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High-Performance Liquid Chromatographic Determination of Plasma Theophylline Levels in the Presence of Caffeine and Dietary Xanthine Metabolites

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A simple, sensitive, selective and specific reversed-phase high performance liquid chromatographic method to determine theophylline in human plasma is described here. Method involved a fast sample extraction step with a short chromatographic run time and required only 0.05 ml of plasma sample for each determination. Concentration as low as 0.2 µg/ml can be measured with acceptable precision. Satisfactory linearity was observed over the range of 0.2-50 µg/ml for theophylline. Inter and intra-day precision for theophylline indicated a relative standard deviation (RSD) of < 3%. Theophylline was resolved satisfactorily not only from etophylline but also from other dietary xanthines, their metabolites and other co-administered drugs. Separation of 3-methyl xanthine and baseline resolution of paraxanthine from theophylline was also achieved. This method is sufficiently simple, rugged and robust to be used for pharmacokinetic studies and especially for drug monitoring in pediatric patients as it requires only a small volume of plasma.

Keywords: Etophylline; HPLC; 3-Methyl xanthine; Paraxanthine; Theophylline

INTRODUCTION

Theophylline is a bronchodilator used extensively in the treatment and management of chronic asthma and obstructive airway disease [1]. It has a narrow therapeutic range (10-20 µg/ml) [2-5], below which it exhibits sub-therapeutic effect and above which it exhibits adverse/toxic effects [6-7]. Hence it is necessary that plasma theophylline levels be monitored, for which a fast and sensitive analytical method is required [8].

Techniques like spectrophotometry [9], immunoassays [10-12], isotachophoresis [13], thin layer chromatography [14], gas and liquid chromatographic techniques [15-22], for determining theophylline levels in biological fluids have been reported. Spectrophotometric determination of theophylline [23] requires large (5.0 ml) volume of plasma and is time consuming. Determining concentrations less than 5 µg/ml may involve appreciable error. Some gas chromatographic assays for theophylline [24-26] are sensitive (measure 1 µg/ml) and require only 1.0 ml of plasma, but drawbacks like co-extraction of theophylline with dietary xanthines and other metabolites, multiple extractions, derivatization and lengthy assay time [24] are seen.

Many high pressure liquid chromatographic (HPLC) assays have been reported (27, 28). Detection upto 2 µg/ml by ion-exchange [27] is possible, but 30 min is required per run and only a few samples can be analyzed each day. Some liquid-chromatographic methods [28] measure 2.5-25 µg/ml. But many of

these methods are associated with disadvantages such as lengthy sample preparation [19], poor chromatography [29] (bad peak shape, low column efficiency, poor resolution of theophylline from plasma peaks and other metabolites), high retention time [18] and need of macrosamples [30].
The reported methods emphasize the advantages of HPLC with respect to sensitivity, specificity and the use of micro samples. Most of the methods differ from one another in sample preparation or in the chromatographic system used. The sample can be injected directly into the chromatograph [31,32], or by precipitation with trichloroacetic acid [33], by solvent denaturation with acetonitrile [32], by molecular filtration [30], or by evaporation and reconstitution of the extracted drug [34] in a suitable solvent. These methods often involve expensive materials and chemicals, large sample volumes, tedious and lengthy process or special care to maintain characteristics of the analytical system.

Although several approaches have been tried upon for the separation of certain dietary xanthines and other metabolites that interfere with theophylline estimation, the potential interference of paraxanthine (1,7-dimethyl xanthine), an important metabolite of caffeine, has not been dealt upon much, especially with respect to satisfactory baseline separation and resolution of theophylline from paraxanthine [27,35], in a reasonable time. With paraxanthine exhibiting an UV absorption and retention time identical to that of theophylline, it often results in wrong elevation of plasma theophylline levels upto 3 µg/ml. Only very few investigators have attempted to solve his problem [36-39]. Similarly 3-methyl xanthine (3-MX) an important metabolite of theophylline, although less potent than theophylline may contribute to certain extent to the therapeutic effects of theophylline [40,41]. This type of elevation, in times, can lead to fatal outcome.

The present work describes a fairly rapid, convenient and specific HPLC method for the determination of theophylline in microsamples of plasma (0.5 ml of plasma from each patient sufficient), with a relatively short and simple sample preparation and a shorter chromatogram run time. It is specific in that there is no interference from other medications or xanthine metabolites and baseline resolution of theophylline from 1,7-dimethyl xanthine is achieved along with separation and detection of 3-methyl xanthine.

Material and Methods

Chemicals
Standard theophylline and the internal standard (I.S) i.e. 7-(β-hydroxyethyl) theophylline (BHET) also known as etophylline, were obtained from German Remedies (Mumbai, India). HPLC grade tetrahydrofuran, acetonitrile and methanol were purchased from Ranbaxy Chemicals (New Delhi, India). HPLC grade water from Milli-Q system (Millipore, Bedford, USA) was used throughout. Blank human plasma, from healthy volunteers, was obtained from a tertairy care hospital (Bangalore, India). Analytical grade potassium dihydrogen phosphate, perchloric acid, triethylamine and orthophosphoric acid were purchased from Qualigens Fine Chemicals (Mumbai, India).

Apparatus and chromatographic conditions

Shimadzu liquid chromatograph (LC 10, Shimadzu corporation, Kyoto, Japan) equipped with an LC-10AT VP 230 CE dual pump, a variable wavelength SPD-10A VP 230 CE 2001 UV detector, a degasser, a 50 µl Rheodyne 7725i injector loop and a data recorder system (CSW station, version 1.7 DLL, USA) was used. All separations were achieved on a reversed phase Luna ODS 2 C_{18} column (5 µm, 250mm x 4.6mm I.D, Phenomenex, California, USA). The mobile phase used was elution buffer (0.05 M potassium dihydrogen phosphate with 1 ml of triethylamine, adjusted to pH 4.6 with orthophosphoric acid), acetonitrile, methanol and tetrahydrofuran (86:8:5:1, v/v/v). Elution was carried out at a flow rate of 1.5 ml/min, at ambient temperature. Detection was at a wavelength of 273 nm with a detector sensitivity setting of 0.02 a.u.f.s.

Standard solution and internal standard solution preparation

20 µg/ml solution of 7-(β-hydroxyethyl)theophylline (BHET) in elution buffer was found to be satisfactory as an internal standard (I.S), for the concentration range studied. Standard theophylline spiked plasma was used for calibration. To 50 µl of drug-free plasma in a micro-centrifuge tube, 25 µl of standard theophylline (concentration range: 0.2-50 µg/ml) in elution buffer and 25 µl I.S. solution (20 µg/ml) were added. To this mixture 25 µl of 70 % perchloric acid was added and vortex-mixed for 60 s, to precipitate the proteins. The mixture was centrifuged at 10 000 g for 4 min and a 50 µl aliquot of the clear supernatant was injected into the chromatograph. The standard calibration curve was determined based on peak area ratios of standard theophylline to that of I.S verses the concentration range studied. The acceptance criteria for the calibration curve to be considered as a standard curve was a correlation coefficient of 0.99 or more. Also, the back calculated standard concentration must be within 15% of the normal value except at the LOQ, for which the maximum acceptable deviation was 20% [42].

Sample preparation

To 50 µl of patient's plasma sample in a micro-centrifuge tube, 25 µl I.S. solution (20 µg/ml) and 25 µl of buffer were added. To this mixture 25 µl of 70 % perchloric acid was added and vortex-mixed for 60 s, to precipitate the proteins. The mixture was centrifuged at 10 000 g for 4 min and a 50 µl aliquot of the clear supernatant was injected into the chromatograph. The theophylline concentration in the patient's plasma was determined based on peak area ratios of theophylline to that of I.S, from the standard curve.

Extraction Recovery

The recovery was assessed using the quality control samples of concentrations of 0.2, 0.8, 4, 10 and 20 µg/ml for theophylline and at 20 µg/ml for the I.S. (BHET). Five replicates of each of the concentrations were extracted and injected. Five injections of each of the concentrations, containing standard theophylline and I.S in aqueous solution (unextracted), were injected directly. Recovery of theophylline and BHET (I.S) was determined by comparing the mean peak areas of extracted samples to the mean peak areas of unprocessed samples.

Selectivity

Ten blank plasma samples, randomly collected from healthy human volunteers, were extracted and chromatographed in order to determine to what extent the plasma components and other endogenous

Fig. 2: Theophylline plasma concentration-time profile, 300 mg slow release theophylline

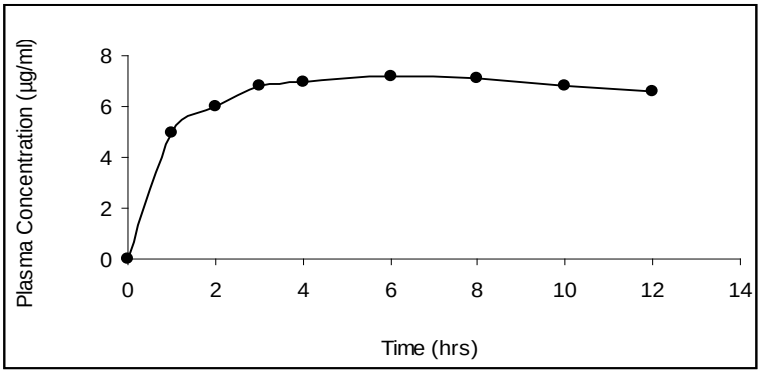

Table 1
Extraction recovery of the assay for theophylline

Amount added (µg/ml)	Concentration recovered (µg/ml) (mean ± S.D, n=5)	Recovery (%)
0.2	0.19 ± 0.05	95.0
0.8	0.73 ± 0.08	91.2
4.0	3.85 ± 0.11	96.3
10.0	9.70 ± 0.25	97.0
20.0	18.7 ± 1.20	93.5

Table 2
Reproducibility and accuracy of theophylline in plasma – Within-day reproducibility

Concentration (µg/ml)	Within-day reproducibility (n = 5)	R.S.D. (%)	Accuracy (%)
	Concentration recovered (µg/ml) (mean ± S.D)		
0.2	0.18 ± 0.004	2.22	90.0
0.8	0.70 ± 0.015	2.14	87.5
4.0	3.80 ± 0.040	1.05	95.0
10.0	9.90 ± 0.094	0.94	99.0
20.0	19.30 ± 0.36	1.86	96.5

Table 3
Reproducibility and accuracy of theophylline in plasma – Between-day reproducibility

Concentration (µg/ml)	Between-day reproducibility (n = 5)	R.S.D. (%)	Accuracy (%)
	Concentration recovered (µg/ml) (mean ± S.D)		
0.2	0.195 ± 0.005	2.56	97.5
0.8	0.75 ± 0.010	1.33	93.7
4.0	3.70 ± 0.020	0.54	92.5
10.0	9.40 ± 0.040	0.42	94.0
20.0	19.0 ± 0.090	0.47	94.5

substances may contribute to interference with respect to theophylline or the internal standard determination.

Reproducibility

Reproducibility of the method was investigated by performing repeated analysis of theophylline and determining the inter-day and intra-day precision and accuracy of the method for theophylline. Intra-day reproducibility assessment involved analyzing five replicates of each of the five quality control samples, on the same day. Inter-day reproducibility assessment involved analyzing five replicates of each of the five quality control samples on five different days.

RESULTS AND DISCUSSION

Chromatographic separation

The chromatograms resulting from the injections of blank plasma, standard theophylline (10 µg/ml) spiked plasma sample and a patient's plasma sample are represented in Fig. 1. Theophylline and the I.S. were separated completely and resolved satisfactorily, as per the described chromatographic conditions, at the therapeutic range of theophylline and the retention times were 8.5 and 10.1 min, respectively. Comparison of Fig. 1A with Fig. 1C, 1D and 1E illustrates lack of interference from plasma matrix and other endogenous constituents. Also, the peaks obtained were good shaped.

The ratio of methanol and acetonitrile to the phosphate buffer was found to be critical for better separation. Slight change in the ratio brought about significant variations in the retention time and separation. Thus the percentage of solvents selected for the mobile phase was based on a compromise between baseline resolution and chromatographic analysis time. Also, variation in the pH of the mobile phase was found to influence theophylline separation significantly. Maximum difference in the retention times of theophylline and other endogenous metabolites was observed at pH 4 to pH5.

Linearity

The standard calibration curves showed satisfactory linearity [correlation coefficient (r^2) = 0.9961], in terms of peak area ratio of theophylline to I.S., over the concentration range investigated (0.2-50 µg/ml). The mean linear regression equation of the standard calibration curves for theophylline was $y = 9.4433x - 0.064$ where y represents peak area ratio of theophylline to I.S and x represents concentration of theophylline.

Sensitivity and detection limits

Variation in the pH of the mobile phase was found to influence theophylline separation significantly and in turn the sensitivity of detection. Maximum difference in the retention time and higher peak area ratio of theophylline to that of I.S was observed with the mobile phase adjusted to pH 4.6. Similarly, a detector sensitivity setting of 0.02 a.u.f.s indicated a higher peak area ratio of theophylline to that of I.S compared to other sensitivity settings of 0.04, 0.08, 0.1 and 1.00 a.u.f.s. Using the criterion of detectability as three times the system noise (3:1) the limit of detection (LOD) for theophylline in plasma was 0.025 µg/ml. Limit of quantification (LOQ) was found to be 0.2 µg/ml.

Extraction recovery

Using 70 % perchloric acid as the extraction solvent good extraction efficiency was achieved. The absolute recovery was expressed as the percentage of the directly injected standard solutions

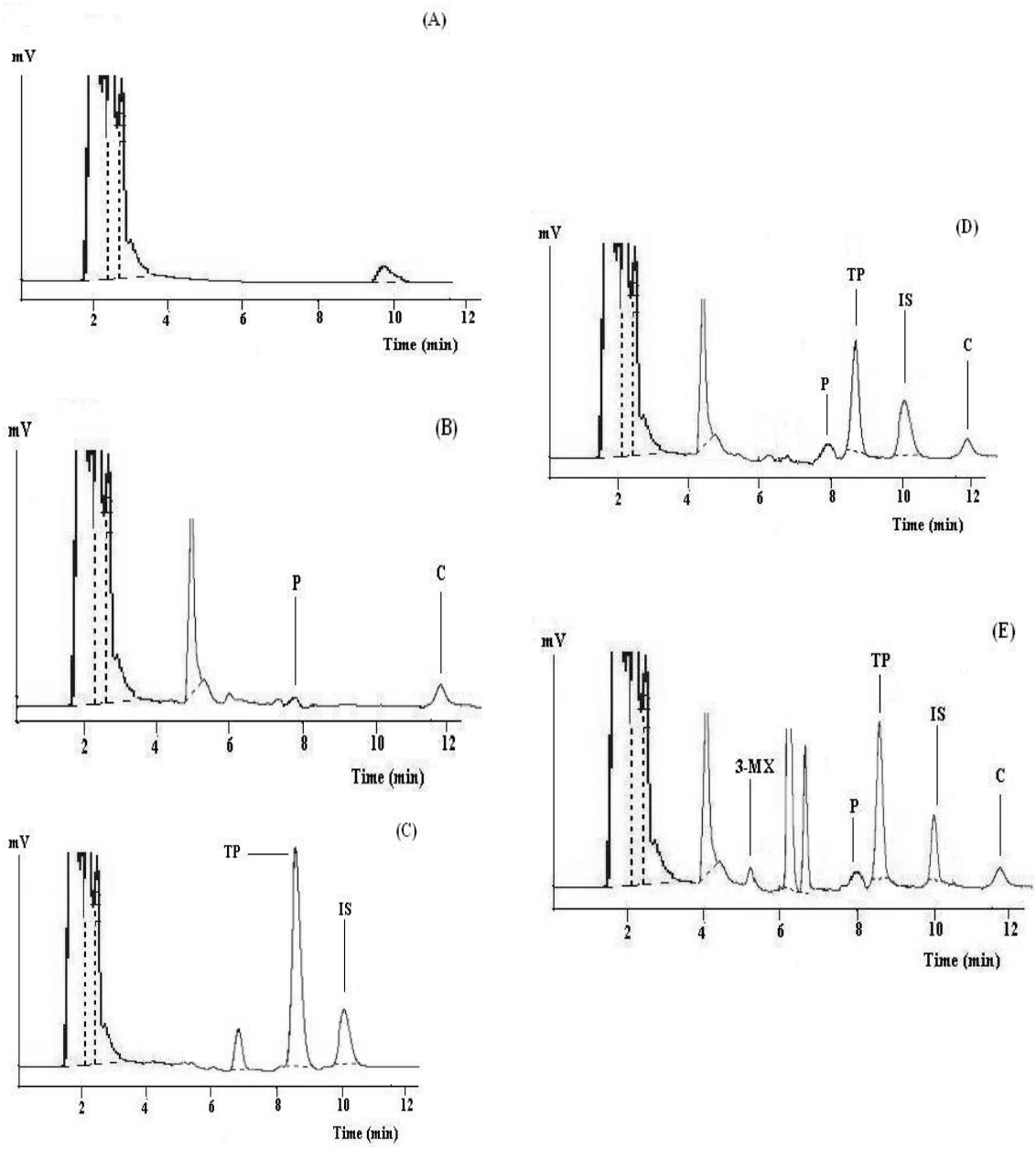

Fig. 1: Chromatograms of: (A) blank human plasma from a volunteer abstained from xanthine-containing food and beverages; (B) blank human plasma from a coffee consumer; (C) blank human plasma (of a volunteer abstained from xanthine-containing food and beverages) spiked with standard theophylline (10 µg/ml) and I.S. (10 µg/ml); (D) blank human plasma (of a coffee consumer) spiked with standard theophylline (10 µg/ml) and I.S. (10 µg/ml); (E) plasma sample of a patient after administration of theophylline. Peaks: 3-MX = 3-methyl xanthine; P = paraxanthine; TP = theophylline; I.S = internal standard (BHET); C = caffeine. .

of theophylline and I.S. The extraction recovery was in the range of 91 to 97 % for the concentrations of theophylline investigated. The assay recoveries appeared to be independent of theophylline concentration (Table 1). The recovery of internal standard, BHET, was 86±1.2 % at the concentration (20 µg/ml) used. Thus this method of protein precipitation and extraction using perchloric acid eliminated the arduous and time consuming extraction steps of evaporation and reconstitution without compromising the sensitivity. Further it helped in reducing processing and analysis time.

Selectivity

The resulting chromatograms were essentially free from endogenous interference. No interfering peaks were noticed at the retention times of theophylline or internal standard (fig. 1A & 1B), in the ten randomly selected blank human plasma samples used for analysis. Potential interference by some co-administered drugs such as aspirin, ampicillin, paracetamol, cephalosporins, sulphamethoxazole, ibuprofen, caffeine, atorvastatin, carbamazepine, metformin, phenytoin, glimepiride, propranolol, phenobarbital, quinidine and nimuselide was not observed for theophylline and 7-(β-hydroxyethyl)theophylline analysis.

The resolution of theophylline from paraxanthine is a major problem in the analysis of theophylline. Paraxanthine interference is of serious concern in the therapeutic monitoring of theophylline in patients who consume caffeine-containing foods, beverages and medications. The amount of paraxanthine in serum or plasma may vary with the kind and the amount of food or beverage. This has to be overcome by chromatographic separation of theophylline. Use of the mobile phase, as per the above mentioned ratio, proposed here resulted in baseline resolution of theophylline and paraxanthine. The small peak eluting immediately before theophylline in fig. 1D and 1E is paraxanthine, which indicates a satisfactory baseline resolution of theophylline and paraxanthine.

A previous study mentions to the use of acetate buffer-methanol-tetrahydrofuran system as the mobile phase [37]. But these had disadvantages like insufficient baseline resolution, possible interference from other drugs, longer retention time [37]. Complete resolution of the methylxanthines could not be obtained using this mobile phase system [37] on the 5-µm reversed-phase column.

Hence acetonitrile was incorporated along with methanol, phosphate buffer and tetrahydrofuran in the mobile phase of the proposed method. Acetonitrile because of its lower polarity and viscosity resulted in higher elution efficiency and lower column pressure compared to using methanol alone. An optimal mixing ratio of the mobile phase components was determined to provide better resolution and reasonable analysis time. As a result, use of the proposed method could overcome the disadvantages of previous methods [37]. The inclusion of acetonitrile must have been responsible for the better resolution of theophylline and paraxanthine. Elimination of interference from the co-administered drugs may be partly attributed to the extraction procedure used in the pretreatment of plasma samples, and partly to the use of acetonitrile and tetrahydrofuran in the mobile phase which allows for better resolution of theophylline from co-extracted compounds and methanol for good shaped peaks. Similarly, 3-methyl xanthine (3-MX), although much less potent than theophylline may contribute to

certain extent to the therapeutic effects of theophylline. Hence it is necessary to determine the 3-MX in order to know the exact concentration of theophylline that is present in plasma, especially in patients in whom a small increase in theophylline dose may indicate manifold increase its clinical response. Applying the proposed method it was possible to separate 3-MX (fig 1E) and thereby determine the exact plasma theophylline concentration.

Reproducibility

The proposed method was found to be precise and reproducible within the same day and also on different days, with respect to analysis of theophylline in human plasma. Precision is represented as relative standard deviation (R.S.D). All the concentrations investigated indicated a R.S.D. of less than 3 % (<15%) (Tables 2 and 3).

The within-day and between-day studies, carried out using five replicates of the five quality control samples of theophylline, indicated that the accuracy of theophylline determination in plasma to be within acceptable limits (<15%) (Table 2 and 3).

Analysis of patient samples

The proposed method was further evaluated to determine whether this method can be applied for the routine monitoring of plasma theophylline concentrations in asthma patients. Plasma samples from ten patients, who were on oral dose of slow release theophylline (300 mg aminophylline), were obtained and theophylline concentration-time profiles were constructed upto 12 h (mean concentration–time profile of theophylline of ten subjects - Fig. 2). This indicated that the proposed can be satisfactorily applied for analysis of patient samples. The theophylline concentrations, its concentration-time profile and pharmacokinetics obtained were in agreement with reported literatures [2-7].

CONCLUSION

The described HPLC assay is convenient for the estimation of theophylline and etophylline in human plasma. It involves a simple protein precipitation and extraction procedure for sample preparation. It requires only 50 µl of plasma sample for each determination and has a short run time with increased sensitivity and selectivity, for theophylline, caffeine and their metabolites. Data indicates good reproducibility, accuracy, linearity and sufficient quantification of theophylline over a wide concentration range of 200–50,000 ng/ml (0.2-50 µg/ml). It overcomes the problem of resolving paraxanthine from theophylline and also the determination of 3-methyl xanthine. In particular this method would be of use for the pharmacokinetic studies and routine drug monitoring in pediatric patients due to limited availability of the plasma. In conclusion, this work describes a simple and selective HPLC method for the estimation of theophylline along with etofylline, caffeine and dietary xanthine metabolites in plasma.

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