Medical Journal of the
Islamic Republic of Iran

Volume 13
Number 3
Fall 1378
November 1999

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF THEOPHYLLINE IN HUMAN SERUM

H. TAJERZADEH* AND S. SADRAY

From the Biopharmacy Division, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, I.R. Iran, 14155/6451.

ABSTRACT

A simple, rapid and sensitive HPLC method for the determination of theophylline (T) in human serum has been developed. An isocratic system consisting of a μ Bondapak C_{18} column, mobile phase of methanol, phosphate buffer (22:78, pH=4.5), and a flow rate of 1.4 mL/min was used. The eluent was detected by UV at 275 nm at room temperature. 8-Chlorotheophylline (8-CT) was used as an internal standard. The serum samples deproteinated by methanol containing 8-CT and the supernatant was injected into the HPLC system. The retention times of 5.7 and 8.1 min were found for T and 8-CT, respectively. The linearity was checked in the range of 0.2-30 μg/mL. Relative standard deviation for both inter-day and intra-day precision analysis was less than 5%. No interference was observed from endogenous serum components. Specificity was shown against some commonly co-administered drugs. Simple and fast sample preparation, small sample volume (250 μL), precision, reproducibility, specificity, sensitivity and high percentage recovery (98%) make the method to be practically useful for T monitoring in asthmatic patients.

MJIRI, Vol. 13, No. 3, 191-194, 1999

Keywords: Theophylline; Reversed-phasechromatography; 8-Chlorotheophylline; Therapeutic drug monitoring.

INTRODUCTION

Theophylline is widely accepted as an effective bronchodilator in the treatment of asthma, apnea, and obstructive lung diseases.[1,2] In general, the disparity between the concentration-effect relationship of beneficial and adverse effects provides partial justification for monitoring serum theophylline concentrations.[3] Although in recent years several HPLC methods have been developed to quantitate T in biological fluids,[4-13] many of them either suffer from having time consuming sample preparations or long chromatogram run times, both of which are not adequate for TDM. The present study describes a simple, rapid and sensitive isocratic HPLC method for the determination of T in human serum without solvent extraction. Experimental conditions, linearity, sensitivity, recovery, reproducibility and drug interference studies are discussed.

* **Corresponding author:** Tel: 021 6112325, Fax: 6419539.

MATERIAL AND METHODS

Materials

Theophylline (1,3-dimethylxanthine (T)) and 8-

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Table I. Intra-day and inter-day precision and accuracy of the assay.

Spiked Concentration (μg/mL)	Inter-day precision			Intra-day precision		
	Mean ±SD*	RSD (%)	Accuracy (%)	Mean ±SD*	RSD (%)	Accuracy (%)
0.2	0.22 ±0.02	9.1	110.0	0.21 ±0.02	9.5	105.0
1.0	1.03 ±0.08	7.8	103.0	1.07 ±0.05	4.7	107.0
5.0	4.97 ±0.11	2.2	99.4	4.89 ±0.19	3.9	97.8
10.0	9.89 ±0.14	1.4	98.9	9.85 ±0.43	4.3	98.5
20.0	19.88 ±0.33	1.7	99.4	19.90 ±0.55	2.8	99.5
	Mean	4.4	102.1	Mean	5.0	101.5

* Mean ±SD of determinations.

chlorotheophylline (8-CT) were obtained from Sigma (Pool, UK). HPLC grade methanol was obtained from Merck (Darmstadth, Germany). All other reagents were of analytical grade.

Instruments

The HPLC system consisted of a model 600E Waters solvent delivery system and a model 486 Waters variable wavelength (UV) detector (Waters Associates, Milford, MA, USA), a reverse phase column (10 μm, μBondapak C_{18}, 30 cm × 4.6 mm i.d.) and a C18 Guard pak (Millipore, Waters Associates, Milford, MA, USA). Injections were made by means of a Waters Model U6K injector. Data analysis was performed with a Waters 746 computing integrator.

Chromatographic conditions

The mobile phase consisted of sodium and potassium dihydrogen phosphate, 15 mM of each (2:1), and methanol (78:22 v/v, pH = 4.5). Flow rate was 1.4 mL/min and analysis was carried out at room temperature. The elutant was detected at 275 nm at a sensitivity of 0.01 absorbance unit full scale (a. u. f. s.) and chart speed was set at 0.25 cm/min.

Sample preparation

To 250 μL of either serum from a subject or spiked serum, 0.5 mL of methanol containing 5 μg/mL 8-CT was added. The mixture was vortexed for 15 sec and then centrifuged for 7 min at 10000 rpm. The aliquot of 25 μL of the filtered supernatant was injected into the HPLC system.

Standard curves

A working solution of 200 μg/mL of theophylline in water was prepared using a stock solution of 5 mg/mL in methanol. 4 mL of drug-free serum was spiked with 1 mL of working solution to obtain a solution of 40 μg/mL, then serially diluted with serum to yield concentrations of 0.2, 0.8, 1, 2, 5, 10, 20 and 30 μg/mL. The standard curve was

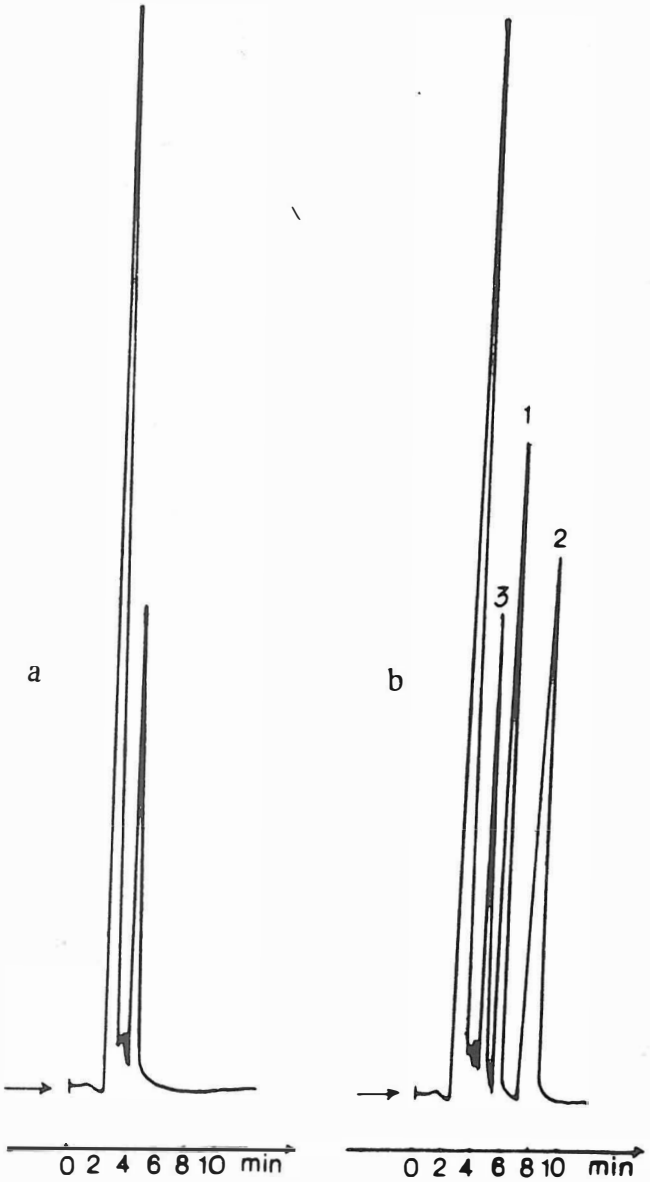

Fig. 1. Chromatograms: a: blank serum b: patient serum (1) theophylline; (2) 8-chlorotheophylline; (3) endogenous substance.

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Table II. Drugs tested for potential interference in chromatography.

Drug	Retention time (min)	Drug	Retention time (min)
Analytes		**Interfering**	
Theophylline	5.7	Ampicillin	8.4
8-Chlorotheophylline	8.1	Cephalexin	7.7
		Erythromycin	8.9
		Dextromethorphan	8.8
Not interfering*		Procainamide	10.3
Acetaminophen	2.9	Propranolol	14.2
Aspirin	3.4	Ranitidine	13.5
Amikacin	*	Salbutamol	12.9
Beclomethasone	*	Sulphamethoxazole	12.2
Betamethasone	22.0	Theobromine	4.8
Bromhexin	*	Trimethoprim	10.4
Caffeine	9.5	Verapamil	14.6
Captopril	14.9		
Chloramphenicol	14.4		
Cimetidine	12.1		
Furosemide	17.5		
Hydrocortisone	16.0		
3-Methylxanthine	2.1		
Nifedipine	13.2		
Nitroglycerine	*		
Phenytoin	15.4		
Phenobarbital	15.6		
Prednisolone	17.2		

* not eluted from the column within 15 min.

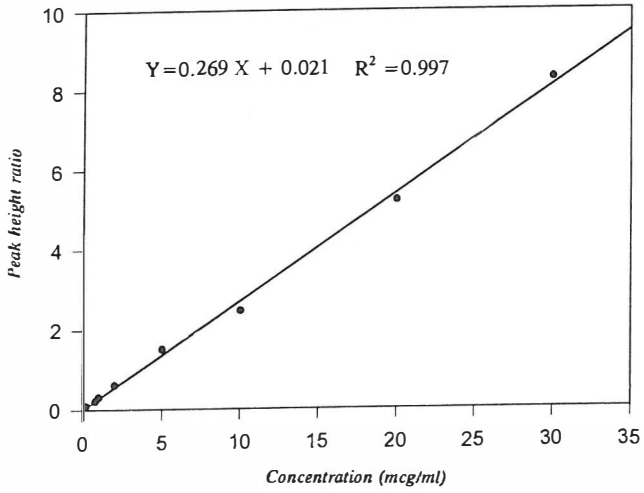

Fig. 2. Standard curve for theophylline in human serum.

constructed using peak height ratio of T to the internal standard against concentration.

Recovery

The percent recovery was calculated using peak height ratio of T extracted from serum with that of the aqueous standards.

Precision

The intra-day and inter-day precision was determined using triplicate analysis of spiked serum samples at concentrations of 0.2, 1, 5, 10 and 20 µg/mL.

Specificity

Serum obtained from different subjects (n=5) was analyzed using the described procedure. The possible interferences from normal serum constituents were examined by inspecting the chromatograms. A number of drugs were tested for their potential interferences in the chromatographic elution by injecting aliquots of stock solutions of these compounds.

RESULTS AND DISCUSSION

A number of HPLC methods reported in the literature [4-13] were evaluated for the analysis of T in serum, but none provided satisfactory results in terms of sensitivity and simplicity. This necessitated the development of an improved, sensitive plasma analytical HPLC method which would be simple, rapid and reproducible. The optimum solvent mixture, composed of methanol: sodium dihydrogen phosphate buffer (22: 78 v/v, pH=4.5), provided proper baseline separation, and no significant alterations in peak

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shape and retention times were observed with a column having been used continuously for at least one month (n>300). Figure 1 shows the chromatograms of T and 8-CT in a sample and blank serum. The linearity of each concentration relative to the internal standard in human serum is shown in Fig. 2. The linearity of the assay was demonstrated by multiple analysis of the standard curves in serum (n=3), indicating good linearity and reproducibility at the range of 0.2-30 µg/mL at R^2=0.997 (Fig. 2). The intercepts were not significantly different from zero, indicating negligible interference.

A variety of serum protein precipitants, namely 67% perchloric acid and K_2CO_3 as neutralizing agent, saturated HCl with ammonium sulphate, ethyl acetate and acetonitrile were evaluated; methanol appeared to be the most appropriate. The assay recovery of more than 98% was obtained using concentrations of 0.2-20 µg/mL. The inter-day and intra-day precision and accuracy of the method at five different concentrations of T in serum are summarized in Table I. The low relative standard deviation (RSD) which ranged from 1.4% to 9.1% demonstrated the good precision of the method. Both the RSDs (below 15%) and the accuracy (±15%) of the method were within acceptable limits as recommended recently for analytical method validation.[14]

The limit of quantification, the lowest concentration on the standard curve with acceptable precision (RSD <20%), was found to be 0.2 µg/mL and the limit of detection, the lowest concentration of theophylline that can be reliably differentiated from background levels, was found to be 2 ng/mL.

The specificity of the assay in serum was established by analyzing blank serum samples of different subjects (n=5). There were no endogenous peaks that co-eluted with the T or 8-CT peaks. Assay interference from other commonly used drugs was evaluated by injecting methanolic solutions of the drugs (5 µg/mL) into the chromatographic system and recording their retention times. Drugs were considered non-interfering if their retention times were significantly different from those of T and 8-CT. Table II lists the drugs that were tested for their potential interferences and their retention times.

This method has been applied successfully for the determination of T in serum samples in patients in a study of population pharmacokinetics of T (Masih Daneshvari Hospital, Tehran, Iran) which will be published elsewhere.

In conclusion, although a number of HPLC methods have been published for the determination of T in serum, but either due to long sample preparation or time consuming chromatographic run times and/or specific system requirements, they were not adequate for clinical application. Therefore, the present method, with simple sample preparation and short chromatographic run times (less than one hour for five sample quantitation), high accuracy, sensitivity and recovery is suitable for routine clinical monitoring of serum levels in patients and pharmacokinetic studies.

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