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Determination of theophylline and its metabolites in biological samples by liquid chromatography–mass spectrometry

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Abstract

Liquid chromatography–mass spectrometry (LC–MS) is a powerful tool for analysis of drugs and their metabolites. We used a column-switching system in combination with atmospheric pressure chemical ionization LC–MS (LC–APCI–MS) for the determination of theophylline and its metabolites in biological samples. The separation was carried out on a reversed-phase column using methanol–20 m*M* ammonium acetate as a mobile phase at a flow-rate of 1 ml/min in 30 min. In the mass spectrum, the molecular ions of these drugs and metabolites were clearly observed as base peaks. This method is sufficiently sensitive and accurate for the pharmacokinetic studies of these drugs.

Keywords: Column switching; Theophylline; Caffeine

1. Introduction

Theophylline is a xanthine derivative and pharmacologically similar to other xanthine drugs, theobromine and caffeine. However, these drugs differ in the intensity of their actions. Theophylline competitively inhibits phosphodiesterase, which degrades cyclic AMP (cAMP) and increased concentration of intercellular cAMP mediate the pharmacologic effects of the drug. Theophylline directly relaxes smooth muscle and is widely employed in the treatment of bronchial asthma. The range of effective plasma theophylline concentrations is comparatively narrow, and various side effects occur when plasma levels exceed 20 mg/ml. The rate of metabolism of theophylline varies considerably from one individual to another and, therefore, effective and safe theophylline therapy requires dose optimization by measuring plasma levels. As some of the theophylline metabolites, such as 3-methylxanthine and 1,3-dimethyluric acid, are known to have bronchodilator activity, serious side effects been observed following their accumulation. Recently, Kizu et al. reported sustained high plasma concentration of caffeine after intake of canned beverages [1]. Caffeine is metabolized by the same enzyme cytochrome P450 in liver microsomes as theophylline [2,3]. In the case of plasma sample from patients, the metabolites of caffeine sometimes interfere with the detection of theophylline and its metabolites. The analysis of theophylline and its metabolites is most often performed by UV detection, which can be very complicated due to the presence of endogenous compounds in biological fluids that also absorb UV. An accurate and a selective method for determination of plasma levels of the drugs has been required.

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LC–MS provides an important tool for making analytical methods more selective. We have investigated analysis of drugs in biological fluids by using LC–MS [4–6]. In this paper, we describe the simultaneous determination of theophylline, caffeine and its metabolites using a column-switching method in combination with atmospheric pressure chemical ionization LC–MS (LC–APCI–MS). This column-switching system has the great advantage that laborious pretreatments are not required, because the target compounds including metabolites can be separated from endogenous compounds in plasma samples on line. This method provides an important tool for the pharmacokinetic studies of these drugs.

2. Experimental

2.1. Material and reagents

The reagents used were obtained from commercial sources as follows: theophylline (1,3-dimethylxanthine; M.w.=180.2), 1,3-dimethyluric acid (M.w.=196.2), 3-methylxanthine (M.w.=166.1), 1-methylxanthine (M.w.=166.1), 1-methyluric acid (M.w.=182.1), caffeine (M.w.=194.2), 1,7-dimethylxanthine (paraxanthine; M.w.=180.2), theobromine (3,7-dimethylxanthine; M.w.=180.2), 1,7-dimethyluric acid (M.w.=196.2), 1,3,7-trimethyluric acid (M.w.=210.2) and β-hydroxyethyltheophylline (M.w.=224.2) (Sigma Co Ltd., St. Louis, MO, USA). The structures of these compounds are shown in Fig. 1. Water was distilled and passed through a Milli-Q purification system (Millipore, Bedford, MA, USA). All other chemicals and solvents were of analytical-reagent grade.

2.2. Apparatus

LC–MS system (Hitachi M-1200H, Tokyo, Japan) with a quadrupole mass spectrometer and a column-switching system consisting of two HPLC pumps

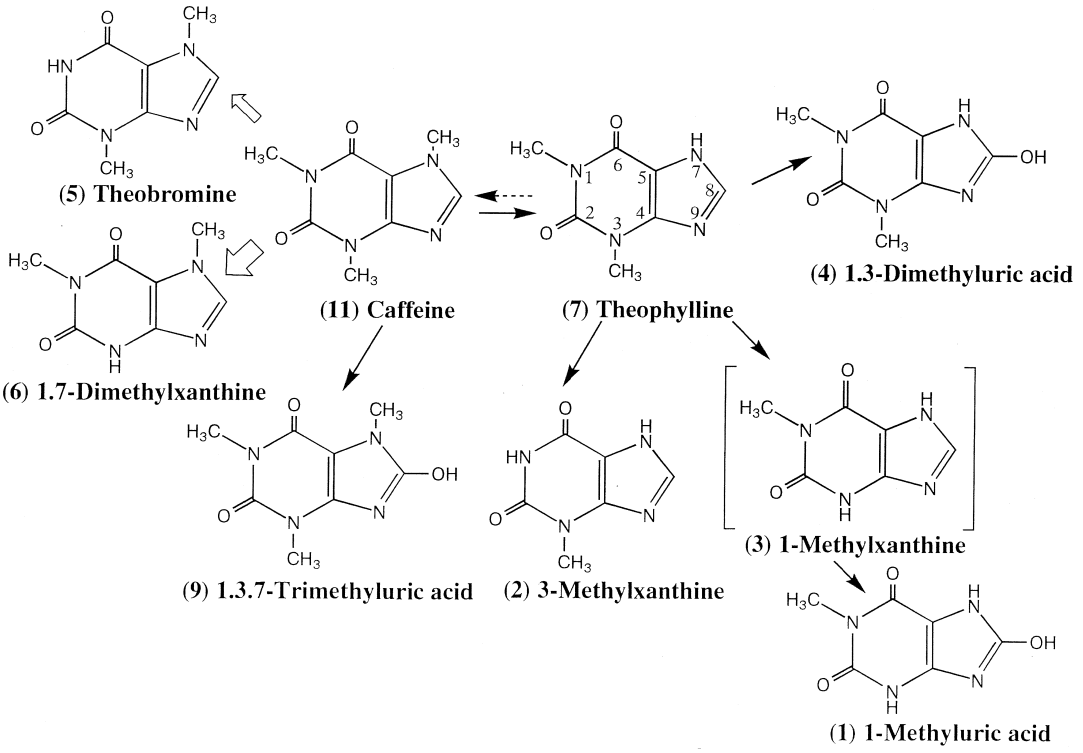

Fig. 1. The metabolic pathway of theophylline and caffeine.

(Hitachi L-7100) which were in operation under a controller (Hitachi M-1211) were used. Liquid chromatography–three dimensional quadrupole mass spectrometry (LC–3DQMS) system (Hitachi M-8000) equipped with sonic spray ionization (SSI) interface was used.

2.3. APCI mode

2.3.1. HPLC conditions

The analytical column was Mightysil RP18 (150× 4.6 mm I.D., Kanto Chemicals, Tokyo, Japan) and the effluent was monitored by an UV detector (Hitachi L-7400). The pre-column was CAPCELL PAK-ODS.UG120 (35×4.6 mm I.D., Shiseido, Tokyo, Japan). The mobile phase for the analytical column was methanol–20 m*M* ammonium acetate (pH 4.8) (1:9) and the eluate was monitored at 275 nm. The flow-rate was 1 ml/min and the column temperature was 25°C.

2.3.2. Mass spectrometry conditions

The drift voltage was 50 V and multiplier voltage was 1800 V. The desolvation temperature was 400°C and nebulizer temperature was 200°C.

2.4. SSI mode

2.4.1. HPLC conditions

The analytical column was GH–C_{18} (150×4.0 mm I.D., Hitachi, Tokyo, Japan) and the effluent was monitored with an UV detector (Hitachi L-7400). The mobile phase was methanol–20 m*M* ammonium acetate (pH 4.8) (1:9) and the eluate was monitored at 275 nm. The flow-rate was 0.5 ml/min and the column temperature was 40°C.

2.4.2. Mass spectrometry conditions

The drift voltage was 40 V. The sampling aperture was heated at 110°C and shield temperature was 270°C.

2.5. Sample preparation

Sample solutions were prepared by dissolving known amounts of the drugs in the mobile phase or human plasma. To remove proteins prior to injection, a plasma sample was pretreated with solid-phase extraction as described in the previous paper [5,6].

Standard samples for calibration were prepared as follows: A known quantity of theophylline 1,3-dimethyluric acid, 3-methylxanthine, 1-methylxanthine and caffeine in the concentration range 1–10 μg was added to 200 μl of blank plasma samples containing 20 μg of β-hydroxyethyltheophylline. Calibration curves were constructed by plotting the peak-area ratios between the each compound and the internal standard versus the amount of each compound in the spiked plasma standard.

Plasma samples were collected from patient who was being treated with aminophylline (theophylline ethylenediamine; Neophyllin, Eisai Co. Ltd, Tokyo, Japan). A 1 ml volume of the plasma containing 20 μg of the internal standard was loaded to a Sep-Pak C_{18} (Waters, Milford, MA, USA) cartridge. After the same treatments as described above, the residue was dissolved in 100 μl of the eluent.

2.6. Column-switching method

In the column-switching system, the washing solvent of the pre-column was methanol–20 m*M* ammonium acetate (2:98) and the backflush solvent introduced into the analytical column was methanol–20 m*M* ammonium acetate (1:9). The flow-rate was 1 ml/min and the eluate was monitored at 275 nm.

The schematic illustrations of column-switching procedures are shown in Fig. 2. The column-switching method procedures were follows; the plasma sample was directly injected onto the pre-column and washed (valve position; V1). After 1.5 min, the fraction containing the target substances was backflushed from pre-column into the analytical column by switching (valve position; V2).

3. Results and discussion

There have been several reports for the determination of theophylline and its major metabolites by HPLC [1,7–9]. Many of these reports used a reversed-phase column and UV detection for the HPLC determination of the drugs. However, since too many components had UV absorption in biological sample such as plasma, these methods are somewhat less

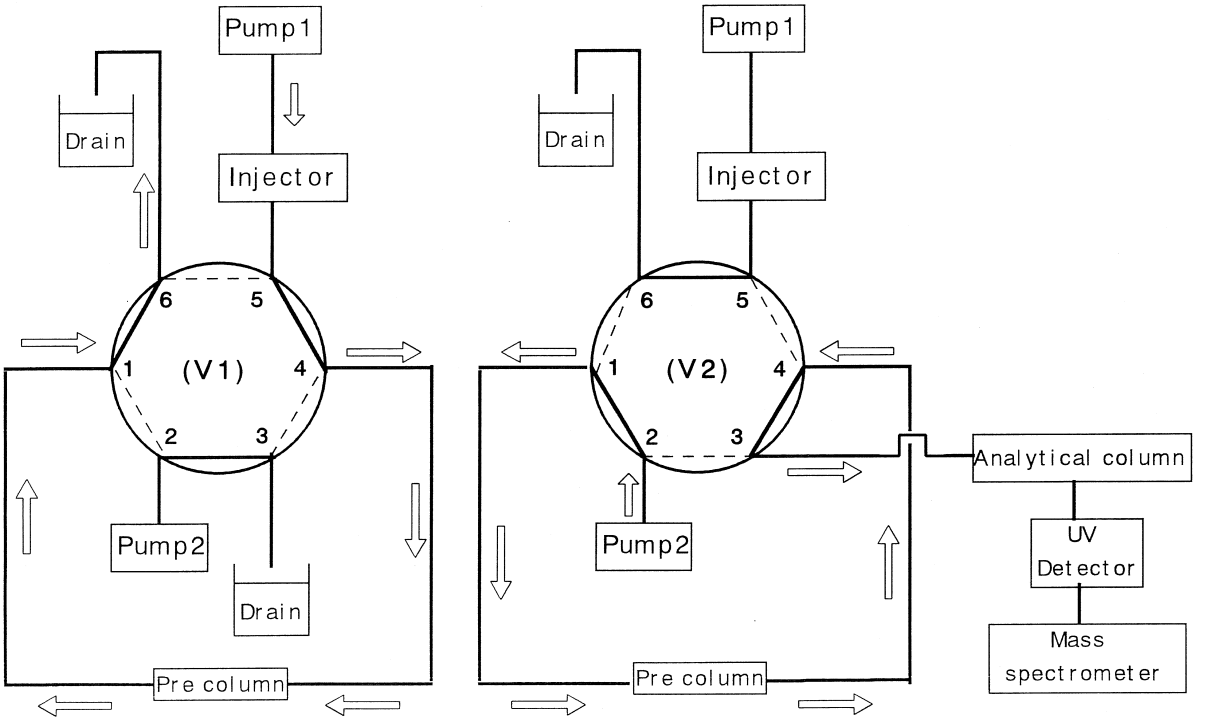

Fig. 2. A schematic diagram of column-switching system.

selective. On the other hand, mass spectrometry (MS) can perform detection based on molecular weight, so LC–MS is a powerful tool for the simultaneous analysis of drugs and metabolites in biological sample.

3.1. Mass chromatograms and mass spectra

Fig. 3a and b show total ion chromatogram (TIC) and mass chromatograms, under positive ion conditions and under negative ion conditions respectively, using LC–APCI–MS of a mixture of 1-methyluric acid (peak No. 1), 3-methylxanthine (2), 1-methylxanthine (3), 1,3-dimethyluric acid (4), theobromine (5), 1,7-dimethylxanthine (6), theophylline (7), 1,7-dimethyluric acid (8), 1,3,7-trimethyluric acid (9), β-hydroxyethyltheophylline (10) and caffeine (11). The well resolved chromatograms were obtained with methanol–20 m*M* ammonium acetate (1:9) as the eluent at a flow-rate of 1 ml/min. The mass spectra under positive ion conditions are shown in Fig. 4. The spectra are almost the same as that obtained by direct analysis. The protonated molecular ions $[M+H]^+$ of the 3-methylxanthine, 1-methylxanthine, theobromine, 1,7-dimethylxanthine, theophylline, 1,7-dimethyluric acid, β-hydroxyethyltheophylline and caffeine were clearly observed at *m/z* 167, 167, 181, 181, 181, 197, 225 and 195, respectively, as base peaks. The molecular ion $[M+H]^+$ of 1,3-dimethyluric acid (4) and fragment ion as a base peak were observed at *m/z* 197 and 169, respectively. The fragment ion should correspond to that formed by the demethylation of 1,3-dimethyluric acid at position 1 and 3, accompanied by protonation.

Fig. 5 shows the mass spectrum of a mixture of theophylline and its metabolites under negative ion conditions. The molecular ions $[M-H]^-$ of 3-methylxanthine, 1-methylxanthine, 1,3-dimethyluric acid, theophylline, 1,7-dimethyluric acid and 1,3,7-trimethyluric acid, were clearly observed at *m/z* 165, 165, 195, 179, 195 and 209, respectively, as base peaks. The molecular ion $[M-H]^-$ of 1,7-dimethylxanthine (6) and fragment ion as a base peak were

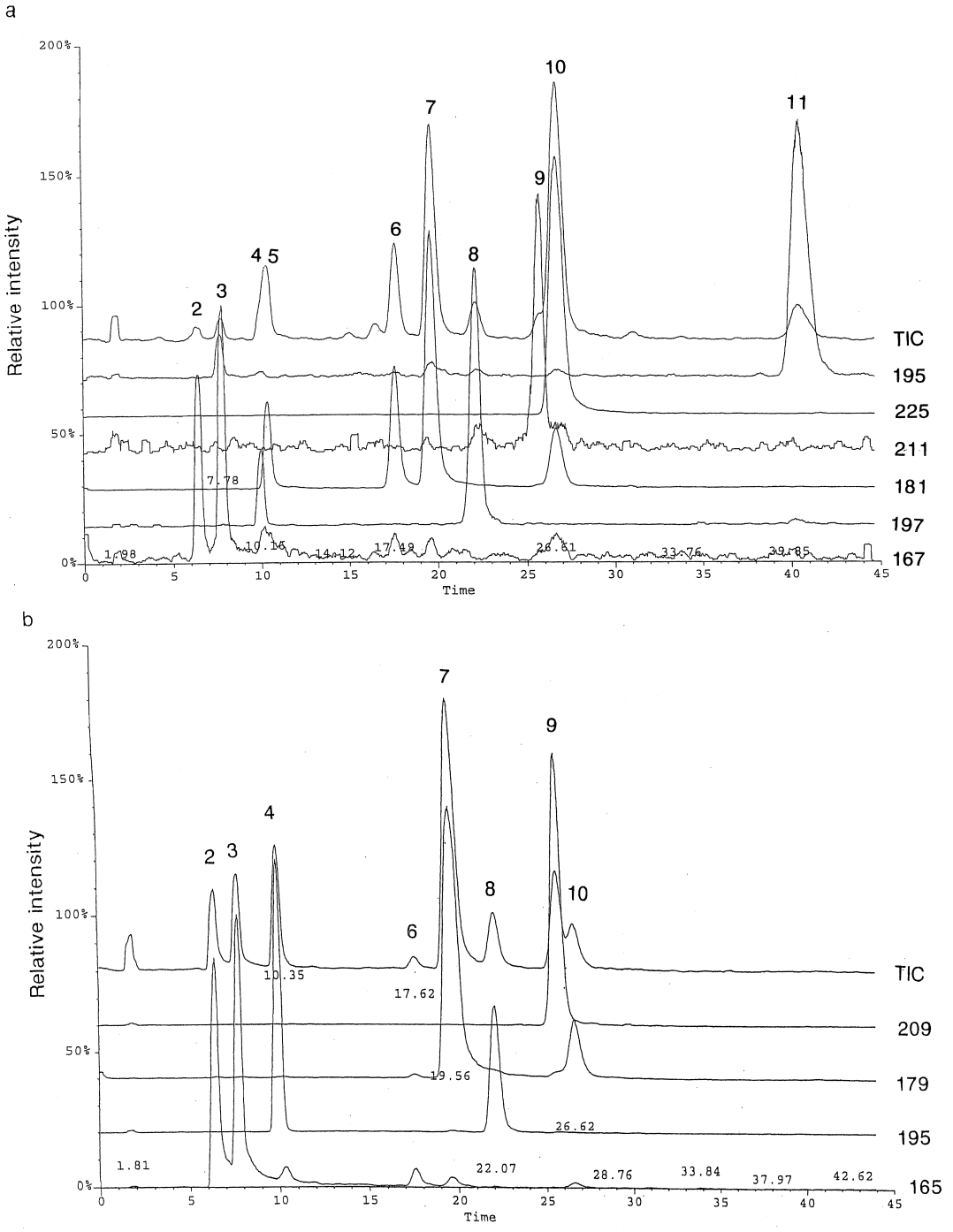

Fig. 3. Total ion chromatogram (TIC) and mass chromatograms of a mixture of theophylline and its metabolites, (a) under positive ion conditions, and (b) under negative ion conditions. Chromatographic conditions are described in Experimental. Injection volume is 50 μl containing 3.8 μg of each compound except 7.7 μg of 1,7-dimethyluric acid and 1-methyluric acid.

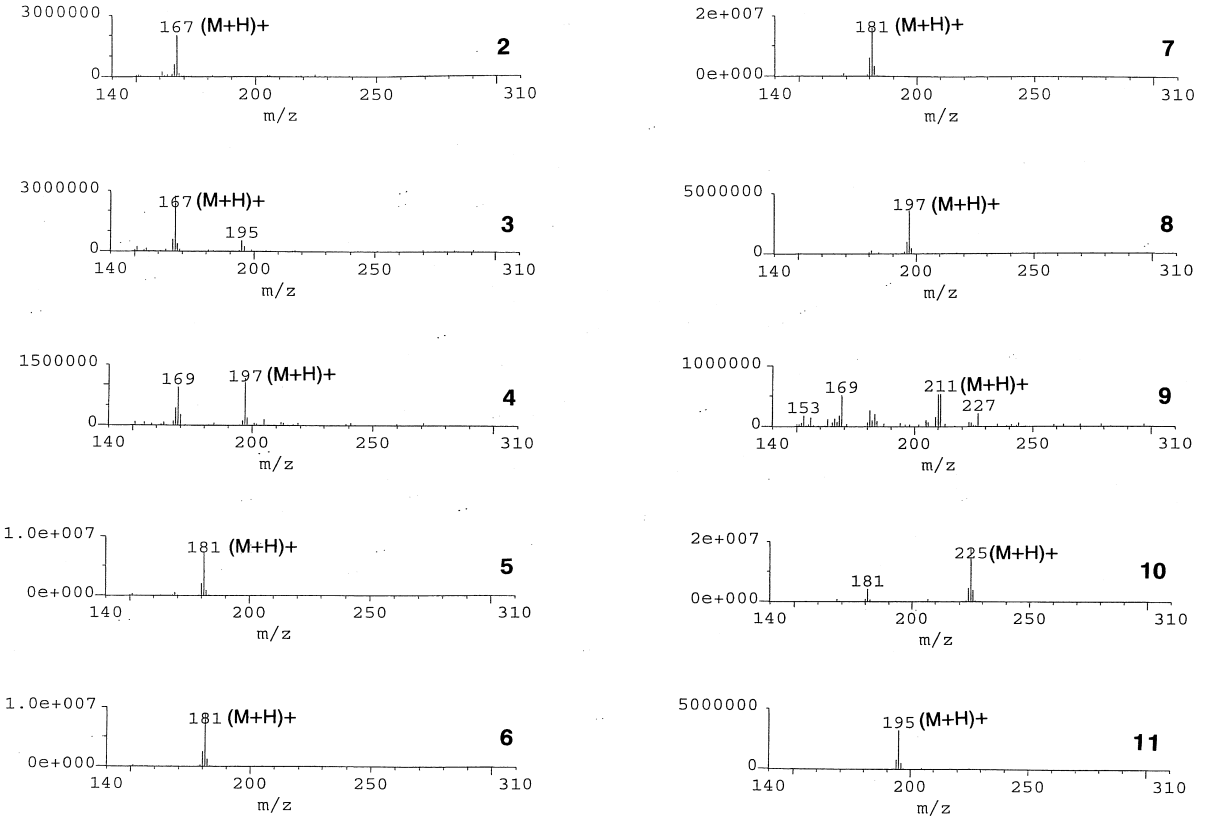

Fig. 4. Mass spectra of 3-methylxanthine (2), 1-methylxanthine (3), 1,3-dimethyluric acid (4), theobromine (5), 1,7-dimethylxanthine (6), theophylline (7), 1,7-dimethyluric acid (8), 1,3,7-trimethyluric acid (9), β-hydroxyethyltheophylline (10) and caffeine (11) under positive ion conditions.

observed at *m/z* 179 and 165, respectively. The fragment ion should correspond to that formed by the demethylation of 1,7-dimethylxanthine at position 7. Base peak of β-hydroxyethyltheophylline (10) was observed at *m/z* 179 under negative ion conditions. The ion should be due to the fragment ion by the loss of hydroxyethyl moiety at position 7.

The ion intensities of 3-methylxanthine, 1-methylxanthine, 1,3-dimethyluric acid, theophylline, 1,7-dimethyluric acid and 1,3,7-trimethyluric acid (peak Nos. 2, 3, 4, 7, 8 and 9, respectively) under negative ion conditions are higher than those under positive ion conditions. However, ion intensities of 1,7-dimethylxanthine and β-hydroxyethyltheophylline (6 and 10) under positive ion conditions are higher than those under negative ion conditions. Theobromine and caffeine (5 and 11) are not observed under negative ion conditions.

3.2. Calibration curves and precision

The peak areas were calculated on the selected-ion chromatograms of theophylline, caffeine and β-hydroxyethyltheophylline (internal standard) at *m/z* 181, 195 and 225 under positive conditions, respectively. Under negative conditions the peak areas were calculated on the selected-ion chromatograms of 1,3-dimethyluric acid, 3-methylxanthine, 1-methylxanthine and β-hydroxyethyltheophylline (internal standard) at *m/z* 195, 165, 165, and 179, respectively. The linear relationship calculated between the peak-area ratio (theophylline, caffeine, 1,3-dimethyluric acid, 3-methylxanthine or 1-methylxanthine/β-hydroxyethyltheophylline: *R*) and the amount (*x* μg) of the drugs in plasma and the correlation coefficients (*r*) were as follows:

Under positive conditions:

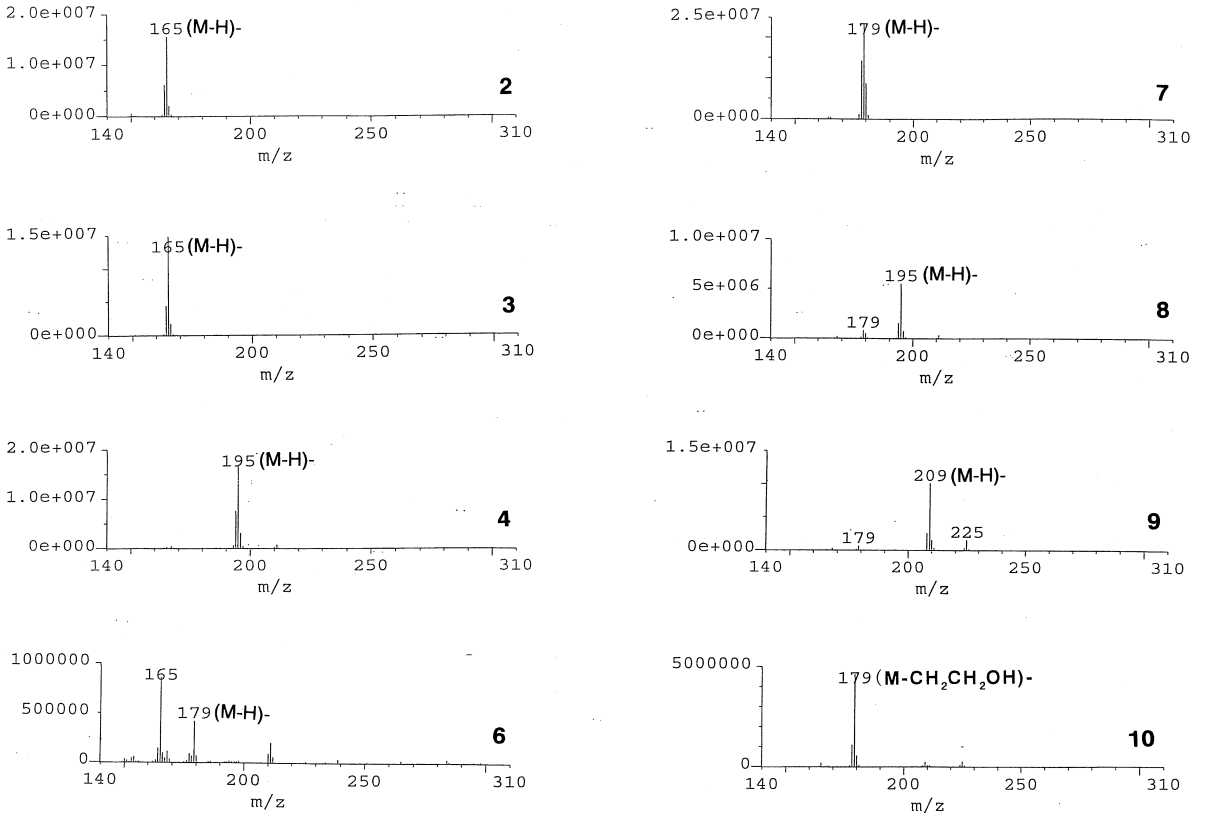

Fig. 5. Mass spectra of 3-methylxanthine (2), 1-methylxanthine (3), 1,3-dimethyluric acid (4), 1,7-dimethylxanthine (6), theophylline (7), 1,7-dimethyluric acid (8), 1,3,7-trimethyluric acid (9) and β-hydroxyethyltheophylline (10) under negative ion conditions.

theophylline; $R = 1.61x + 0.004$ $(r^2 = 0.995)$

caffeine; $R = 2.71x + 0.026$ $(r^2 = 0.963)$

Under negative conditions:

1,3-dimethyluric acid; $R = 0.70x - 0.007$ $(r^2 = 0.958)$

3-methylxanthine; $R = 1.02x + 0.145$ $(r^2 = 0.969)$

1-methylxanthine; $R = 8.22x + 0.117$ $(r^2 = 0.977)$

The lower limit of quantification of theophylline was 0.5 ng at a signal-to-noise ratio of 3. The recoveries of the analytical procedure theophylline, caffeine, 1,3-dimethyluric acid, 3-methylxanthine and 1-methylxanthine from plasma were 100%, 100%, 86.4%, 99.6% and 94.2%, respectively. The present method is sufficiently sensitive and accurate to measure pharmacokinetic parameters. The sensitivity of the present method for theophylline is almost as the same as that of the conventional HPLC method using UV detection from comparing UV chromatogram under the same condition. The precision of the method was established from 5 assays using the LC–MS. The C.V. values of the retention times were less than 1% and those of the peak areas on total ion chromatograms were less than 4%.

3.3. Determination of plasma levels of theophylline and its metabolites

Fig. 6 shows UV and total ion chromatogram (TIC) of an extract of plasma sample obtained from

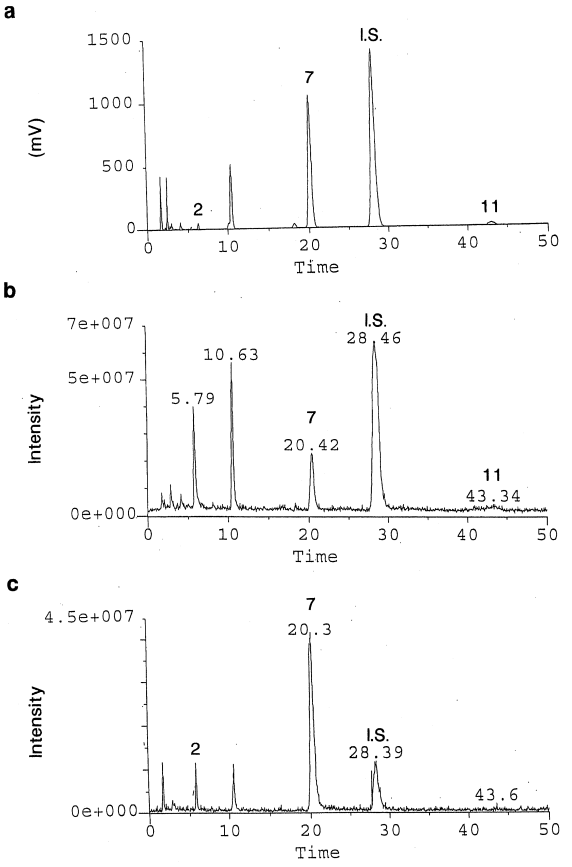

Fig. 6. UV (a) and total ion chromatograms (TIC) of an extract of plasma sample obtained from a patient administered theophylline, (b) under positive ion conditions, and (c) under negative ion conditions. Injection volume is 20 μl.

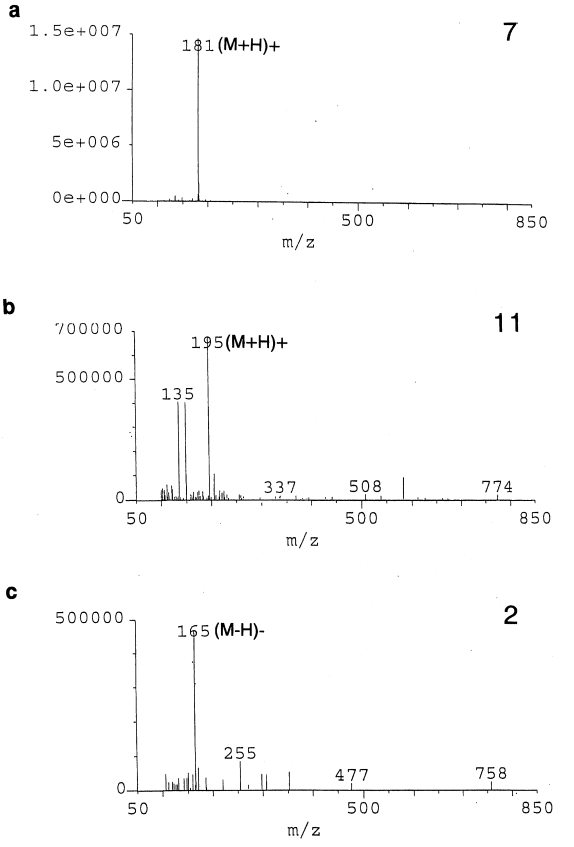

Fig. 7. Mass spectra of theophylline (7), caffeine (11) and 3-methylxanthine (2).

a patient administered theophylline orally, under positive ion and under negative ion conditions. The well resolved chromatograms were obtained without any influence of endogenous compounds in plasma. The peaks of 3-methylxanthine, theophylline and caffeine were identified with retention times of 6.34, 20.42 and 43.34 min, respectively. The molecular ions $[M+H]^+$ of theophylline and caffeine were clearly observed at m/z 181and 195, respectively, as base peaks, shown in Fig. 7. The molecular ions $[M-H]^-$ of 3-methylxanthine was clearly observed at m/z 165 as base peaks. The concentrations of the components as determined by the present method, were 0.93, 7.65 and 0.42 μg/ml, respectively.

The peak with retention time 10.54 was observed in both of UV and TIC chromatograms shown in Fig. 6. The base peak was observed at m/z 205 under positive ion conditions. It did not correlate with theophylline and was presumed, from MS-spectrum, to be L-tryptophan [1].

The metabolites of caffeine and endogenous compounds in plasma interfere with the detection of theophylline, and for this reason a high selective method like the one presented here is required. Theophylline and caffeine share common metabolites, such as theophylline itself and 1-methyluric acid. Therefore, it is important to restrict caffeine intake when pharmacokinetic studies are performed.

3.4. Column-switching method

Biological sample such as plasma cannot be applied directly in LC–MS system, a powerful tool

Table 1
Precision of the LC–MS assay using column-switching system

Compound	Peak area ($10^{\times 6}$) mean±S.D.	C.V. (%)
3-Methylxanthine	11.68±0.13	1.13
1-Methylxanthine	15.20±0.18	1.17
1,3-Dimethyluric acid	3.40±0.13	3.72
Theobromine	3.14±0.11	3.63
1,7-Dimethylxanthine	13.71±0.06	0.44
Theophylline	13.70±0.15	1.06
1,7-Dimethyluric acid	15.30±0.20	1.32
1,3,7-Trimethyluric acid	7.64±0.14	1.84
β-Hydroxyethyltheophylline	26.56±1.13	4.25
Caffeine	17.16±0.10	0.56

for the pharmacokinetic studies of these drugs. Then we examined the column-switching method with LC–MS.

This system used two columns, an analytical column for separation of target compounds and a pre-column for pretreatment. Direct injection of an extract of human plasma spiked with a standard mixture of theophylline and its metabolites was possible with LC–MS. The well-resolved chromatograms were obtained and the mass spectra are almost the same as that obtained by the direct analysis. There was no disturbance from plasma components.

The precision of the method was established from 5 assays using the LC–MS with the column-switching system. The mean values and C.V. are shown in Table 1. The C.V. values of the peak areas on total ion chromatograms were less than 5%.

Column-switching method can also be used for the concentration of target compounds in biological fluids using trapping column in the concentrating

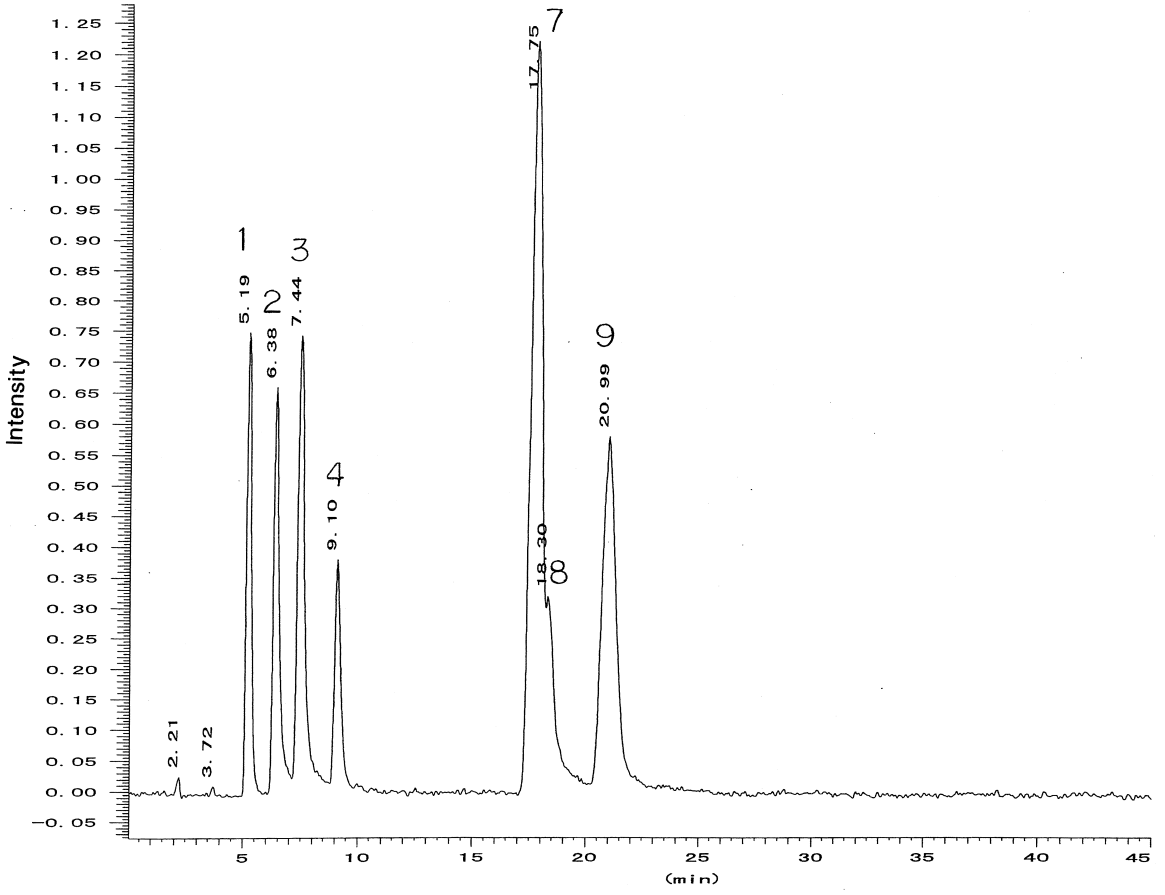

Fig. 8. TIC of a mixture of the theophylline and its metabolites by the SSI mode under negative ion conditions.

mode. We determined the concentrations of theophylline and its metabolite in human blood sample using solid-phase extraction in this report. The determination of their plasma levels using a column-switching system in combination with LC/MS is in progress in our laboratory.

3.5. SSI mode

To improve the less sensitivity for plasma level determination of 1-methyluric acid, we examined the SSI interface mode of LC–MS. SSI method is very powerful for polar analytes which exist as ions in solution [10].

Fig. 8 shows TIC of a mixture of the theophylline and its metabolites by the SSI mode under negative ion conditions. The molecular ion $[M-H]^-$ of 1-methyluric acid was clearly observed at m/z 181 as a base peak. Theobromine, 1,7-dimethylxanthine, β-hydroxyethyltheophylline and caffeine were not observed under negative ion conditions.

The ion intensity of theophylline under negative ion conditions was higher than that under positive ion conditions. The uric acid groups were relatively preferred to negative ion conditions. 1-Methyluric acid was not sensitive enough for plasma level determination by APCI mode.

4. Conclusions

Knowledge of the pharmacokinetics of drugs should be necessary in clinical practice. LC–MS is a powerful tool for analysis of drugs and their metabolites. We used LC–MS for determination of plasma levels of theophylline and its metabolites. A column-switching system in combination with LC–MS should be a very powerful tool for direct analysis of the drugs and its metabolites in biological samples. The present method is useful for the pharmacokinetic studies of theophylline, its metabolites and related drugs.

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