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Research Article

A RELIABLE REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR A FREQUENTLY PRESCRIBED BRONCHODILATOR - THEOPHYLLINE

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ABSTRACT

A high performance liquid chromatography method has been developed that allows quantification of concentrations of theophylline in human serum. Separation was achieved by HPLC on a Princeton SPHER C_{18} column with a mobile phase consisted of a mixture of 15 % of acetonitrile and 85 % of 0.5 % triethylamine buffer (pH 3.5) delivered on a isocratic programme. Optimum detection was at 275 nm. The assay was linear over the concentration range of 5.0 – 50.0 ng/ml. Both intraday and inter-day precision data showed reproducibility (R.S.D.≤8.0). Stability studies showed theophylline was stable in serum for up to 6 h after thawing; the samples were also stable for up to 6 h after preparation. This method is proposed as a means of therapeutic drug monitoring of theophylline in patients with chronic obstructive pulmonary diseases (COPD).

Keywords: Theophylline; Serum; HPLC-UV Detection.

INTRODUCTION

Therapeutic drug monitoring (TDM) of COPD drug is necessary to optimize the patient's clinical outcome by managing their medication regimen with the assistance of measured drug concentration. Theophylline has been introduced in the therapy of chronic airway disease several decades ago[1]. Theophylline has maintained an important role as a potent and useful bronchodilator, which may be due to the reliable relationship of the pharmacological effect with plasma theophylline concentrations. However, the use of theophylline is often restricted by its narrow therapeutic range (5–20 mcg/mL)[2,3] and various adverse effects occur when plasma levels exceed 20 mcg/mL[4]. The rate of metabolism of theophylline varies considerably from one individual to another. As a consequence of the variation of pharmacokinetics between patients, it is necessary to monitor concentration of drugs in individual patients to ensure the maximum clinical response and to avoid undesirable side effects. For the analysis of theophylline in plasma samples, spectrophotometry[5,6], immunoassays[7-9], capillary electrophoresis (CE)[10-13] and chromatographic methods[14-22] have been introduced. The immunological and spectrophotometric methods would be inappropriate for determination of the combination of different methylxanthines. Only chromatographic and CE methods can be applied to differentiate and measure these drugs, simultaneously. But most of the clinical laboratories are not equipped with CE methods. The chromatographic methods are more widely accessible and capable of being implemented in clinical laboratories with standard high performance liquid chromatography (HPLC) instrumentation. In the present study, an optimized sample preparation technique for quantitative and reproducible recovery of theophylline from human serum is described. A validated HPLC method for subsequent quantification is reported and it's suitability in the monitoring of theophylline in serum is demonstrated. These improved techniques should aid the TDM of theophylline in COPD patients. In the present work, we report a new isocratic reversed phase HPLC-UV method for the measurement of theophylline in human serum.

MATERIALS AND METHOD

Chemicals and Materials

Theophylline (99.25 % w/w) and Caffeine (99.85 % w/w) were obtained from Ranbaxy. Triethylamine was obtained from Merck. Acetonitrile, HPLC grade was purchased from Rankem. Dichloromethane, analytical grade was purchased from Merck. Water used filtered with a Milli – Q water processing system.

Preparation of standard solution

Separate standards stock solutions of theophylline (1 mg/ml) and internal standard caffeine (1 mg/ml) were prepared in acetonitrile. From the stock solutions standards containing 100 µg/ml of theophylline were prepared. The internal standard stock solution was used to prepare 50 µg/ml of caffeine. All stock solutions were stored at 8°C until use and were stable for 6 months. Each spiked serum sample was processed as described in sample preparation.

Sample preparation

A 0.5 ml aliquot of human serum sample was mixed with 50 µl of internal standard working solution (Caffeine) and were added and mixed. The resulting solution was vortexed and extracted with Dichloromethane. The upper organic layer was separated, evaporated and the drug was reconstituted using 0.5 ml of the mobile phase and analyzed. The sample chromatogram is presented in Figure 1.

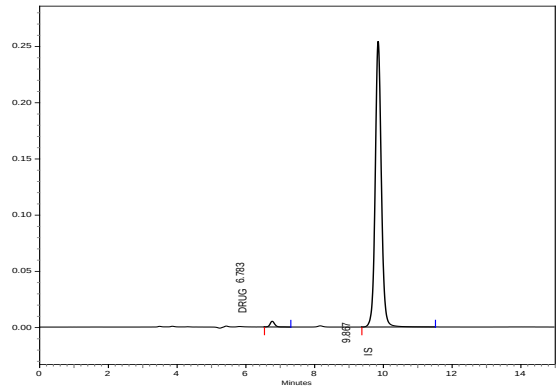

Fig 1: It shows typical chromatogram of sample.

Chromatographic system

The analysis was performed on a system consisting of a Shimadzu Model LC – 10 AT VP, SPD 10 A UV & Visible detector, SCL 10 A VP System controller. Chromatographic separation was achieved isocratically, at room temperature, on a Princeton SPHER 5µ C18 (2) 100A 250 x 4.60 mm column. The mobile phase consisted of 15 % of acetonitrile and 85 % of triethylamine buffer, pH 3.5. Flow rate was 0.8 ml/min. The ultra violet detector was set at 275 nm. The standard chromatogram is presented in Figure 2.

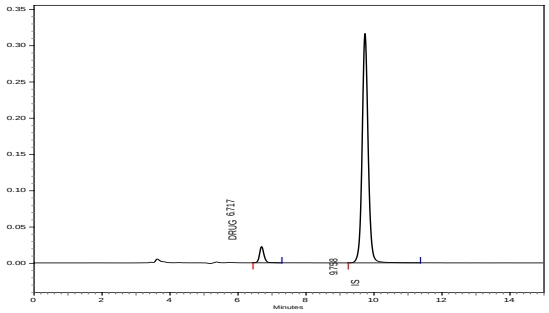

Fig 2: It shows typical chromatogram of standard and internal standard.

APPLICATION

The method was used for therapeutic drug monitoring in about 20 COPD patients presently under the treatment of theophylline. The study was carried out after approval of protocol from Institutional Ethical Committee of J.S.S. College of pharmacy, Ooty. Venous samples were withdrawn from theophylline patients collected in centrifuge tubes and processed by centrifugation to obtained serum. The samples were analyzed using the above-described HPLC method.

RESULTS AND DISCUSSION

The procedure yielded excellent separation and symmetrical peaks for the theophylline and internal standard. Representative chromatograms of blank plasma and spiked with theophylline are presented in Figure 3. and Figure 4. respectively.

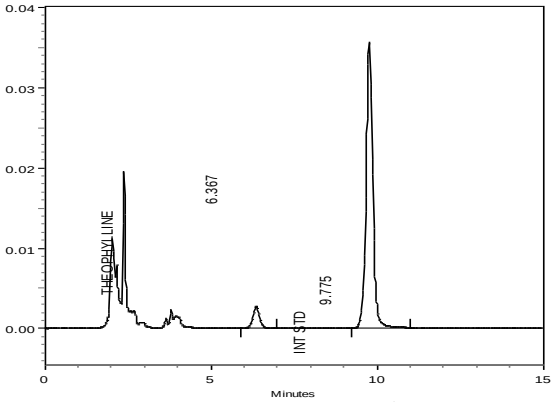

Fig 3: It shows typical chromatogram of blank serum.

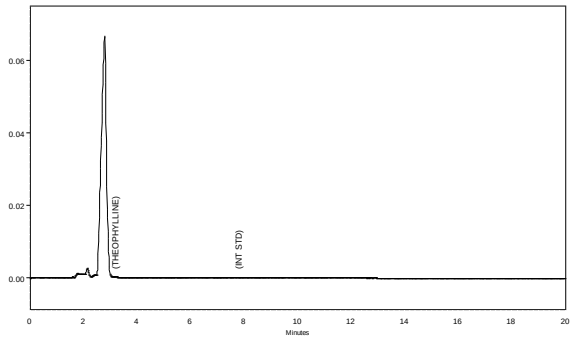

Fig 4: It shows typical chromatogram of spiked serum.

Under the described chromatographic conditions, retention times were 6.78 min (theophylline) and 9.86 min (internal standard). The complete elution was obtained in less than 15 min. The system suitability studies are presented in Table 1.

Table 1 shows system suitability studies.

Parameters	Theophyline
Linearity range	50-500 (ng/ml)
Regression equation Y = mx + c	0.007 × -0.0015
Correlation coefficient	0.9976
Theoretical plate/meter	25132
Resolution factor	2.71
Asymmetric factor	0.85
LOD (ng/ml)	5.0
LOQ (ng/ml)	10.0

Calibration curves were linear in the range of 50 ng/ml and 500 ng/ml of theophylline with correlation coefficient r^2= 0.9976. The precision and accuracy of the assay are summarized in Table 2. The inter-assay and intra-assay coefficients of variation (CVs) for theophylline were within the limit. Liquid – liquid extraction (LLE) was used because of its high efficiency, selectivity and simplicity. Extraction recovery was found to

Sensitivity

Under the experimental conditions used, the detection limit (LOD) was approximately 5ng/ml for all drugs, at a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) is 10 ng/ml.

Specificity

The method was evaluated for specificity by analyzing 8 different batches of drug free human serum to check the interference of peaks of endogenous compounds of serum. All the different batches of serum tested were found to be free from interfering compounds at the retention times of the drugs and the internal standard.

Table 2: Shows Intra-day and Inter-day Precision of Theophylline in Human Serum

	Intra-day			Inter-day		
Concentration added (ng/ml)	Measured concentration (mean ± S.D.)	CV (%)	Accuracy	Measured concentration (mean ± S.D.)	CV (%)	Accuracy
Theophylline						
50	48.56 ± 0.54	1.11	97.23	48.80 ± 0.58	1.19	97.61
200	198.32 ± 0.87	0.87	99.15	199.09 ± 0.64	0.32	99.54
500	498.86 ± 0.43	0.08	99.77	499.14 ± 0.53	0.10	99.82

Accuracy and precision

The accuracy and precision (presented as relative standard deviation, R.S.D.) of the assay were determined using quality control (QC) samples at 50, 200 and 500 ng/ml. Accuracy (%) was determined by the percentage ratio of measured over spiked QC concentration (mean of measured/ spiked × 100%). Intra-day precision was determined by analyzing replicate aliquots of QCs (n = 5 per each concentration) on the same day. Inter-day precision was determined by repetitive analysis of QC samples (each concentration) on five consecutive days.

Recovery

To investigate the recovery of theophylline by the LLE method, plasma samples were spiked with theophylline at concentrations of 50, 200 and 500 ng/ml. The resulting peak–area ratios (analyte: internal standard) were compared with that of the standards prepared in mobile phase to provide the recovery values. Ion suppression of ionization was evaluated by comparing the absolute peak areas of control plasma extracted and then spiked with a known amount of drug, to neat standards injected directly in the same reconstitution solvent.

Stability

To evaluate sample stability after three freeze–thaw cycles and at room temperature, five replicates of QC samples at each of the low, medium and high concentrations were subjected to three freeze–thaw cycles or were stored at room temperature for 4 h before sample processing, respectively. Five replicates of QC samples at each of the low and high concentrations were processed and stored under auto sampler conditions for 24 h. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples.

CONCLUSION

The HPLC method described herein allows the detection and quantification of a frequently prescribed bronchodilator theophylline. The method was validated as per Guidelines. The method is suitable for therapeutic drug monitoring studies and can also be used for pharmacokinetic studies conducted in humans.

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