Development and Validation of a Reversed-Phase Liquid Chromatographic Method for Analysis of Theophylline in Human Plasma

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1 Introduction

Theophylline is a powerful smooth muscle relaxant frequently used for acute and chronic bronchial asthma and apnea in premature infants [1]. Therapeutic concentrations of theophylline are in the range of 10–20 µg/mL in the plasma of adults [2–4], and toxic signs appear at concentrations higher than 20 µg/mL [5–7].

Numerous analytical methods have been reported for the analysis of theophylline in biological fluids [8–12]. Gas chromatographic procedures involve either multiple solvent extractions or chemical derivatization. High-performance liquid chromatography (HPLC) seems to be the most popular method for the determination of theophylline in biological samples. Gradient elution can resolve many interferences but, in general, it is more time-consuming than isocratic methods.

In the present study, we demonstrated and validated a sensitive and selective HPLC method for the quantitative determination of theophylline in human plasma using an isocratic reverse-phase system for the separation, with a small sample volume and simple sample preparation procedure.

2 Materials and Methods

2.1 Chemicals and Reagents

Theophylline anhydrous (analytical grade), acetonitrile (HPLC grade) and trichloroacetic acid were obtained from BDH (Poole, UK). Acetaminophen (internal standard) was obtained from SIGMA (Poole, UK). Demineralized and double-distilled water was prepared in our laboratory.

2.2 Apparatus and HPLC-conditions

The chromatographic system consisted of a Model 2150 HPLC pump (Pharmacia, Uppsala, Sweden), Model PU4020 variable wavelength UV detector (PYE UNICAM, Cambridge, England) to detect absorbance at 280 nm and Model 7125 injector (Rheodyne, Cotati, CA) with a 20 µL loop. A stainless-steel Hibar column (125 × 4 mm i.d.) prepacked with 5-µm LiChrospher 100 RP-18 (Merck, Darmstadt, Germany) was used. The mobile phase consisted of acetonitrile/acidified distilled water (pH 4) in the 10/90 (*v/v*) ratio. The flow rate was 1 mL/min. Peak-areas were integrated by a Hewlett-Packard Model 3390A integrator (Littlefalls, DE). All analyses were performed at room temperature.

2.3 Sample Preparation

180 µL blank plasma was taken into a microcentrifuge Eppendorf tube and 10 µL of the standard solutions of theophylline and 10 µL of the internal standard solution were added obtaining equivalent plasma concentrations of 0.4, 1, 4, 8, 10, 14, 16, and 20 µg/mL theophylline and 12 µg/mL internal standard. Plasma proteins were precipitated by the addition of 200 µL of 10% trichloroacetic acid solution. The mixture was shaken for 30 s (Vortex, Heidolph REAX 2000). The samples were centrifuged at 3 000 *g* for 10 min in a microfuge (Sigma 112). The supernatant was transferred to a microcentrifuge Eppendorf tube and 50 µL was injected directly into the chromatographic system.

2.4 Validation of Analytical Method

2.4.1 Linearity

A standard curve were generated by spiking a series of drug-free plasma samples with theophylline to produce concentration ranges of 0.4–20 µg/mL. A calibration curve was constructed by plotting the ratios of peak areas against concentration and analyzing by linear regression analysis.

2.4.2 Accuracy and Precision

The accuracy (intra-day variation) was determined by analyzing 3 parallels at concentration of 3, 12, and 18 µg/mL. Inter-day variation was determined at each concentration on 3 different days.

2.4.3 Extraction Efficiency

The recovery of theophylline was determined at concentration of 3, 12, and 18 µg/mL. Three replicates of each standard were extracted by the above mentioned sample preparation and injected into the HPLC system. Three replicates of each standard prepared in aqueous solutions were directly injected. The extraction recovery at each concentration was calculated using the following equation:

$$\text{Recovery} = \frac{\text{peak} - \text{area after extraction}}{\text{peak} - \text{area after direct injection}} \times 100$$
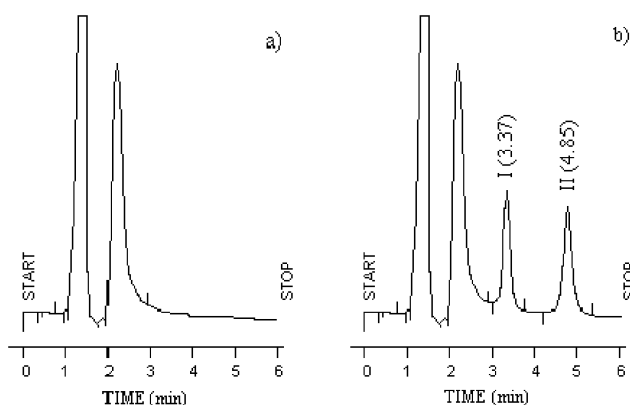
Figure 1. Typical chromatograms for theophylline in human plasma: a) Blank. b) Spiked plasma containing internal standard (I) and theophylline (II).

2.4.4 Stability

In order to assess the stability of theophylline, fresh human samples spiked with a known amount of theophylline were stored at $-20\,°C$ and assayed weekly for a month.

3 Results and Discussion

The resulting chromatograms were essentially free from endogenous interferences because the human plasma was derived from volunteers who had abstained from xanthine-containing foods and beverages for at least 48 h (**Figure 1**). The retention times of internal standard and theophylline were 3.37 min and 4.85 min respectively.

The linearity of the calibration curve was determined by plotting the drug concentration against ratio of drug/internal standard peak area. The regression equation for the calibration curve was $y = 0.12x + 0.00$ and the correlation coefficient (R^2) was calculated to be 1.0. The limit of quantitation using the prescribed experimental conditions was found to be 0.4 µg/mL.

The precision of the assay procedure described for human plasma was assessed by calculating the intra- and inter-day variation for each concentration (Table 1). All intra-and-inter-day coefficients of variation (C.V.) were less than 15% [13]. The

Table 1. Intra- and inter-day variation of theophylline in the analysis of human plasma.

Conc. Added (µg/mL)	Intra-day variation		Inter-day variation	
	Conc. found (µg/mL) (mean ± S.D.)	C.V %	Conc. found (µg/mL) (mean ± S.D.)	C.V %
3.0	3.00 ± 0.03	0.91	2.91 ± 0.15	5.33
12.0	12.20 ± 0.05	0.43	12.04 ± 0.21	1.39
18.0	17.96 ± 0.44	2.62	16.89 ± 0.24	1.44

Table 2. Extraction recovery of theophylline.

Plasma concentration of theophylline (µg/mL)	Recovery of extraction (mean ± S.D., $n = 3$) (%)
3.0	93.38 ± 4.47
12.0	92.77 ± 1.97
18.0	94.17 ± 4.13
Mean	S.D.
93.31	0.70

regression equation for the relationship between concentration found and concentration added was $y = 0.95x + 0.23$ and the correlation coefficient (R^2) was calculated to be 0.99. The extraction recoveries of theophylline are presented in **Table 2**. The assay recovery range was found between 92.77–94.18% (mean 93.31%). The stability of theophylline in plasma at $-20\,°C$ was good. For up to a month no significant reduction of the plasma concentration was observed by two-way analyses of variance (ANOVA) ($p < 0.05$). Besides, the coefficients of variation were less than 3% over a period of 30 days.

A fast and simple HPLC method for the determination of theophylline in plasma is presented. The method's linearity, precision, accuracy, and recovery are excellent. The small sample volume (200 µL) and quantitation limit of 0.4 µg/mL makes this assay a suitable choice for pharmacokinetic, bioequivalence studies and therapeutic drug monitoring.

References

[1] S.L. Goodman, A. Gilman, *The Pharmacological Basis of Therapeutics*, Pergamon Press, New York 1985, p. 589.

[2] L. Hendeles, M. Weimberger, G. Johnson, *Clin. Pharmacokin.* **1978**, *3*, 294.

[3] R. Wyatt, M. Weimberger, L. Hendeles, *J. Pediatr.* **1978**, *92*, 125.

[4] R.I. Ogilvie, *Clin. Pharmacokin.* **1978**, *3*, 267.

[5] G. Milavetz, M. Weimberger, L. Hendeles, *Pediatric Pharmacology and Therapeutics* **1986**, *109*, 351–354.

[6] S.L Sheldon, D.W. Suchard, *Ann. Allergy* **1980**, *44*, 48.

[7] J.P. Finnerty, S.T. Holgate, J.R. Vane, G.A. Higgs, S.A. Marisco (Eds), *Asthma: Basic Mechanism and Therapeutic Perspectives*, Pythagora Press, Rome 1989, pp. 193–214.

[8] M.B. Kester, C.L. Saccar, M.L. Rocci, H.C. Mansmann, *J. Chromatogr.* **1988**, *380*, 99.

[9] S.A. Hotchkiss, J. Caldwell, *J. Chromatogr.* **1987**, *423*, 179.

[10] C.J. Newth, *Br. J. Clin. Pract.* **1984**, *38*, 47.

[11] K.T. Muir, J.H.G. Jonkman, D.S. Tang, *et al.*, *J. Chromatogr.* **1980**, *221*, 85.

[12] M.W.F. Teunissen, L.G.J. De Leede, J.K. Boeijinga, *et al.*, *J. Pharmacol. Exp. Ther.* **1985**, *233*, 770.

[13] P.S. Vinod, K.M. Kamal, *et al.*, *Pharmaceutical Res.* **1992**, *9*, 588–592.