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A simple and rapid HPLC/UV method for the simultaneous quantification of theophylline and etofylline in human plasma

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Abstract

A simple, sensitive and selective high performance liquid chromatography (HPLC) method with ultraviolet detection (272 nm) was developed and validated for the simultaneous quantification of theophylline and etofylline in human plasma. Following rapid sample preparation, the analytes and internal standard (hydrochlorothiazide) were separated using an isocratic mobile phase on a reverse phase C_{18} column. The lower limit of quantification was 100 ng/mL for both theophylline and etofylline with a relative standard deviation of less than 6%. A linear dynamic range of 100–10,000 ng/mL for both theophylline and etofylline was established. This HPLC method was validated with between-batch precision of 2.2–6.0 and 1.4–3.7% for theophylline and etofylline, respectively. The between-batch accuracy was 94.3–98.0 and 95.4–98.2%, respectively. Stability of theophylline and etofylline in plasma was excellent, with no evidence of degradation during sample processing (autosampler) and 30 days storage in a freezer. This validated method is simple and rugged enough to be used in pharmacokinetic studies.
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1. Introduction

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 μg/mL) [2,3] and various adverse effects occur when plasma levels exceed 20 μg/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.

Etofylline or hydroxyethyltheophylline is a bronchodilator, and is normally applied in combination with theophylline. The pharmacological actions of the etofylline are generally considered to be similar to those of theophylline [5]. Unlike other xanthine derivatives, etofylline does not convert into theophylline in the body. Etofylline is mainly excreted unchanged by the kidneys and only a small fraction of it is metabolized by hydroxylation [6]. This offers a wide therapeutic window and combination of etofylline and theophylline (Deriphylline®) exhibits less frequent adverse side effects than an equivalent dose of theophylline alone. Therefore, the monitoring of etofylline and theophylline levels in plasma is required for therapeutic use and toxic control.

For the analysis of theophylline and etofylline in plasma samples, spectrophotometry [7,8], immunoassays [9–11], capillary electrophoresis (CE) [12–15] and chromatographic methods [16–24] 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) instrumen-

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tation. Theophylline alone or theophylline with other drugs or with its metabolites in plasma has been determined by HPLC.

In this investigation, a simple plasma pretreatment and HPLC method is developed and validated for the simultaneous quantification of theophylline and etofylline in human plasma. The proposed method also can be applied to pharmacokinetic research and clinical monitoring of these methylxanthines. The proposed HPLC method was applied to the dose dependent pharmacokinetic study of theophylline and etofylline.

2. Materials and methods

2.1. Chemicals

Theophylline and etofylline were obtained from German Remedies (Mumbai, India). Hydrochlorothiazide (internal standard, I.S.) was from Torrent Research Centre (Ahmedabad, India). HPLC grade LiChrosolv methanol and LiChrosolv acetonitrile were from Merck (Darmstadt, Germany). Drug-free human plasma, containing EDTA as an anticoagulant, was obtained from Nizam Institute of Medical Sciences (Hyderabad, India). Ammonium acetate, trichloro acetic acid and acetic acid were purchased from Merck (Mumbai, India). HPLC grade water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

2.2. Chromatography

The integrated high performance liquid chromatography system (LC 2010C, Shimadzu Corporation, Kyoto, Japan) was equipped with a quaternary pump, a degasser, an autosampler, an injector with a 100-μL loop, a column oven, a UV detector and a data system (Class VP version 6.12). The separation of compounds was made on an Intertsil C_{18} column (5 μm, 150 mm × 4.6 mm i.d., GL Sciences Inc., Japan) at 30 °C temperature. The mobile phase was a mixture of 10 mM ammonium acetate buffer/methanol/acetonitrile (86:7:7, v/v/v) pumped at a flow-rate of 1.0 mL/min. Detection was set at a wavelength of 272 nm.

2.3. Sample preparation

Plasma sample (0.5 mL) was pipetted into micro-centrifuge tubes, then 25 μL of I.S. working solution (75 μg/mL) was added and the mixture was vortex mixed for 10 s. Then 50 μL of trichloro acetic acid (35%) was added drop by drop to the sample carefully to ensure complete precipitation of the proteins while vortex-mixing for 1 min. The sample was centrifuged using micro-centrifuge at 8000 rpm for 5 min at 25 °C. Then clear supernatant was transferred into injector vials and a 100-μL aliquot was injected into chromatographic system.

2.4. Method validation

2.4.1. Calibration and control samples

Standard stock solutions of theophylline (4 mg/mL), etofylline (4 mg/mL) and I.S. (1 mg/mL) were prepared in methanol. The I.S. working solution (75 μg/mL) was prepared by diluting stock solution with diluent (water/methanol, 50:50, v/v). Working solutions (0.2 mL) were added to drug-free plasma (9.8 mL) as a bulk, to obtain both theophylline and etofylline concentrations of 100, 200, 500, 1000, 2000, 3000, 5000 and 10,000 ng/mL as a single batch at each concentration. The quality control samples were prepared as a bulk on an independent weighing of standard drugs, at concentrations of 100 ng/mL (LLOQ), 300 ng/mL (low), 4000 ng/mL (medium) and 8000 ng/mL (high) for both theophylline and etofylline as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in micro-centrifuge tubes (Tarson, 2 mL) and stored in the freezer (<−50 °C) until analyses.

A calibration curve was constructed from eight non-zero samples covering the total range (100–10,000 ng/mL for both theophylline and etofylline), including LLOQ. The calibration curves were generated using the analytes to I.S. peak area ratios by weighted ($1/x^2$) least-squares linear regression on 5 consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r^2) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification [25].

2.4.2. Selectivity

Five randomly selected blank human plasma samples were carried through the protein precipitation and chromatographed to determine the extent to which endogenous plasma components may contribute to interference with the analytes or the internal standard. The results were compared with LLOQ for theophylline and etofylline.

2.4.3. Recovery

Recovery of theophylline and etofylline was evaluated by comparing the mean peak areas of five extracted low, medium and high quality control samples to mean peak areas of five neat reference solutions (unprocessed). Recovery of hydrochlorothiazide (I.S.) was evaluated by comparing the mean peak areas of five extracted quality control samples to mean peak areas of five neat reference solutions (unprocessed) of the same concentration.

2.4.4. Accuracy and precision

Within-batch accuracy and precision evaluations were performed by repeated analysis of theophylline and etofylline in human plasma. The run consisted of a calibration curve plus five replicates of each LLOQ, low, medium and high quality control samples. Between-batch accuracy and precision were assessed by analysis of samples consisting of a calibration curve and five replicates of LLOQ, low, medium and high quality control samples for theophylline and etofylline on 3 separate days. The overall precision of the method expressed as percent coefficient of variation (%CV) and accuracy of the method expressed as percent to true value.

2.4.5. Stability

The short-term stability was examined by analyzing replicates of the low and high plasma quality control samples at room temperature for 24 h. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h, refrozen for 12–24 h. Autosampler stability of theophylline and etofylline was tested by analysis of processed and reconstituted low and high plasma QC samples, which are stored in the autosampler tray for 24 h. Stability of theophylline and etofylline in human plasma was tested after storage in a freezer (<−50 °C) for 30 days. The stability of standard solutions was also tested at room temperature for 3 h, 20 h and upon refrigeration (4 °C) for 1 month. For each concentration and each storage condition, five replicates were analyzed in one analytical batch. The concentration of theophylline and etofylline after each storage period was related to the initial concentration as determined for the samples that were freshly prepared and processed immediately.

3. Results and discussion

3.1. Separation

Fig. 1 shows the representative chromatograms of blank plasma, at LLOQ for theophylline (100 ng/mL) and etofylline (100 ng/mL) and plasma samples spiked with theophylline (4000 ng/mL) and etofylline (4000 ng/mL). The analytes, theophylline, etofylline and I.S., were well separated from co-extracted material under the described chromatographic conditions at retention times of 6.6, 7.9 and 9.4 min, respectively. The peak shapes were good and completely resolved one from another at therapeutic concentrations of theophylline and etofylline. No interference with constituents from the plasma matrix was observed.

3.2. Linearity and sensitivity of the assay

The peak area ratio of theophylline and etofylline to I.S. in human plasma was linear with respect to the analyte concentration for both over the range 100–10,000 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and none). The best fit for the calibration curve could be achieved with the linear equation $y = mx + c$ with a $1/x^2$ weighing factor. The mean linear regression equation of calibration curve for theophylline was $y = 0.0004(\pm 0.00)x + 0.0129(\pm 0.0080)$ and for etofylline was $y = 0.0003(\pm 0.00)x + 0.0010(\pm 0.0012)$, where y is the peak area ratio of the analyte to the I.S. and x is the concentration of the analyte. The correlation coefficient (r^2) for both theophylline and etofylline was above 0.999 over the concentration range used. These calibration curves were suitable for generation of acceptable data for the concentrations of the analyte in the samples during between-batch and within-batch validations (Tables 1 and 2).

LLOQ was established as 100 ng/mL for both theophylline and etofylline, respectively. The mean response for the analytes peak at LLOQ was more than 10-fold greater than the mean response for the peak in five blank human plasma samples at the retention time of the analytes.

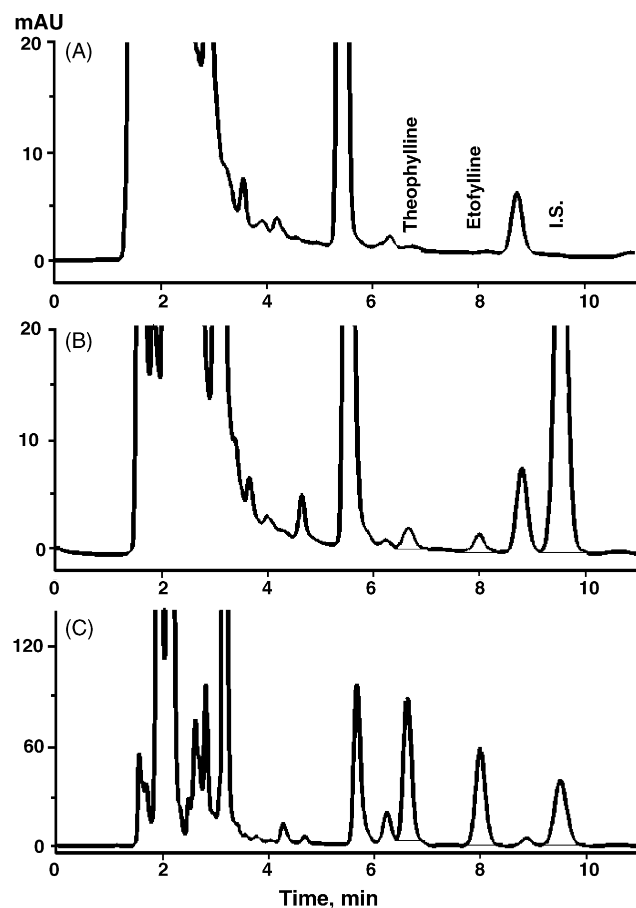

Fig. 1. Chromatograms of: (A) blank human plasma; (B) spiked human plasma sample at LLOQ for theophylline (100 ng/mL) and for etofylline (100 ng/mL); (C) human plasma sample spiked with theophylline (4000 ng/mL), etofylline (4000 ng/mL) and I.S. Approximate retention times: theophylline = 6.6 min; etofylline = 7.9 min; I.S. = 9.4 min.

3.3. Extraction efficiency

The optimized protein precipitation extraction with trichloro acetic acid eliminated the laborious extraction steps of evaporation and reconstitution involved in generic solid phase and liquid–liquid extraction methods without compromising the sen-

Table 1
Precision and accuracy data of back-calculated concentrations of calibration samples for theophylline in human plasma

Concentration added (ng/mL)	Concentration found (mean ± S.D., n = 5; ng/mL)	Precision (%)	Accuracy (%)
100	97 ± 6	6.0	96.6
200	196 ± 6	3.4	98.1
500	486 ± 8	1.8	97.1
1,000	1057 ± 48	4.5	105.7
2,000	2054 ± 51	2.5	102.6
3,000	3065 ± 83	2.7	102.2
5,000	5159 ± 113	2.2	103.2
10,000	9718 ± 140	1.5	97.2

Table 2
Precision and accuracy data of back-calculated concentrations of calibration samples for etofylline in human plasma

Concentration added (ng/mL)	Concentration found (mean ± S.D., $n = 5$; ng/mL)	Precision (%)	Accuracy (%)
100	97 ± 4	3.5	97.3
200	197 ± 6	2.9	98.5
500	495 ± 9	1.9	98.9
1,000	1043 ± 11	1.1	104.3
2,000	2018 ± 15	0.7	100.8
3,000	2993 ± 32	1.1	99.7
5,000	5064 ± 42	0.8	101.2
10,000	9900 ± 85	0.9	98.9

sitivity, which further resulted in reduced processing and analysis time.

The extraction recovery at low, medium and high quality control samples for theophylline was 86.3 ± 1.9, 95.5 ± 1.1 and 93.9 ± 0.8% and for etofylline was 88.2 ± 1.6, 95.3 ± 1.1 and 95.6 ± 0.8%, respectively. It indicates that extraction recovery of theophylline and etofylline is independent of concentration. The recovery of internal standard, hydrochlorothiazide was 57.6 ± 0.6% at the concentration used in the assay (75 μg/mL). Recovery of theophylline and etofylline were high where as recovery of I.S. was low, but the recoveries of the analytes are consistent, precise and reproducible.

3.4. Selectivity

There were no interfering peaks present in five different randomly selected samples of drug free human plasma used for analysis at the retention times of either analytes or internal standard. There was no interference of theophylline, etofylline and hydrochlorothiazide analysis by other potentially co-administered drugs such as paracetamol, nicotinamide, ibuprofen, caffeine, aspirin, ampicillin, loratadine, atorvastatin, clopidogrel, metformin, glimepiride, venlafaxine, celecoxib, naproxen and nimuselide.

3.5. Precision and accuracy of the assay

The results shown in Tables 3 and 4 indicate that the assay method is reproducible for replicate analysis of theophylline and etofylline in human plasma within the same day and also on different days.

The accuracy values for between-batch and within-batch studies at the LLOQ and at low, medium and high concentrations of theophylline and etofylline in plasma were within acceptable limits (<15%, $n = 3$) (Tables 3 and 4).

3.6. Stability

The stock solutions were stable for at least 1 month when stored at 4 °C. The stability experiments were aimed at testing all possible conditions that the samples might experience after collection and prior the analysis. These were performed as described in Section 2.4.5. All stability results are summarized in Table 5. Three freeze–thaw cycles and 24 h room temperature storage for low and high quality controls samples indicated that theophylline and etofylline were stable in human plasma under these conditions. QC samples were stable for at least 30 days if stored frozen at <−50 °C. Testing of autosampler stability of quality control samples indicated that theophylline and etofylline were stable when kept in the autosampler for up to 24 h.

3.7. Dilution integrity

The dilution integrity was also conducted to assess whether the upper concentration limit (10,000 ng/mL) can be extended. Quality control samples (in five replicates) at concentration 40,000 ng/mL were diluted by five times with blank plasma,

Table 3
Accuracy and precision of the HPLC method for determining theophylline concentrations in plasma samples

Concentration added (ng/mL)	Within-batch ($n = 5$)			Between-batch ($n = 3$)		
	Concentration found (mean ± S.D.; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± S.D.; ng/mL)	Precision (%)	Accuracy (%)
100	90 ± 3	3.4	89.7	98 ± 6	6.0	98.0
300	265 ± 7	2.5	88.3	289 ± 15	5.4	96.3
4000	3935 ± 118	3.0	98.3	3776 ± 170	4.5	94.3
8000	7990 ± 47	0.6	99.8	7804 ± 178	2.2	97.5

Table 4
Accuracy and precision of the HPLC method for determining etofylline concentrations in plasma samples

Concentration added (ng/mL)	Within-batch ($n = 5$)			Between-batch ($n = 3$)		
	Concentration found (mean ± S.D.; ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± S.D.; ng/mL)	Precision (%)	Accuracy (%)
100	95 ± 2	1.9	95.3	97 ± 3	3.7	97.4
300	273 ± 2	0.6	90.9	286 ± 9	3.3	95.4
4000	3834 ± 17	0.5	95.8	3910 ± 76	1.9	97.7
8000	7933 ± 85	1.1	99.1	7857 ± 107	1.4	98.2

Table 5
Stability of theophylline and etofylline in human plasma

Sample concentration ($n = 5$; ng/mL)	Theophylline			Etofylline		
	Concentration found (ng/mL)	Precision (%)	Accuracy (%)	Concentration found (ng/mL)	Precision (%)	Accuracy (%)
Short-term stability for 24 h in plasma						
300	293	3.1	97.6	282	2.8	93.9
8000	7576	1.4	94.6	7621	1.5	95.2
Three freeze and thaw cycles						
300	315	2.3	104.9	290	2.1	96.7
8000	7913	1.2	98.8	7928	0.5	99.1
Autosampler stability for 24 h						
300	304	1.8	101.5	290	1.1	96.9
8000	8090	2.6	101.1	7894	2.7	98.6
30 days stability in a freezer						
300	285	2.3	95.3	295	1.5	98.4
8000	7775	0.5	97.1	7806	0.5	97.5

and the assay precision and accuracy were determined in a similar manner as described above. For theophylline and etofylline, the mean concentration found was $40,515 \pm 3.7$ and $39,862 \pm 2.8$ ng/mL and bias was 3.2 and 1.9%, respectively. The results suggested that samples whose concentrations were greater than the upper limit of the standard curve could be reanalyzed by appropriate dilution.

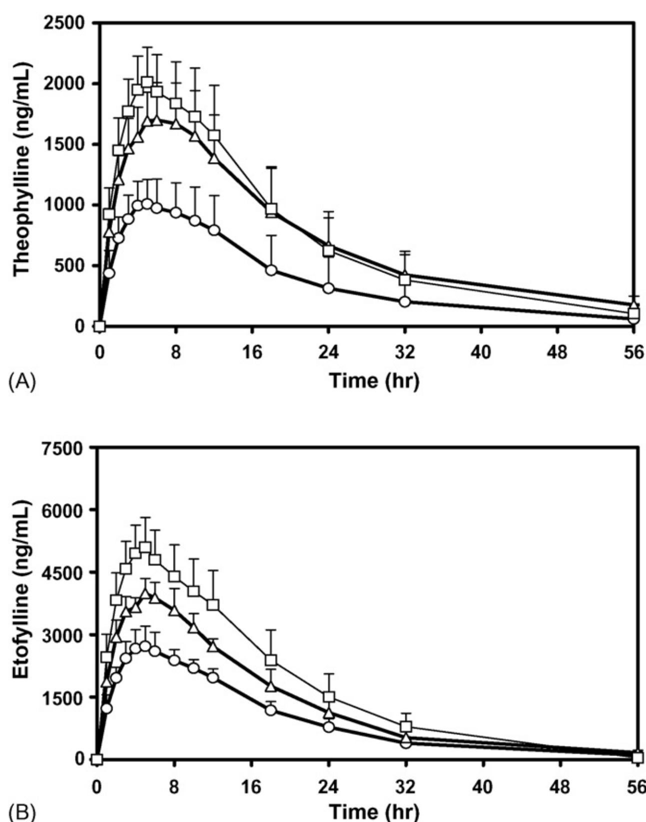

Fig. 2. Mean concentration vs. time profiles over 56 h of: (A) theophylline and (B) etofylline in human plasma from six subjects receiving a single oral dose of 300, 450 and 600 mg of Deriphylline under fasting conditions (circles—300 mg; triangles—450 mg; squares—600 mg).

3.8. Application to clinical study

The present HPLC method was employed to determine the pharmacokinetic parameters of theophylline and etofylline in the plasma samples of clinical subjects. After a single oral dose of Deriphylline (300, 450 and 600 mg) to 18 healthy subjects, concentration versus time profiles were constructed up to 56 h. Each Deriphylline tablet contains etofylline 77% and theophylline 23%. Fig. 2 shows mean concentration–time profiles of theophylline and etofylline in six subjects each receiving a single oral dose of 300, 450 and 600 mg Deriphylline under fasting conditions. In literature there were no reports on the pharmacokinetics of etofylline in human plasma where as the pharmacokinetics of theophylline are in agreement with the reported literature [2,3].

4. Conclusions

The described HPLC/UV method employs the protein precipitation procedure for sample preparation and is convenient for the simultaneous quantification of theophylline and etofylline from human plasma samples. The validation data demonstrates good precision and accuracy. The validated method allows quantification of both theophylline and etofylline in 100–10,000 ng/mL range, and fully validated as per FDA guidelines [25]. Because of the straightforward sample preparation procedure, a sample throughput of 50 per 10 h is routinely achieved. In conclusion, this paper describes a very simple and selective HPLC method for the simultaneous quantification of theophylline and etofylline suitable to monitor plasma concentrations during clinical pharmacokinetic studies in humans.

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