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AUTOMATED DETERMINATION OF DRUGS IN SERUM BY COLUMN-SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. SEPARATION OF THEOPHYLLINE AND ITS METABOLITES

KOJIRO MATSUMOTO*, HARUHITO KIKUCHI and HISAMI IRI

Department of Clinical Laboratories, School of Medicine, Keio University, 35 Shinanomachi, Shinjuki-ku, Tokyo 160 (Japan)

and

HIROAKI TAKAHASI and MASUO UMINO

Scientific Engineering, Tosoh Corporation, 2743-1, Hayakawa, Ayase-shi, Kanagawa 252 (Japan)

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SUMMARY

The automated determination of theophylline and related compounds in human serum by column-switching high-performance liquid chromatography, including direct injection of serum samples, is described. TSK pre-column BSA-ODS and TSK gel ODS-80TM were used in the pre-column and analytical column, respectively. Serum samples of 20 μl were directly injected on to the pre-column. After washing out serum proteins from the pre-column with 0.1 *M* NaH_2PO_4 at a flow-rate of 1.0 ml/min for 3.5 min, the effluent from the pre-column was introduced on to the analytical column by a column-switching device. The analysis was performed by stepwise gradient elution using 10 and 18% methanol in 0.1 *M* NaH_2PO_4. Theophylline and nine derivatives could be determined simultaneously within 40 min. The recovery of these compounds from serum was 95–103%. The linearity (1.0–50 μg/ml theophylline) and reproducibility (coefficient of varietion less than 2.0%) were sufficient for drug monitoring at the lower and upper limits of therapeutic concentrations of theophylline.

INTRODUCTION

Theophylline (1,3-dimethylxanthine) and its derivatives are frequently used for the treatment of acute and chronic bronchial asthma and apnea in premature infants. Therapeutic concentrations of theophylline are in the range 5–20 μg/ml in the serum of adults, and toxic signs appear at concentrations higher than 20

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μg/ml. The pharmacological activity of theophylline is highly correlated with its concentration in serum and inter-individual variations are considerable during drug clearance [1, 2]. Therefore, dosages must be individualized for optimized therapy by the determination of the concentrations of the drugs in serum.

In adults, theophylline is metabolized to 1-methyluric acid, 1,3-dimethyluric acid and 3-methylxanthine by demethylation and oxidation. In premature infants, theophylline is methylated to caffeine (1,3,7-trimethylxanthine), which is more effective than theophylline itself for the treatment of apnea [3–5]. Various environmental factors and genetic polymorphism influence the clearance of theophylline from plasma [6, 7]. Therefore, simultaneous monitoring of theophylline and its metabolites is necessary for clinical assessment of the treatment.

Analytical methods currently used for the determination of theophylline in clinical laboratories are based on immunological methods, which only permit the determination of theophylline and not of its metabolites. On the other hand, high-performance liquid chromatography (HPLC) permits the simultaneous determination of these compounds although it requires sample pre-treatment such as deproteinization or extraction [8–11]. However, there are very few HPLC methods for the determination of xanthine-related compounds including theophylline and its major metabolites [6, 9].

The purpose of this investigation was to develop an automated HPLC system for the simultaneous determination of the compounds in human serum without any hazardous pre-treatments. Using the HPLC system described here, theophylline and nine derivatives in human serum could be determined within 40 min.

EXPERIMENTAL

Materials

Reagents. Theophylline, caffeine theobromine, paraxanthine (1,7-dimethylxanthine), dyphylline [7-(2,3-dihydroxypropyl)theophylline], 1,3-dimethyluric acid, 1-methylxanthine, 3-methylxanthine, 1-methyluric acid and 3-methyluric acid were purchased from Sigma (St. Louis, MO, U.S.A.). Methanol was of HPLC grade from E. Merck (Darmstadt, F.R.G.). All other chemicals were of analytical-reagent grade.

Standard solutions. Stock standard solutions of each drug (1.0 mg/ml) were prepared by dissolution in 0.1 *M* sodium hydroxide solution and distilled water. Working standard solutions were prepared by diluting the stock solutions with pooled human serum or 50 m*M* NaH_2PO_4 to give concentrations of 0.1–50 μg/ml.

Biological samples. Blood samples were obtained from normal subjects and patients in our laboratory. Serum was prepared in the usual manner.

Apparatus

A TOSO CP-8000 instrument (Tosoh, Tokyo, Japan) equipped with a PT-8000 column-switching device and an AS-48 autosampler were used. TSK pre-column BSA-ODS (35 mm × 4.6 mm I.D.) and TSK gel ODS-80TM (150 mm × 4.6 mm I.D.) were used as the pre-column and the analytical column, respectively.

Procedures

Immunological assay for theophylline. Theophylline was determined by a homogeneous enzyme-multiplied immunoassay technique (EMIT) using DuPont Aca III (DuPont, Wilmington, DE, U.S.A.) and by fluorescence polarization immunoassay (FPIA) using ABBOTT-TDx (Abbott Labs., Irving, CA, U.S.A.).

Chromatographic conditions. After injecting 20 μl of serum samples on to the pre-column, which had been previously equilibrated with 0.1 *M* NaH_2PO_4, the column was washed for 3.5 min with 0.1 *M* NaH_2PO_4 at a flow-rate of 1.0 ml/min. After washing for 3.5 min, the pre-column was eluted in the back-flushing mode to minimize the volume of effluent containing the substances. The substances adsorbed on the pre-column were introduced on to the analytical column with 10% methanol in 0.1 *M* NaH_2PO_4 for 4.0 min by switching the six-port valve. After introducing the substances on to the analytical column, the six-port valve was returned to the original position. The pre-column was washed with 18% methanol in 0.1 *M* NaH_2PO_4 for several minutes and then equilibrated with 0.1 *M* NaH_2PO_4 for the next injection. The chromatographic parameters for the analytical column were as follows: stepwise gradient from 10% methanol in 0.1 *M* NaH_2PO_4 (0–23 min) to 18% methanol in 0.1 *M* NaH_2PO_4 (23–40 min), with a flow-rate of 1.0 ml/min. The effluent was monitored at 273 nm.

RESULTS

Pre-treatment conditions

Chromatograms of standard samples and drug-free human serum are shown in Fig. 1. Theophylline and its metabolites were gradually eluted from the pre-column by 0.1 *M* NaH_2PO_4 (Fig. 1A). After changing the eluent to 10% methanol in 0.1 *M* NaH_2PO_4 at 20 min, theophylline and related compounds such as theobromine, dyphylline and caffeine were easily eluted. On the other hand, excess serum proteins and hydrophilic substances (front peaks) were eluted from the pre-column by 0.1 *M* NaH_2PO_4 within about 3.5 min (Fig. 1B). The main unknown peak in serum was observed at a retention time of ca. 7.5 min, and after changing the eluent to 10% methanol in 0.1 *M* NaH_2PO_4 several minor peaks could also be detected. 3-Methyluric acid was retained more selectively on the pre-column at pH 5.0 in phosphate buffer (pH range 3.0–7.0), but serum proteins were eluted with an almost constant efficiency at pH above 5.0.

The effects of washing time with 0.1 *M* NaH_2PO_4 on the recovery of theophylline and related compounds are summarized in Fig. 2. 3-Methyluric acid was completely retained on the TSK pre-column BSA-ODS for 3.5 min. The other compounds were retained even after washing for 5 min.

Analytical column loading time

The overall recoveries of each drug for various column connection times are shown in Fig. 3. 3-Methyluric acid, 1-methyluric acid, 3-methylxanthine, 1-methylxanthine and 1,3-dimethyluric acid were completely introduced to the analytical column within the first 1.5 min, but the other compounds were introduced incompletely within the connection time. For maximum and constant recoveries of all the compounds, the column connection time was set at 4 min.

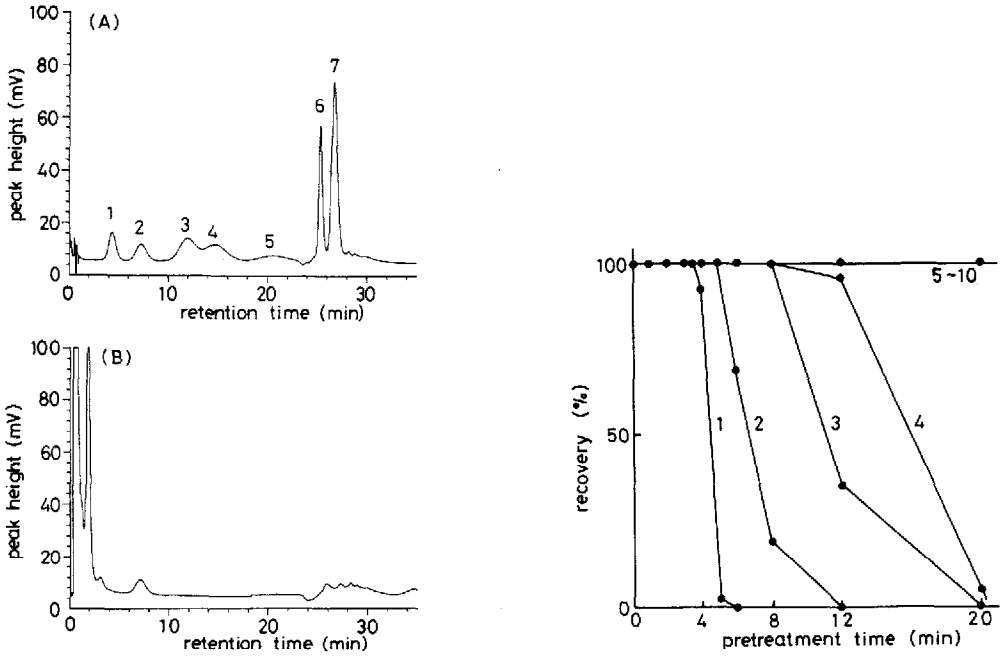

Fig. 1. Elution of theophylline and related compounds from the pre-column. Column, TSK pre-column BSA-ODS (35 mm×4.6 mm I.D.); eluent, 0.1 *M* NaH_2PO_4 (0–20 min) and 10% methanol in 0.1 *M* NaH_2PO_4 (20–40 min); flow-rate, 1.0 ml/min; detection, 273 nm. (A) 20 μl of 10 μg/ml of each compound in 50 m*M* NaH_2PO_4; (B) 20 μl of drug-free human serum. Peaks: 1 = 3-methyluric acid; 2 = 1-methyluric acid; 3 = 3-methylxanthine; 4 = 1-methylxanthine; 5 = 1,3-dimethyluric acid; 6 = theobromine; 7 = paraxanthine, theophylline dyphylline and caffeine.

Fig. 2. Recovery of theophylline and related compounds from the pre-column at different washing times. A 20-μl volume of standard solution containing 20 μg/ml of each authentic compound was injected on to the pre-column and then the column was washed with 0.1 *M* NaH_2PO_4. At different times as indicated, the eluent was changed to 10% methanol in 0.1 *M* NaH_2PO_4. The effluent from the pre-column was introduced on to the analytical column and the substances were separated as described in Fig. 4. (1) 3-Methyluric acid; (2) 1-methyluric acid; (3) 3-methylxanthine; (4) 1-methylxanthine; (5) 1,3-dimethyluric acid; (6) theobromine; (7) paraxanthine; (8) theophylline; (9) dyphylline; (10) caffeine.

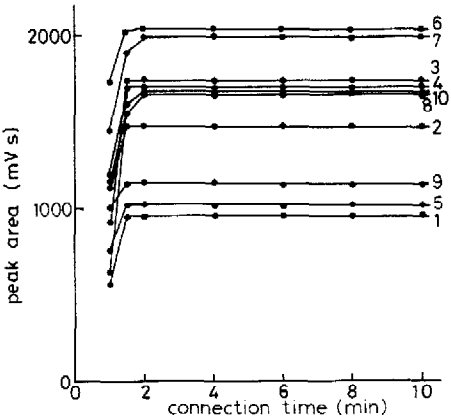

Fig. 3. Recovery of theophylline and related compounds from the pre-column for different pre- and analytical column connection times. A 20-μl volume of standard solution containing 20 μg/ml of each authentic compound was injected on to the pre-column. After washing for 3.5 min with 0.1 *M* NaH_2PO_4, the pre- and analytical columns were connected for various periods of time as indicated. Elution programmes as in Fig. 4. Numbers as in Fig. 2.

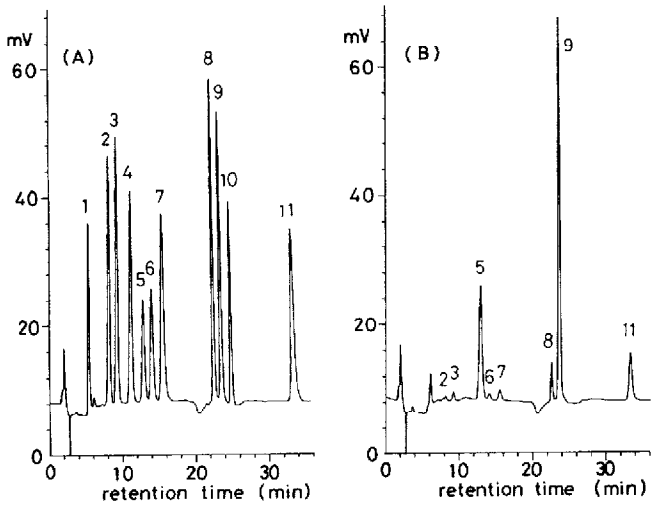

Fig. 4. Chromatograms of theophylline and related compounds. Pre-treatment column: TSK pre-column BSA-ODS; eluent, 0.1 *M* NaH_2PO_4; flow-rate, 1.0 ml/min. Analytical column: TSK gel ODS-80TM; eluent, 10% methanol (0–23 min) and 18% methanol (23–36 min) in 0.1 *M* NaH_2PO_4; flow-rate, 1.0 ml/min; column connection time, 4 min; detection, 273 nm. (A) 20 μl of 10 μg/ml of each compound in 50 m*M* NaH_2PO_4; (B) 20 μl of serum from patients treated with theophylline. Peaks: 1 = 3-methyluric acid; 2 = 1-methyluric acid; 3 = 3-methylxanthine; 4 = 1-methylxanthine; 5 = unknown; 6 = 1,3-dimethyluric acid; 7 = theobromine; 8 = 1,3-dimethylxanthine; 9 = theophylline; 10 = dyphylline; 11 = caffeine.

Separation of theophylline and its related compounds

Chromatograms of authentic theophylline and related compounds in pooled human serum and serum from a patient treated with theophylline are shown in Fig. 4. The authentic substances were clearly separated from each other within 35 min (Fig. 4A). In the patient's serum, not only theophylline but also its metabolites were detected (Fig. 4B). The unknown peak (peak 5) in serum was eluted at a position between 1-methyluric acid and 1,3-dimethyluric acid.

Reproducibility and recovery

The within-run reproducibility was determined in the same concentration range by ten analyses of samples containing 5, 10 and 20 μg/ml theophylline and related compounds in pooled human serum. As shown in Table I, the precision expressed as the coefficient of variation (C.V.) was 0.27–1.67% at each concentration. The recovery of the substances from serum in this HPLC system with a pre-column was determined by comparing the peak areas with those obtained for 50 m*M* NaH_2PO_4 solutions containing known concentrations of the drugs using an HPLC system without a pre-column. The recovery of the substances was 95–103% at all the concentrations examined (Table II).

Linearity and sensitivity

Good linear regressions between the concentrations and the corresponding peak areas were obtained (data not shown). No positive constant bias was detected in

TABLE I

WITHIN-RUN REPRODUCIBILITY ($n=10$)

Compound	Coefficient of variation (%)		
	5 μg/ml	10 μg/ml	20 μg/ml
3-Methyluric acid	1.17	3.23	0.58
1-Methyluric acid	0.96	0.61	0.67
3-Methylxanthine	1.03	0.38	0.43
1-Methylxanthine	1.18	0.38	0.27
1,3-Dimethyluric acid	1.02	0.42	0.55
Theobromine	1.54	0.78	0.40
1,7-Dimethylxanthine	1.69	1.02	0.72
Theophylline	1.30	0.62	0.61
Dyphylline	0.77	0.44	0.86
Caffeine	1.37	0.61	1.67

TABLE II

RECOVERIES OF THEOPHYLLINE AND RELATED COMPOUNDS IN HUMAN POOLED SERA BY THE PRESENT METHOD

Compound	Recovery (%)			
	2.5 μg/ml	5.0 μg/ml	10.0 μg/ml	20.0 μg/ml
3-Methyluric acid	96.9	98.1	94.5	98.0
1-Methyluric acid	95.5	99.7	103.7	100.7
3-Methylxanthine	100.4	102.5	101.3	98.3
1-Methylxanthine	100.9	100.7	100.7	96.4
1,3-Dimethyluric acid	95.8	98.1	96.1	95.9
Theobromine	98.9	99.8	100.3	97.2
1,7-Dimethylxanthine	99.6	97.3	97.8	95.4
Theophylline	99.6	103.2	100.9	96.6
Dyphylline	99.2	100.4	99.5	95.1
Caffeine	96.7	98.0	99.2	95.3

the calibration graphs of theophylline and its metabolites. Caffeine and paraxanthine were detected at less than 0.5 μg/ml in pooled human serum. The detection limits of these substances by this method (calculated as 20 mV s peak area) were 0.2 μg/ml.

Comparison of HPLC method with immunological methods

Correlations of the theophylline concentrations obtained by our HPLC method with those obtained by the DuPont Aca III method and the ABBOTT-TDx method are shown in Fig. 5. The correlations obtained were $n=76$, $r=0.982$, $y=1.02x-0.08$ (DuPont Aca method) and $n=58$, $r=0.992$, $y=0.91x-0.11$ (ABBOTT-TDx method).

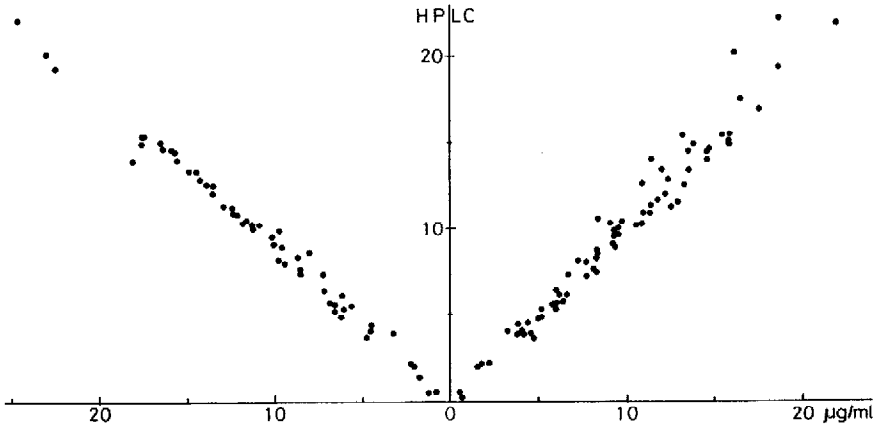

Fig. 5. Correlation of the values of theophylline in patients' sera by the present method with those obained by two immunological methods. Right, DuPont Aca method ($n=76$, $r=0.982$, $y=1.02x-0.08$); left, ABBOTT-TDx method ($n=58$, $r=0.992$, $y=0.91x-0.11$).

DISCUSSION

For the determination of theophylline in serum by HPLC, the serum needs to be deproteinized by using trichloroacetic acid, a solvent or molecular permeation before injection of the sample on to the column. Further, an additional internal standard is necessary for correction of drug recoveries from biological fluids.

Recently, multi-dimensional HPLC techniques with a column-switching device have been used for semi-automated pre-treatment [12–20]. A fully automated HPLC method involving direct injection of biological fluids has been developed by using a gel permeation [21] or a modified reversed-phase column [22–24] as the pre-treatment column. Although a gel permeation column system is generally applied to remove macromolecules such as serum proteins, large amounts of small molecules are introduced on to the analytical column and column switching needs to be carefully controlled in order to obtain a sufficient recovery and reproducibility.

With the use of a column-switching method incorporating a modified reversed-phase column, macromolecules and small hydrophilic molecules can be easily eluted and small molecules with similar hydrophobicity to those being determined can be selectively introduced on to the analytical column. Column-switching techniques with the use of a modified reversed-phase column as the pre-treatment column can be successfully applied to relatively hydrophilic substances such as theophylline and its metabolites. Owing to the complete recoveries and sufficient reproducibilities obtained, the values obtained by our method did not necessitate correction by using internal standards. Moreover, the simultaneous progress of pre-treatment and separation in this system reduced additional analytical times required for pre-treatment. Both the pre-column and analytical columns proved to function satisfactorily for 500 successive injections of 20-μl serum samples. However, the filters in the end-fittings of the columns should be changed after every 50 injections. With the use of TSK gel ODS-80TM, theophylline was

well separated from paraxanthine and other compounds in human sera. There was no positive bias of the theophylline concentrations determined by the present method compared with those obtained by two immunological methods. Further, the concentrations of theophylline in patients' sera determined by the present method agreed well with those obtained by the two immunological methods. The amount of theophylline metabolites in the patients' sera was very small, but the amounts of caffeine and paraxanthine were significant in all the sera examined. The clinical significance of these metabolites for therapeutic drug monitoring should be studied further. The amounts of 1-methyluric acid, 3-methylxanthine, 1,3-dimethyluric acid, paraxanthine and caffeine in patients' sera were 1.52 ± 0.75, 3.69 ± 1.49, 4.28 ± 1.49, 3.13 ± 1.74 and $3.75 \pm 1.57\%$ (mean $\pm$ S.D.) of that of theophylline. In 1, 5, 5, 6 and 7 cases out of 63, the concentrations in patients' sera of 1-methyluric acid, 3-methylxanthine, 1,3-dimethylxanthine, paraxanthine and caffeine, respectively, exceeded 10% of those of theophylline. As most of the cases studied were outpatients, theophylline metabolism should be further studied in patients with controlled environmental conditions. Our method will be useful for studies of the inter-individual or genetic variation of theophylline metabolism.

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