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An isocratic high-performance liquid chromatographic system for simultaneous determination of theophylline and its major metabolites in human urine

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Abstract

A simple and highly selective isocratic high-performance liquid chromatography method is presented for the simultaneous determination of theophylline and its major metabolites in human urine using β-hydroxyethyl theophylline as an internal standard. The method utilizes direct injection of diluted urine samples followed by separation and quantitation by reversed-phase isocratic elution and ultraviolet detection. The assay is accurate and reproducible with a sensitivity of 1 μg ml^{-1} for theophylline and 0.5 μg ml^{-1} for its metabolites. The assay was employed for the analysis of theophylline and its major metabolites in urine following the oral administration of theophylline to four healthy volunteers.

Keywords: Theophylline major metabolites; 3-Methylxanthine; 1-Methyluric acid; 1,3-Dimethyluric acid; Urine; Reversed-phase ion-pair chromatography

1. Introduction

Theophylline is a widely used bronchodilating agent in the treatment of chronic obstructive pulmonary diseases. In humans, about 10% of the drug is eliminated unchanged by renal excretion whilst the remainder is extensively metabolized to 3-methylxanthine (3-MX), 1-methyluric acid (1-MU) and 1,3-dimethyluric acid (1,3-DMU) [1-3]. These metabolites arise from the 8-oxidation (1,3-DMU), 1-demethylation (3-MX), and consecutive 3-demethylation and oxidation (1-MU) of theophylline [4]. In addition, 7-N-methylation of theophylline, giving rise to caffeine, occurs in neonates [5,6]. Conversion of theophylline to caffeine has also been claimed to occur in adults following multiple dose administration of theophylline [7].

There is great inter-individual variation in both the pharmacokinetic profile and clinical responses to theophylline in human populations. The pharmacokinetic variation arises from inter-subject differences in metabolism [8], and hence it is important to differentiate between the factors responsible for such variations. Therefore, a simple, sensitive and reproducible simultaneous assay method for theophylline and its metabolites is important.

Although in recent years several high-performance liquid chromatography (HPLC) methods have been developed to quantitate theophylline and its metabolites in urine and serum/plasma, but many of them suffer from having time consuming sample preparation, long chromatogram run time and/or poor resolution [1,3,9-20].

The present study describes an isocratic HPLC method for the simultaneous determina-

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tion of theophylline and its major metabolites, which is used in the evaluation of the urinary excretion of theophylline and its metabolites following oral single dose administration to normal subjects.

2. Experimental

2.1. Materials

Theophylline (1,3-dimethylxanthine, 1,3-DMX), caffeine (1,3,7-trimethylxanthine, 1,3,7-TMX), β-hydroxyethyl theophylline (BHET) and tetrabutylammonium hydrogen sulphate (TBA) were obtained from Sigma (Poole, UK). 3-methylxanthine (3-MX), 1-methyluric acid (1-MU) and 1,3-dimethyluric acid (1,3-DMU) were purchased from Fluka (Buchs, Switzerland). HPLC grade tetrahydrofuran, acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Sodium acetate trihydrate and acetic acid were all analytical grade from BDH (Poole, UK).

Xanthine-free urine was collected from a normal volunteer who had abstained from all methylxanthine-containing foods and beverages for 48 h.

2.2. Instruments

The HPLC system consisted of a model 600E Waters solvent delivery system and a model 486 Waters variable wavelength ultraviolet (UV) detector (Waters Association, Milford, MA, USA). A reversed-phase column (5 μm, Ultrasphere-ODS, 15 cm × 4.6 mm i.d.) (Altex, Berkeley, CA, USA) and a precolumn (4.0 cm × 2.5 mm i.d.) of Lichrosorb RP-2 (10 μm) were used. Injections were made by means of a Waters Model U6K injector. Data analysis was performed with a Waters 746 computing integrator.

2.3. Chromatographic conditions

The mobile phase consisted of sodium acetate (10 mM), acetonitrile and tetrahydrofuran (96.9:3:0.1, v/v/v) containing tetrabutylammonium hydrogensulphate (5 mM) (pH* 4.7). All separations were carried out at room temperature and a flow-rate of 1.5 ml min^{-1}, detector sensitivity of 0.03 absorbance units and a chart speed of 0.25 cm min^{-1}. Eluted peaks were detected at 280 nm.

2.4. Standard preparation

Xanthine-free urine samples were loaded with theophylline, caffeine, 3-MX and 1-MU (1–50 mg l^{-1}) and 1,3-DMU (5–100 mg l^{-1}) and stored at −20°C until analysis.

2.5. Sample preparation

Urine samples were diluted 1:10 with an aqueous solution of the internal standard, β-hydroxyethyl theophylline, 5 mg l^{-1}, (I.S. was used in order to minimize the possible injected volume variation). After mixing and centrifugation of the sample at 14 000 rpm for 2 min, 25 μl of the supernatant was injected into the column without further treatment. A 10 min lagtime between injections was used to equilibrate the column. The concentration of theophylline and its metabolites were determined using the peak-height ratios of compounds to the internal standard.

2.6. Human studies

Four healthy volunteers (three females and one male) aged between 24 and 33 years participated in the investigation after giving their informed consent and approving the study by the Ethical Committee of the Ministry of Health and Medical Education. After 48 h abstention from methylxanthine-containing foods and beverages, each volunteer received 375 mg of theophylline with 100 ml water after an overnight fast. Urine samples were collected, the volumes and pH values recorded, and aliquots frozen at −20°C until analysis.

3. Results

Fig. 1 is a typical chromatogram of theophylline and its metabolites (1-MU, 3-MX, 1,3-DMU and caffeine) in aqueous solution. The chromatograms of a blank urine and a urine sample taken from a volunteer 4 h after taking a 375 mg dose of theophylline is shown in Fig. 2 (Parts A and B respectively).

The retention times and capacity factors for theophylline and its metabolites are presented in Table 1. Capacity factors for each compound were calculated from the equation $(t_R - t_0)/t_0$, where t_R is the retention time of the compound and t_0 is the retention time of an unretained compound.

3.1. Calibration curves

The linearity of calibration curves was studied at the concentrations of 2–5 mg l^{-1} for 3-MX, 1-MU, theophylline and caffeine, and 5–100 mg l^{-1} for 1,3-DUM. Each standard curve showed good linearity over the range of concentrations examined ($r^2 \geq 0.982$).

3.2. Limit of detection

The limit of detection with a signal-to-noise ratio of 2 was 0.5 mg l^{-1} for 3-MX, 1-MU and 1,3-DMU, and 1 mg l^{-1} for theophylline and caffeine.

3.3. Assay recovery and reproducibility

The assay recoveries of theophylline and its metabolites were studied at concentrations of 2–5 mg l^{-1} which were more than 99% for each compound. The intra-day and inter-day assay relative standard deviations for each of these compounds are summarized in Table 2. For inter-day analysis, samples were analyzed every other day for eleven days. All intra-day relative standard deviations were less than 4.6%, while inter-day values were less than 5.8%.

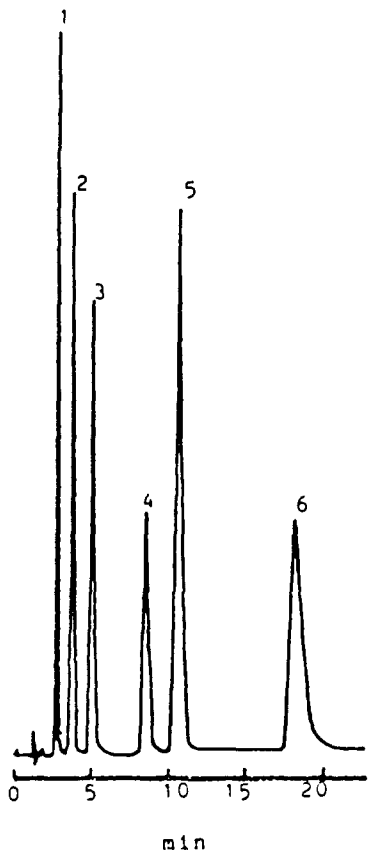

Fig. 1. Chromatogram of theophylline and its metabolites in aqueous mixture. Chromatographic conditions as described in text. 0.03 a.u.f.s. peaks: (1) 3-MX; (2) 1-MU; (3) 1,3-DMU; (4) theophylline; (5) β-hydroxyethyl theophylline (I.S.); (6) caffeine.

3.4. Recovery of theophylline and its metabolites in 0–48 h urine samples

The total recovery of theophylline and its metabolites in 0–48 h urine collected from the volunteers given 375 mg theophylline was found to be 84.5 ± 3.5%. Table 3 shows details of the excretion of theophylline and its metabolites.

4. Discussion

Several HPLC assay methods have been developed for simultaneous quantitation of theophylline and its metabolites in urine [1,9–18], but many of them suffer either from time consuming sample preparation or lack of sufficient specificity and sensitivity. The method proposed by Thompson et al. [1] in 1974 is time consuming, since methylxanthine and methyluric acid fractions must be separated before injection on to the HPLC column. Similarly, Grygiel et al. [11] described an assay which requires two separate HPLC runs for theophylline and its metabolites. In addition, this method is unsatisfactory owing to co-elution and carry-over with compounds having long retention times. Aldridge et al. [12] proposed a gradient elution system following organic extraction of urine samples using chloroform–isopropanol (85:15); however, the extraction efficiency of methyluric acids was low, especially that of 1-MU which was only 36%. Muir et al. [13] developed a method which requires an ion-pair gradient elution, and is therefore both lengthy and complicated. The method published by Tang-Liu and Riegelman [14] describes the most sensitive and selective assay to date, but requires a pump that can automatically vary its rate of delivery of an individual solvent. Most of these methods use a gradient elution system, but very few isocratic assays for quantitation of theophylline and its metabolites have been reported [17,18]. The latter isocratic methods involve extraction which is time consuming and can result in

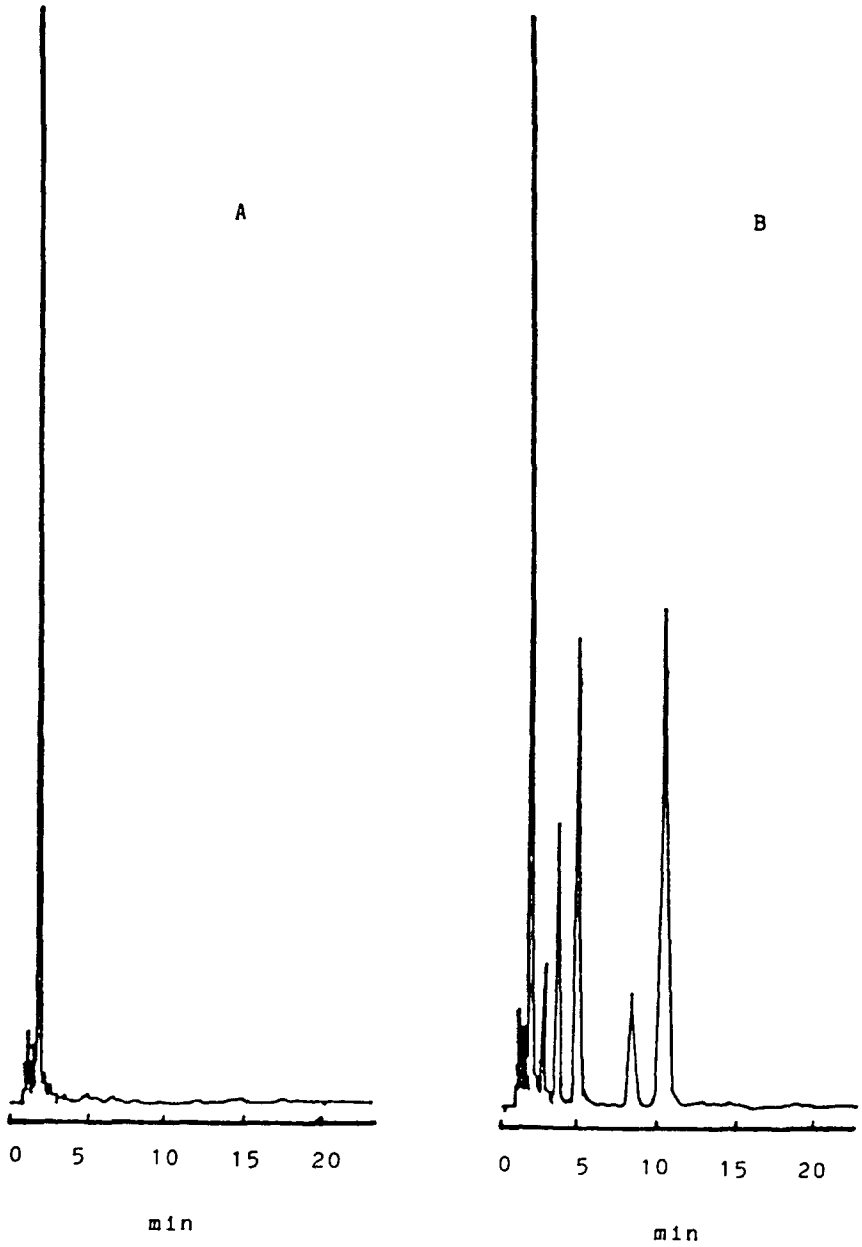

Fig. 2. Chromatograms of: (A) a xanthine-free urine sample from a subject having abstained from all xanthine-containing foods and beverages for 48 h and (B) a urine sample collected 4 h following oral ingestion of 375 mg theophylline. Chromatographic conditions as described in text; 0.03 a.u.f.s. peak identification as in Fig. 1.

differential loss of the compounds of interest. Because of the wide range of polarities of the various methylxanthines and uric acids, it is difficult to obtain satisfactory recoveries of all compounds by the same method. However, the method proposed by Kester et al. [17] requires a constant temperature of 55°C to be maintained with a column oven during the run time. Owing to the large difference in physicochemical properties of the methylxantines and the methyluric acids, simultaneous quantitation of theophylline and its metabolites under isocratic conditions without using an ion pair was rather impossible. In the absence of the counter ion, 1-MU was not retained or separated in the isocratic method, and when the concentration of acetonitrile in the mobile phase was reduced to overcome this problem, there was an unacceptable increase in the retention times for theophylline and caffeine.

Addition of the TBA counter ion to the mobile phase enabled adequate retention and separation of the early eluting compounds without an unacceptable increase in the reten-

Table 1
Chromatographic retention data of theophylline and its metabolites

Compound	Retention time (min)	Capacity factor (k')
3-MX	2.7	0.68
1-MU	3.7	1.31
1,3-DMU	4.9	2.06
Theophylline	8.5	4.31
BHET [a]	10.07	5.68
Caffeine	20.8	12.0

[a] β-Hydroxyethyl theophylline.

tion time for theophylline and caffeine. The reason for the latter is that the approach of theophylline and caffeine (nonpolar methylxanthine derivatives) to the nonpolar sites in the stationary phase is restricted.

The effect of different concentrations of TBA (0.5–10 mM) on retention time was studied. The lower concentrations of TBA caused a small reduction in the retention time of 1-MU whilst a marked increase in the retention time of theophylline and caffeine was observed. Therefore, a concentration of 5 mM of TBA was used throughout the study as an optimum concentration.

The effect of mobile phase pH* on retention times was also studied. A pH* of 4.7 was found to result in optimum resolution, which was also in agreement with that reported by Muir et al. [13]. It was suggested that lowering the pH* to 4.5 results in a marked reduction in the retention time and resolution of 1-MU and 1,3-DMU by virtue of reducing the fraction of these molecules in the ionic state, capable of forming ion pairs. Increasing the pH* to 5.0 resulted in the opposite effect.

Table 3
Urinary recovery of theophylline and its metabolites [a] (mean ± SD; $n = 4$)

Compound	% Recovered
Theophylline	13.4 ± 1.5
1-MU	17.8 ± 1.9
3-MX	12.0 ± 1.3
1,3-DMU	41.3 ± 3.2
Total	84.5 ± 3.5

[a] Values represent the amount excreted in 48 h as percentage of theophylline administered dose.

5. Conclusions

An isocratic HPLC assay has been developed for simultaneous quantitation of theophylline and its major metabolites in urine, which utilizes a short and simple sample preparation and shorter chromatogram run time with adequate sensitivity and reproducibility required for pharmacokinetics studies. This method was used satisfactorily for the determination of the pharmacokinetics parameters of theophylline and its major metabolites in our laboratory. In the work presented here, the recovery of theophylline and its major metabolites in 48 h urine collected after oral administration of the drug was similar to the results reported in the literature [3,16,20–22].

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Table 2
Intra-day and inter-day reproducibility of the assay ($n = 6$ at each concentration)

Concentration (mg l^{-1})	Relative standard deviation (%)							
	3-MX		1-MU		1,3-DMU		Theophylline	
	a	b	a	b	a	b	a	b
5	4.0	5.5	3.9	4.8	4.3	5.3	3.8	4.7
10	3.8	4.1	3.0	4.3	3.9	5.7	4.0	5.1
25	2.8	3.9	3.8	4	4.6	5.8	4.1	4.6
50	2.4	2.8	4.3	4.0	3.5	4.7	3.4	3.8
100	2.4	3	4.4	4.5	2.7	4.9	3.7	3.8

[a] Intra-day relative standard deviation.
[b] Inter-day relative standard deviation.

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