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Separation and determination of theophylline from paraxanthine in human serum by reversed-phase high-performance liquid chromatography

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Abstract: A sensitive and highly specific method for the determination of theophylline in serum by high-performance liquid chromatography (HPLC) has been developed. Theophylline was completely separated from paraxanthine, a major metabolite of caffeine which has been known to interfere with most isocratic reversed-phase HPLC methods, with a mixture of acetonitrile/tetrahydrofuran/acetate buffer (10 mM, pH 5.0; 5:1:94, v/v %) as the mobile phase using a C_{18} bonded reversed-phase column. Neither the other xanthine and uric acid derivatives nor numerous drugs tested were found to interfere. The proposed method was applied to therapeutic monitoring utilizing its excellent precision, reproducibility and high specificity for theophylline.

Keywords: *Theophylline; reversed-phase high-performance liquid chromatography; paraxanthine; caffeine; interference.*

Introduction

Theophylline has been used extensively for the treatment of reversible airway obstruction and also neonatal apnea. The pharmacological efficacy and toxicity of theophylline have been recognized to be highly correlated with its serum or plasma concentrations [1–4]. Because of its narrow therapeutic range, i.e. 10–20 μg ml^{-1} for bronchial asthma and 6–11 μg ml^{-1} for neonatal apnea [3, 5–7], and large inter-individual variation in the clearance of the drug [8–10], dosage must be individualized in order to optimize the therapy based on the measurement of theophylline concentration in serum.

A large number of analytical techniques are available for the determination of serum theophylline. These include several kinds of immunoassay methods [11–15] which have the advantage of rapidity and simplicity in operation. They have, however, the disadvantage of cross reactivity with analogous compounds, resulting in falsely elevated concentrations, especially, in the case of uremics [16]. In newborns, theophylline is metabolized to caffeine [17–19] which is also effective in the treatment of apnea [20, 21].

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Therefore, the concurrent measurements of theophylline and caffeine are important in the management of neonatal apnea [19, 22]. From these points of view, high-performance liquid chromatography (HPLC) is advantageously applicable since it offers simultaneous analysis, sufficient specificity and accuracy.

While numerous HPLC methods have been reported for theophylline analysis, it has been shown that the peak resolution of theophylline from paraxanthine (1,7-dimethylxanthine), a major metabolite of caffeine [23, 24], is poor on most reversed-phase HPLC systems [25–27]. To eliminate this problem, ion-pairing reversed-phase HPLC has been used [28, 29]. However, this method is generally time-consuming. It takes a significant amount of time to reach equilibrium between the stationary and mobile phases at the initiation of analysis.

This report describes the separation of theophylline from paraxanthine using isocratic reversed-phase HPLC with a modified mobile phase. The method is comparable to, and somewhat superior in terms of analytical performance to previously reported methods [30–32] which have also demonstrated the separation of these compounds using isocratic reversed-phase HPLC. The advantage of a highly selective method for assaying theophylline is stressed in comparison with a common reversed-phase HPLC method which has been often used for the determination of theophylline but shows poor peak resolution for these methylxanthines [25–27].

Experimental

Reagents

Sources of xanthine and uric acid derivatives were as follows: theophylline, theobromine, xanthine, hypoxanthine and uric acid (Nakarai Chemicals, Kyoto, Japan), 7-(2-hydroxyethyl)theophylline and 8-chlorotheophylline (Tokyo Kasei, Tokyo, Japan), 1-methyluric acid, 3-methylxanthine and paraxanthine (Sigma, St. Louis, MO, USA), 1-methylxanthine and 1,3-dimethyluric acid (Fluka AG, Buchs SG, Switzerland), caffeine (Japanese Pharmacopoeia grade). Acetonitrile and tetrahydrofuran were of HPLC grade (Nakarai Chemicals). All other reagents used were of analytical grade.

Serum samples

In order to evaluate precision and reproducibility of the method, spiked serum samples were prepared at concentrations of 5, 10, 15 and 20 $\mu g\ ml^{-1}$ of theophylline, and of 1 and 10 $\mu g\ ml^{-1}$ of paraxanthine or caffeine. Blank serum was obtained from healthy volunteers abstaining from methylxanthine-containing foods and beverages for at least 36 h.

Blood samples were withdrawn from 10 healthy male volunteers and 10 hospitalized patients with chronic obstructive pulmonary disease (COPD), receiving aminophylline infusion and oral preparations of theophylline on separate occasions. The healthy volunteers abstained from methylxanthine-containing foods and beverages for at least 36 h prior to the administration of theophylline and during blood collection. The patients were allowed to consume any foods or beverages. The serum specimens were stored at −20°C until analysis.

Apparatus

A Model LC-2 HPLC system (Shimadzu, Kyoto, Japan) was used, equipped with a Model SIL-1A sample injector, a Model SPD-1 variable-wavelength UV detector and a

Chromatopack-E1A integrator. A reversed-phase column, Cosmosil $5C_{18}$ (4.6 mm i.d. × 150 mm, Nakarai Chemicals), was used throughout the study.

Mobile phase

After adjusting to the desired pH with acetic acid, 10 mM sodium acetate solution was filtered through a membrane filter (0.45 μm pore size, Millipore Type HA, Millipore, Bedford, MA, USA). The mobile phase for the proposed method (M-HPLC) was 10 mM acetate buffer (pH 5.0)/acetonitrile/tetrahydrofuran (94:5:1, v/v %). As a comparative assay method (C-HPLC), a mixture of 10 mM acetate buffer (pH 4.0)/acetonitrile (91:9, v/v %) was used as the mobile phase, which was similar to that reported previously [25, 26]. Both mobile phases were degassed by sonication for 15 min prior to use.

Assay procedure

The following assay procedure was common for both M-HPLC and C-HPLC.

To 100 μl of serum in a 1.5 ml capped test tube (Eppendorf, Hamburg, FRG) was added 300 μl of chloroform/isopropanol (1:1, v/v %) containing 4 μg ml^{-1} of 7-(2-hydroxyethyl)theophylline as internal standard. After vortexing for 60 s, the sample was centrifuged for 5 min at 10,000 rpm. To a glass tube (10 × 75 mm), 200 μl of the organic layer was transferred and evaporated to dryness under a nitrogen stream. The residue was reconstituted with 50 μl of the mobile phase, and 20 μl of the solution was injected into the chromatograph. The flow rate of the mobile phase was 1.0 ml min^{-1} (approximately 80–90 kg cm^{-2}) at ambient temperature. The UV detector was operated at a wavelength of 280 nm and 0.08 AUFS. Quantification was obtained from the ratios of peak areas of each methylxanthine to those of the internal standard.

Results

Chromatograms

Chromatograms of methylxanthines extracted from spiked serum are shown in Fig. 1. Theophylline and paraxanthine were clearly separated on the chromatogram obtained by M-HPLC, as shown in Fig. 1(A). Retention times were 6.9, 7.8, 9.6 and 13.9 min for paraxanthine, theophylline, 7-(2-hydroxyethyl)theophylline (internal standard) and caffeine, respectively. For comparison, theophylline and paraxanthine could not be resolved by C-HPLC. The two compounds were eluted as a single peak with a retention time of 6.4 min [Fig. 1(B)].

Calibration curves

Calibration curves for theophylline, and also for caffeine and paraxanthine, were simultaneously prepared from serum samples containing these compounds. The plots of peak area ratios versus serum concentrations over the ranges of 1.0–30.0, 1.0–20.0 and 0.5–10.0 μg ml^{-1} for the respective methylxanthines, gave straight lines, with correlation coefficients higher than 0.9999 and y-intercepts not significantly different from zero for all methylxanthines.

Sensitivity

The detection limit of the assay method depends on the sample size and injection volume of the reconstituted sample solution. Based on the procedure described here,

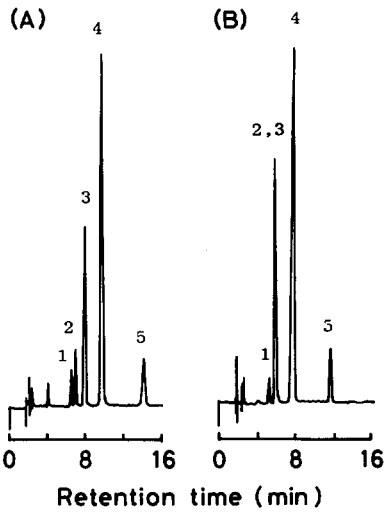

Figure 1
Chromatograms of methylxanthines spiked to blank serum analysed by M-HPLC (A) and C-HPLC (B). Peaks: 1, unknown substance; 2, paraxanthine; 3, theophylline; 4, internal standard; 5, caffeine.

detection limits defined as three times the level of baseline noise were 0.2 μg ml^{-1} for theophylline and paraxanthine, and 0.5 μg ml^{-1} for caffeine.

Accuracy and precision

Table 1 shows the within-day and between-day variabilities of the present method for theophylline analysis. Coefficients of variation and analytical recoveries in both variability studies were <3% and between 97.6–100.2%, respectively. Similar results were also obtained for caffeine and paraxanthine as shown in Table 2.

Correlation of assay results between M-HPLC and C-HPLC

Theophylline in serum samples, obtained from healthy volunteers and patients, was determined by M-HPLC and C-HPLC. Figure 2 shows the correlation of results using the two methods.

For healthy volunteers, a good correlation — correlation coefficient of 0.9982 with a slope of 1.0028 ± 0.0064 (estimated value ±SD), a *y*-intercept of 0.0418 ± 0.0453 and a

Table 1
Precision and reproducibility for theophylline measurement using the proposed method (M-HPLC)

	Added concentration (μg ml^{-1})			
	5	10	15	20
Within-day (n = 10)				
Mean	5.00	10.02	ND*	19.53
SD	0.09	0.12		0.50
CV (%)	1.74	1.19		2.56
Between-day†				
n	61	16	51	15
Mean	4.95	9.85	14.97	19.86
SD	0.13	0.29	0.37	0.47
CV (%)	2.63	2.93	2.50	2.38

*Not determined.
†Performed for an 8-month period.

Table 2
Precision and reproducibility for caffeine and paraxanthine measurements using the proposed method (M-HPLC)

	Added concentration (μg ml^{-1})			
	Caffeine		Paraxanthine	
	1	10	1	10
Within-day (n = 10)				
Mean	1.00	9.95	1.01	10.04
SD	0.02	0.20	0.02	0.23
CV (%)	1.56	1.99	2.18	2.28
Between-day (n = 10)*				
Mean	1.00	9.98	1.01	10.16
SD	0.03	0.24	0.03	0.29
CV (%)	2.95	2.36	2.89	2.84

*Performed once a week for a 2-month period.

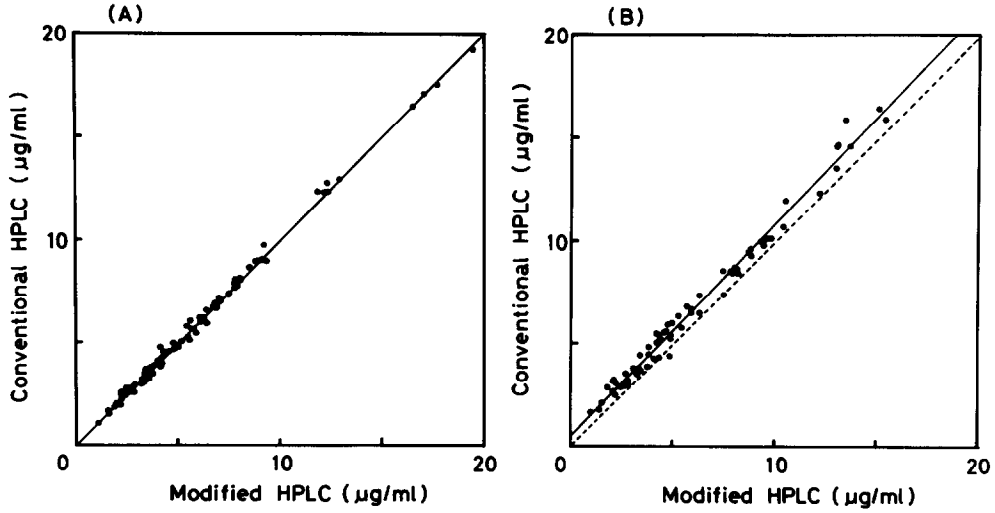

Figure 2
Correlation of serum theophylline concentrations measured by M-HPLC and C-HPLC. (A) Samples obtained from healthy volunteers abstaining from methylxanthine-containing foods and beverages. Regression equation: $y = 1.0028x + 0.0418$ (n = 92, r = 0.9982, $P < 0.001$, Sy/x = 0.224). (B) Samples obtained from patients with COPD consuming normal food and beverage. Regression equation: $y = 1.0670x + 0.4836$ (n = 76, r = 0.9897, $P < 0.01$, Sy/x = 0.602). The dotted line represents the line of unity.

standard error of estimate (Sy/x) of 0.224, was obtained [Fig. 2(A)]. There were no statistically significant differences in the slope and the y-intercept against unity and zero, respectively. Either no peaks or only slight peaks for caffeine and paraxanthine were observed on chromatograms obtained by M-HPLC. With patient samples, a good linear correlation was also obtained between the assay results from both HPLC methods as shown in Fig. 2(B). However, the slope (1.0670 ± 0.0180) and the y-intercept (0.4836 ± 0.1397) of the regression line were significantly different from unity and zero, respectively. Paraxanthine was detected in 65 of 76 samples obtained from patients, with an average concentration of 1.46 μg ml^{-1} and a range of 0.38–2.69 μg ml^{-1} when

determined by M-HPLC. These results indicate that C-HPLC gives higher concentrations of theophylline in serum than M-HPLC due to the poor resolution of theophylline from paraxanthine.

Typical interference from paraxanthine

A female patient, 80 years old, with bronchial asthma, received an intravenous infusion of aminophylline (200 mg as theophylline) for 2 h. This was followed by serial blood sampling in order to determine the pharmacokinetic parameters of theophylline for the construction of an optimum dosage regimen. Serum specimens were assayed by both C-HPLC and M-HPLC. Figure 3 shows the time courses of serum concentrations for each methylxanthine. The concentrations of theophylline obtained by C-HPLC were about 3–4 $\mu g\ ml^{-1}$ higher than those determined by M-HPLC. The patient had also received caffeine-containing medication, because she was suffering from a cold for several days, prior to and during the aminophylline infusion. This was the reason for the high concentrations of caffeine and paraxanthine, and also for the higher theophylline concentration with C-HPLC than with M-HPLC. If the daily dose of theophylline to maintain the 15 $\mu g\ ml^{-1}$ average concentration is calculated on the basis of these results, C-HPLC gives 103 mg day^{-1} in contrast to 213 mg day^{-1} in the case of M-HPLC. This clinical case strongly supports the significance of the need for very high specificity in assay methodology used for the therapeutic monitoring of theophylline.

Other possible interferences with M-HPLC

Various drugs commonly administered with theophylline were tested to examine interference. Each compound listed in Table 3 was spiked to blank serum at a concentration of 100 $\mu g\ ml^{-1}$ and analysed by M-HPLC. None of these compounds interfered with the assay for theophylline. Previously reported reversed-phase HPLC methods for theophylline have been known to suffer from interference from several drugs, including acetazolamide [33], ampicillin [34], cephalothin [35], cefazolin [35, 36], cefotaxime [37], and sulphamethoxazole [38]. With M-HPLC, acetazolamide was eluted immediately after the internal standard. Retention times of cefazolin and cefotaxime

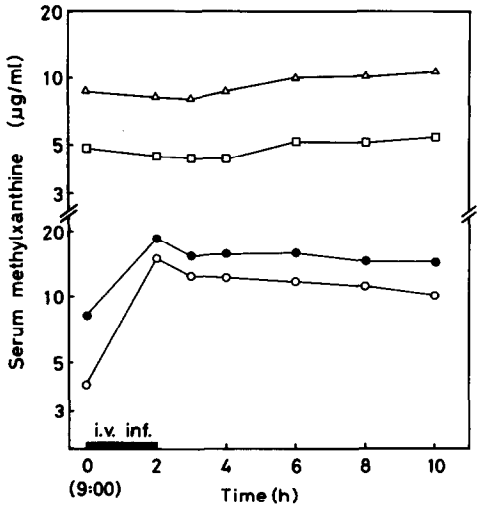

Figure 3
Serum concentration profiles of methylxanthines in an asthmatic patient after intravenous infusion of aminophylline (200 mg as theophylline). Key: ●, theophylline by C-HPLC; ○, theophylline by M-HPLC; □, paraxanthine by M-HPLC; △, caffeine by M-HPLC.

Table 3
Interference-free compounds in serum theophylline assay using the proposed method (M-HPLC)

Acetaminophen	1,3-Dimethyluric acid
Acetazolamide	Hypoxanthine
Ampicillin	Ketotifen
Caffeine	Latamoxef (Moxalactam)
Carbenicillin	1-Methyluric acid
Cefamandole	1-Methylxanthine
Cefazolin	3-Methylxanthine
Cefmenoxime	Paraxanthine
Cefmetazole	Phenacetan
Cefoperazone	Piperacillin
Cefotaxime	Salicylamide
Cefotetan	Salicylic acid
Cefotiam	Sulbenicillin
Cefoxitin	Sulphamethoxazole
Cefsulodine	Theobromine
Ceftizoxime	Tranilast
Cephalothin	Uric acid
8-Chlorotheophylline	Xanthine

were 25.0 and 18.2 min, respectively. Peaks corresponding to ampicillin, cephalothin and sulphametoxazole were not observed.

Discussion

In earlier HPLC methods, a methanol–tetrahydrofuran–acetate buffer system was used as the mobile phase for the separation of theophylline from paraxanthine [30, 31]. However, there were some disadvantages to these methods including insufficient baseline resolution and the possibility of interference from ampicillin [30], much longer retention time and higher pressure on the 5-μm reversed-phase column [31]. While these disadvantages were reduced by the use of a radial compression module with a reversed-phase cartridge [31], the high initial equipment cost and still longer analysis time of 12 min for theophylline may be the limitations for clinical application. Indeed, the preliminary examination indicated that complete resolution of these methylxanthines could not be obtained using this mobile phase system on the 5-μm reversed-phase column used in the present study. Therefore, acetonitrile, in place of methanol, was used as a component of the mobile phase because of its lower polarity and viscosity resulting in higher elution efficiency and lower column pressure compared with using methanol. An optimal mixing ratio of the mobile phase components was determined to provide better resolution and shorter analysis time. As a result, use of the proposed method could overcome the disadvantages of previous methods [30, 31]. The solvent selectivity of the mobile phase system containing acetonitrile rather than methanol must have been responsible for the high resolution of theophylline and paraxanthine.

In another method reported previously [32], a mixture of methanol–acetonitrile–tetrahydrofuran–acetate buffer had been used as the mobile phase to obtain optimal resolution of caffeine and its N-demethylated metabolites, i.e. theobromine, paraxanthine and theophylline. Although the system containing these three organic solvents with different polarities may be effective for the separation of xanthine analogues, no information was provided about interferences from co-administered drugs in clinical

practice because of its application only in animal studies. Despite the lack of methanol in the mobile phase, the present method is comparable to this previous method in terms of peak resolution and analysis time. Moreover, various drugs commonly co-administered with theophylline did not interfere with the determination of theophylline using the proposed method (Table 3). The elimination of such interference may be partly attributed to the extraction procedure used in the pretreatment of serum samples, and the use of acetonitrile and tetrahydrofuran in the mobile phase which allows for better resolution of theophylline from co-extracted compounds.

Interference from paraxanthine is a serious concern for the therapeutic monitoring of theophylline in patients who have difficulty in abstaining from caffeine-containing foods and beverages, and especially for those who have received caffeine-containing medication as shown in Fig. 3. The amount of paraxanthine in serum or plasma may vary with the kind and the amount of food or beverage. For example, the average plasma concentration of paraxanthine was reported to be 3.0 $\mu g\ ml^{-1}$ in healthy volunteers drinking three to four cups of coffee per day [28]. This value is two times higher than the value of 1.46 $\mu g\ ml^{-1}$ obtained in the present study.

In conclusion, the proposed method described here is useful for pharmacokinetic studies and therapeutic monitoring, due to its high specificity for theophylline, excellent sensitivity and reproducibility.

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