		
		Concentration found (μg/ml)	Accuracy (%)	CV (%)	Concentration found (μg/ml)	Accuracy (%)	CV (%)
Theophylline	5	5.9 ± 0.6	118	10.2	5.4 ± 0.7	107	13.5
	10	10.3 ± 1.2	103	11.7	8.3 ± 1.2	82.6	14.9
	20	19.4 ± 2.4	97.0	12.4	16.4 ± 1.5	81.8	9.0
	30	28.6 ± 2.2	95.3	7.7	28.5 ± 3.0	95.1	11.7
	60	60.8 ± 4.1	101	6.7	52.6 ± 6.0	87.7	11.4
Caffeine	5	6.2 ± 0.3	124	4.8	5.7 ± 0.9	114	15.0
	10	10.3 ± 1.2	103	11.7	9.7 ± 1.1	96.5	11.0
	20	18.6 ± 0.4	93.0	2.2	18.7 ± 1.1	93.3	5.8
	30	29.0 ± 1.5	96.7	5.2	25.1 ± 2.9	83.8	11.4
	60	60.8 ± 2.4	101	3.9	53.9 ± 5.5	89.9	10.3

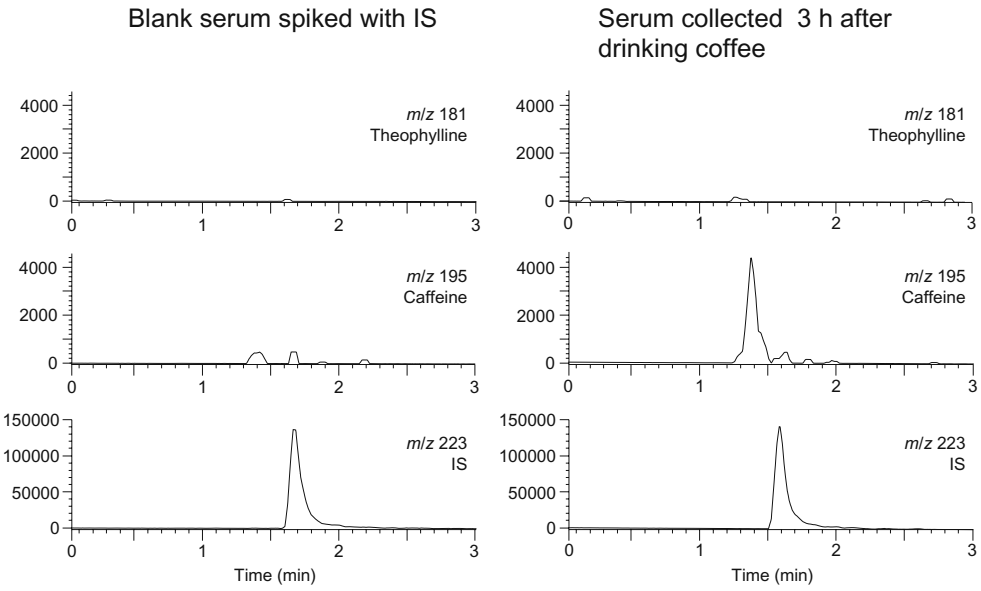

Fig. 4 Typical mass chromatograms at *m*/*z* 181, 195, and 223, showing $[M + H]^+$ ions of theophylline, caffeine, and IS, respectively, in human blank serum spiked with IS (*left column*) and a serum sample collected 3 h after drinking coffee (*right column*)

types of columns, chromatographic peaks of the target compounds appeared within 2 min, resulting in high-throughput analysis using a conventional HPLC instrument (not a new type of ultra-high pressure HPLC instrument). Furthermore, the procedure before the HPLC-MS analysis was only the addition of IS and ultrapure water, which required only about 1 min. Therefore, the total analytical time for a single specimen was as rapid as a few minutes.

In the present study, we established an LC-MS method for analysis of relatively high concentrations of theophylline and caffeine in sera. This is because the therapeutic blood theophylline and caffeine concentrations were reported to be as high as 8–20 μg/ml and 8–15 μg/ml, respectively; their toxic blood levels are 25–30 μg/ml and 20–30 μg/ml, respectively [30]. However, it seems easy to greatly enhance the method sensitivity by adapting the LC-MS conditions. The present system seems to have high potential in applicability to other compounds, and thus is likely to be useful in both fields of forensic toxicology and emergency medicine.

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