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High performance liquid chromatographic method for the determination of cetirizine and ambroxol in human plasma and urine—A boxcar approach

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ABSTRACT

A column switching high performance liquid chromatographic method with estimable sensitivity and accuracy was developed for the determination of cetirizine and ambroxol in human plasma using nebivolol as the internal standard. Plasma samples were prepared by liquid–liquid extraction in methylene chloride and a mixture of diethylether (80:20, v/v). The extracted samples were injected into a multifunctional clean-up column SupelcosilTM LCABZ (50 mm × 4.6 mm, 5 μm particle size) using mobile phase 1 comprising acetonitrile–phosphate buffer (pH 3.5; 20 mM) (20:80, v/v). The eluate of cetirizine and ambroxol were separated to an analytical Kromasil C_8 micro bore column (50 mm × 0.3 mm, 5 μm particle size) via a column switching device. A Kromasil C_{18} analytical column (250 mm × 2.1 mm, 5 μm particle size) was used as a separation column. Mobile phase 2 consisting acetonitrile–triethylamine (0.5%) in phosphate buffer (pH 3.5; 20 mM) (55:45, v/v) was used for the compound elution. The eluents were detected at 230 nm with photodiode array detector. An aliquot of 150 μl of plasma sample was introduced into the pretreatment column via the auto sampler using mobile phase 1 at a flow rate of 0.5 ml/min, column switching valve being positioned at A. The pretreatment column retained cetirizine, ambroxol and nebivolol (IS) in the column leaving the residual proteins of plasma eluted in void volume and drained out. The switching valve was shifted to position B at 7.5 min. Cetirizine, ambroxol and IS were eluted from the pretreatment column between 7. 5 and 11.5 min and introduced to the concentration column. Finally, cetirizine, ambroxol and IS were introduced to the separation column by switching valve using mobile phase 2 at a flow rate of 0.4 ml/min. During the analysis the pretreatment column was washed for the next analysis and resume to the position A. The total run time was 25 min for a sample. The procedure was repeated for urine analysis also. The method was linear from 2 to 450 ng/ml and 7–300 ng/ml for cetirizine and ambroxol respectively in plasma and 1–500 ng/ml and 5–400 ng/ml, respectively for cetirizine and ambroxol in urine. Intra-day and inter-day precision of cetirizine and ambroxol was below 15% in terms of coefficient of variation and accuracy of cetirizine and ambroxol was ranged from 94 to 101.6% and 91.1 to 100.2%, respectively. The method demonstrated high sensitivity and selectivity and therefore, applied to evaluate pharmacokinetics of cetirizine and ambroxol in healthy human volunteer after a single oral administration. Urine samples obtained from healthy human volunteers and clinical subjects with renal impairment have also been analyzed by the method to compare the elimination pattern. The method was precise and accurate for the estimation of cetirizine and ambroxol both in blood and in urine.

1. Introduction

Cetirizine is chemically (±)-[2-[4-[(4-chlorophenyl) phenylmethyl]-1-piperazinyl] ethoxy]acetic acid. Cetirizine (CTZ) is a human metabolite of hydroxyzine an antihistamine. Its principal effects are mediated via selective inhibition of peripheral H_1 receptors. The antihistaminic activity of cetirizine has been clearly documented in a variety of animal and human models. In vivo and ex vivo animal models have shown negligible anti-cholinergic and anti-serotonergic activity.

Ambroxol (AMB) is trans-4-(2-Amino-3,5-dibrombenzyl amino)-cyclohexanol, and is a secretolytic agent used in the treatment of respiratory diseases associated with viscid or excessive mucus. It is a mucoactive drug with secretolytic and secretomotoric

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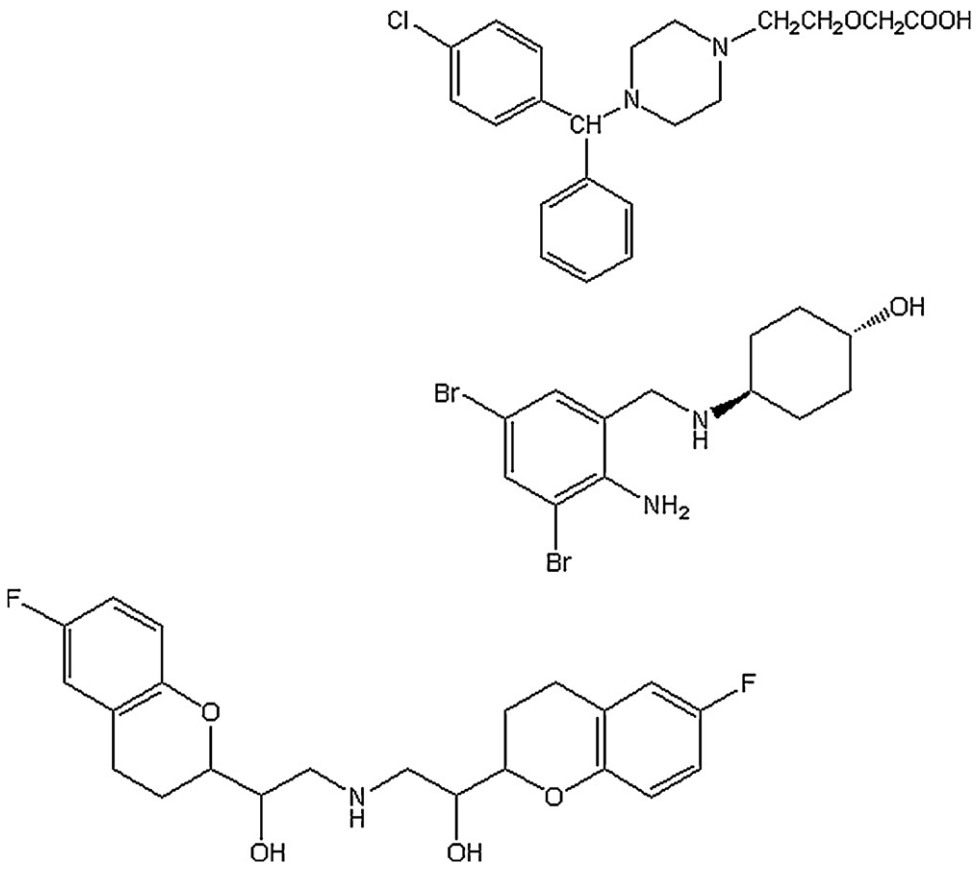

Fig. 1. Chemical structures of CTZ, AMB and NBV (IS).

actions that restore the physiological clearance mechanism of the respiratory tract, which play an important role in the body's natural defense mechanism. It stimulates synthesis and release of surfactant by type II pneumocytes. The surfactant released acts as an anti-glue factor by reducing the adhesion of mucus to the bronchial wall, in improving its transport and in providing protection against infection and irritating agents [1].

The major disadvantage of common HPLC method development in trace analysis is the interfering peaks of endogenous plasma constituents, which mask the peak intensity of analyte under consideration. Due to this effect, estimation of drugs in lower nano grams turns into a failed maneuver and resorts in to LC–MS technique, which is a cost escalating practice. Column switching (boxcar chromatography) is a powerful technique for the separation and cleanup of complex multi component samples with higher accuracy [2].There is a published research article on narrow bore column switching HPLC method for the estimation of CTZ in plasma [3], in vitro estimation of CTZ using HPLC and spectrophotometry [4], estimation of CTZ in human plasma [5–9] and urine by HPLC [10,11], a comparative study between HPLC and CE methods for the determination of cetirizine in human plasma [12], estimation of cetirizine in animal plasma [13,14] and methods to estimate CTZ in plasma by LC/MS technique [15,16]. Similarly, methods have been reported for the estimation of AMB in plasma and urine by HPLC and LC/MS methods [17–24]. Relative UV absorption of AMB than CTZ is less; hence, most of the measurements have been carried out with LC/MS technique. Therefore, there was a need to develop and validate more specific HPLC method for the estimation of CTZ and AMB in blood plasma. A column switching HPLC method was developed with optimal accuracy and sensitivity using nebivolol (NBV) as the internal standard (IS).

Column switching HPLC method has major advantages in terms of sensitivity and specificity achieved by intriguing sample concentration in the column. Also, the technique can be accomplished by integrating additional pump and columns to the usual HPLC instrument with simple operational procedures. The method was applied to pharmacokinetic study samples of CTZ and AMB involving healthy human volunteer and urine samples obtained from a clinical study conducted on healthy human volunteer and subjects with renal impairment.

2. Materials and methods

2.1. Materials

CTZ, AMB and IS (Fig. 1) were obtained from Roxaane Clinical Research Laboratories (Chennai, India). Low volume evaporator Turbovap Lv, (Caliper Life Sciences Inc., USA) and deep freezer U570 (Eppendorf, UK) were used for sample preparation. All solvents were of HPLC grade from Qualigen Chemicals (Mumbai, India) and water was purified by a Milli-Q system (Millipore Corp., USA) all other chemicals were of analytical reagent grade.

2.2. Preparation of standard solutions

Stock solutions of CTZ, AMB and nebivolol IS (1 mg/ml) were prepared in methanol and were serially diluted with mobile phase 1 [Acetonitrile 20% (v/v) in phosphate buffer pH 3.5] to get the working standard solutions for the preparation of calibration curves.

For preparation of calibration graph, standard solutions of CTZ, AMB and IS (50 μl) were spiked to 500 μl of blank plasma in 10 ml glass tube. Quality control samples were prepared by spiking CTZ and AMB standards within the calibration limit.

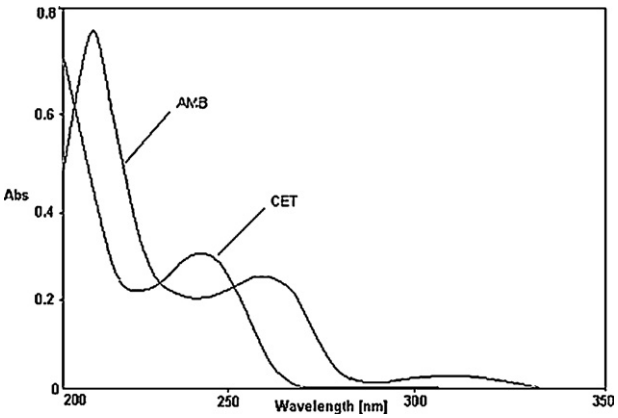

Fig. 2. Overlaid UV spectra of CTZ and AMB.

Standard and QC solutions for urine analysis were prepared by spiking 50 μl each of CTZ, AMB and IS solution to 500 μl of urine.

2.3. Preparation of plasma and urine samples

Standard, QC solutions and plasma samples drawn from human volunteer in aliquot (250 μl) were mixed with 3 ml of methylene chloride and diethylether mixture (80:20, v/v) and vortex-mixed thoroughly for 30 s. After a centrifugation for 15 min at 4000 × *g*, the upper clear portion in each tube was carefully transferred to a 10 ml glass tube and evaporated to dryness under nitrogen gas at 30 °C. The residue in each tube was reconstituted by 500 μl of mobile phase 1.

Similarly, standard solutions, QC solutions and samples obtained from human volunteer spiked in 500 μl of urine were extracted with 500 μl of diethyl ether and evaporated to dryness under nitrogen at 35 °C. The residue then reconstituted with 500 μl of mobile phase 1.

2.4. Micro bore column switching HPLC

CTZ and AMB were determined by column switching HPLC with PDA detection using an automated HPLC (Prominence) series equipped with two pumps LC20AT, PDA detector SPD M20, auto sampler, a column oven, a high-pressure switching valve (FCV-14AH) and a degassing unit (Shimadzu, Japan).

A SupelcosilTM LC ABZ multifunction column (50 mm × 4.6 mm, 5 μm particle size) (Ardmore, USA) was used for selective absorption of CTZ and AMB in plasma. A Kromasil C_8 micro bore analytical column (50 mm × 0.3 mm, 5 μm particle size) (Bohus, Sweden) was used for concentration of CTZ and AMB. A Kromasil C_{18} analytical column (250 mm × 2.1 mm, 5 μm particle size) was used as a separation column. The column temperature was maintained at 45 °C during analysis. Pretreatment and concentration columns were operated at ambient temperature. Pumps 1 and 2 were used to deliver mobile phase 1 at a flow rate of 0.5 ml /min and mobile phase 2 at a flow rate of 0.4 ml/min, respectively. Detection was performed at 230 nm for both plasma and urine sample conveniently and the UV spectra of CTZ and AMB are given in Fig. 2.

2.5. Analytical procedure

A schematic diagram of HPLC system with position of switching valve is shown in Fig. 3. Sample loading and chromatographic separation were performed as follows.

Step 1: Plasma sample (150 μl) was introduced into the pretreatment column via the auto sampler using mobile phase 1 at a flow rate of 0.5 ml/min. At the time of sample injection, the column switching valve was placed in position A. The pretreatment column kept CTZ, AMB and IS retained in the column. Residual proteins of plasma were eluted in void volume and drained out surpassing the analytical column.

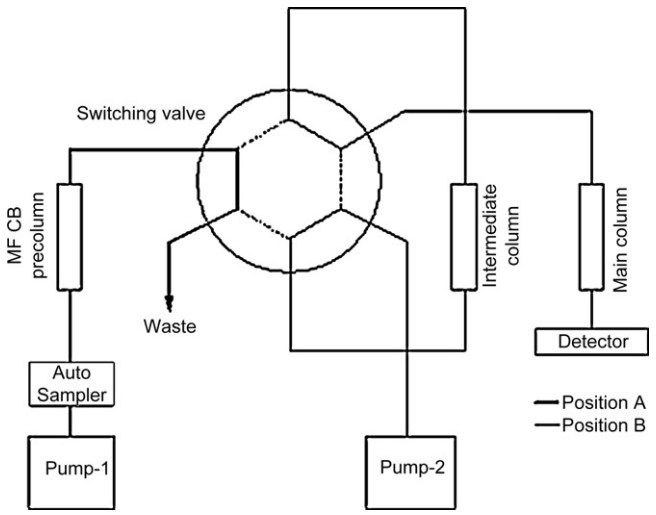

Fig. 3. Schematic diagram of column switching system. (A) Sample loading, precolumn wash and re-equilibrium position and (B) elution, separation phenomena.

Step 2: The switching valve was shifted to position B at 7.5 mn, thus CTZ, AMB and IS were eluted from the pretreatment column between 7. 5 and 11.5 min and introduced to the concentration column by isocratic elution using mobile phase 1 at a flow rate of 0.5 ml/min.

Step 3: Finally, CTZ, AMB and IS adsorbed to the concentration column were introduced to the separation column by switching valve with back-flush technique using mobile phase 2 with a flow rate of 0.4 ml/min at a wavelength of 230 nm. During the analysis the pretreatment column was washed for the next analysis and resume to the position A. The total run time was 25 min for a sample. The procedure was repeated for urine analysis with a detection wavelength of 230 nm.

2.6. Assay validation

2.6.1. Specificity and sensitivity

Specificity was demonstrated by analyzing standard solutions of CTZ and AMB, drug – free plasma, plasma spiked with CTZ and AMB. The procedure was adopted for drug free urine, urine spiked with CTZ and AMB. The limit of quantization (LLOQ) was based on signal to noise ratio (*S*/*N*) 1:10.

2.6.2. Linearity

Plasma standards and standards for urine analysis at six concentrations over linear range were analyzed for five consecutive days to study the linearity of the method. Standard samples were prepared by adding CTZ at concentrations of 2, 50, 150, 300 and 450 ng/ml and AMB 7, 50, 100, 200 and 300 ng/ml along with IS 20 ng/ml to 500 μl of plasma. Similarly for urine sample analysis 1, 75, 150, 250, 350–500 ng/ml of CTZ and 5, 75, 200, 300 and 400 ng/ml AMB were added to 500 μl of urine, extracted and analyzed as described in Section 2.3. Peak area ratios of CTZ and AMB to IS were measured and calibration curve was obtained from least-square linear regression.

2.6.3. Precision and accuracy

The intra-and inter-day precision and accuracy were determined by analyzing plasma and urine samples spiked at 10, 60,

120, 240 ng/ml and 20, 80, 160, 240 ng/ml, respectively for CTZ and AMB on four different days. The coefficient of variation was calculated from the standard deviation (S.D.) to the mean peak area of CTZ and AMB. Accuracy was calculated by comparing the difference between the spiked concentration and the concentration found.

2.6.4. Recovery

Recovery was assessed using CTZ and AMB spiked blank plasma at four different concentrations 10, 60, 120, 240 ng/ml and 20, 80, 160, 240 ng/ml, respectively. It was estimated by comparing the mean peak area ratios of the extracted spiked plasma to the mean peak area ratios of equivalent aqueous standard solutions.

2.6.5. Stability

Stability of CTZ and AMB in plasma and urine under storage condition and experimental conditions of the study was investigated by analyzing plasma spiked with standard drug at three concentrations within calibration range which have been stored at 2 °C for 28 days and a set of the samples which were kept at room temperature for 24 h.

2.6.6. Effect of carryover

As sample carryover can cause serious problems with peak quantification and identification, its assessment becomes important in the validation of a HPLC method. Carryover is recognized as the presence of a small peak that appears when a blank is injected after a sample that produces a large peak. Processed plasma and urine (200 μl) spiked with standard solution of CTZ, AMB and IS at HQC level was injected into the HPLC system and observed for appearance of any peak response.

2.7. Pharmacokinetics of CTZ and AMB in healthy human volunteer

A pharmacokinetic study was performed in male healthy human volunteer ($n = 3$, age range 20–26 yrs) to show the applicability of the newly developed bio-analytical method after a single oral administration of CTZ 5 mg and AMB 30 mg [16,17]. Blood in quantity of 5 ml was collected into heparinized tubes from each volunteer at time points as; 0 h, 0.50, 1, 1.5, 2, 3, 5, 7, 10 and 12 h after oral administration. Samples were kept in deep freezer until the analysis. Plasma separation was carried out by centrifugation at 4000 rpm for 5 min at 25 °C and immediately pretreated according to Section 2.3 and analyzed. The study adheres to the principles of human ethical committee and is approved by the Human Research Ethics Committee (HREC) of Roxaane Reseaarch laboratories, Chennai, India.

2.7.1. Pharmacokinetic data and analysis

The pharmacokinetic study parameters viz., maximum plasma concentration time curve from 0 to the last measured concentration ($AUC_{0-\alpha}$), area under plasma concentration–time curve (AUC_{0-t}) and half life of drug elimination during the terminal phase ($t_{1/2}$) were estimated using non-compartmental analysis method for each plasma concentration–time point using WinNolin professional software version 4.0.1 (Pharsight corporation, USA).

2.7.2. Analysis of urine samples

Urine samples obtained from a clinical study ($n = 6$), that compares excretion pattern of CTZ and AMB in patients with renal impairment and healthy volunteers at time points 0 h (pre dosing) 0.50, 1, 1.5, 2, 3, 5, 7, 10 and 12 h post dosing were collected and pretreated according to Section 2.3 and analyzed [10,11].

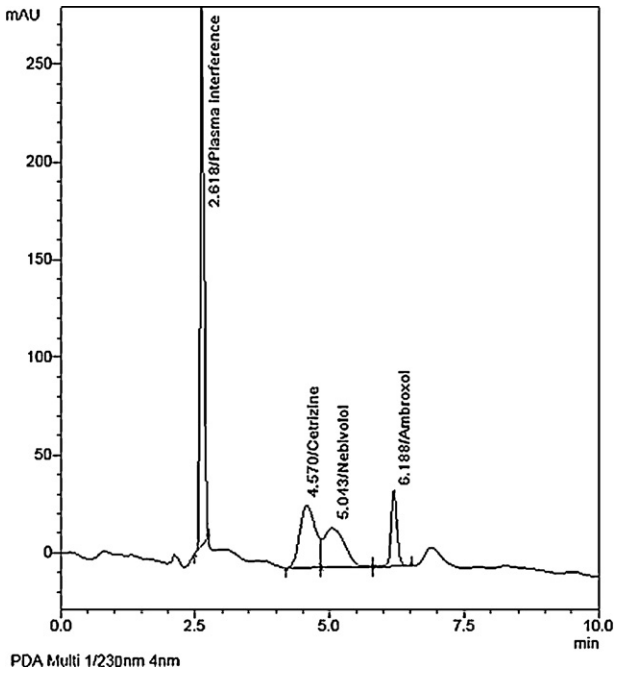

Fig. 4. Chromatogram of CTZ, AMB and IS eluted without column Switching technique.

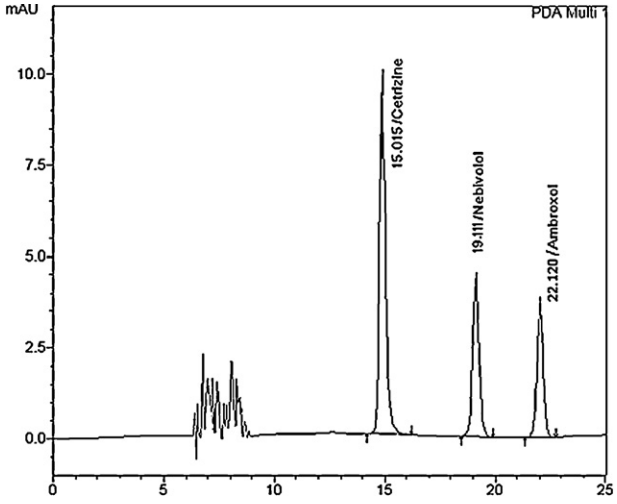

Fig. 5. Chromatogram of CTZ, AMB and IS eluted by column switching technique.

3. Results and discussion

3.1. Column switching system of CTZ and AMB

To determine the elution profile for clean-up procedures of plasma and urine CTZ, AMB and IS of 1000 ng/ml in extracted blank plasma and urine were analyzed in the pretreatment column using the mobile phase 1. CTZ, AMB and IS were eluted around 4.5, 5.0 and 6.1 min, respectively, but separation between CTZ and IS was partially achieved (Fig. 4). Based upon this fact an elution pattern was designed for these compounds with appropriate resolution. In the first step, the matrix interferents were flushed out then CTZ, AMB and IS were introduced to the concentration column from 4 to 7 min in intermediate column. Finally, CTZ, AMB and IS retained on the concentration column were eluted and introduced into the analytical column and analyzed by mobile phase 2 (Fig. 5).

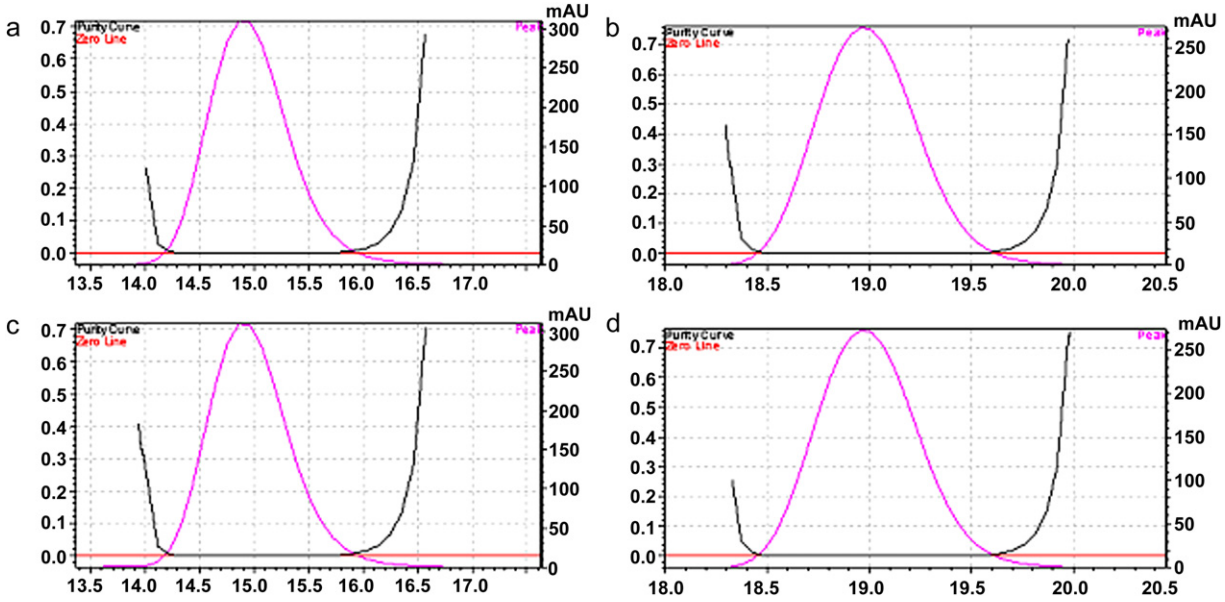

Fig. 6. Peak purity curve of standard solution of (a) CTZ and (b) AMB in plasma; (c) and (d) in urine sample.

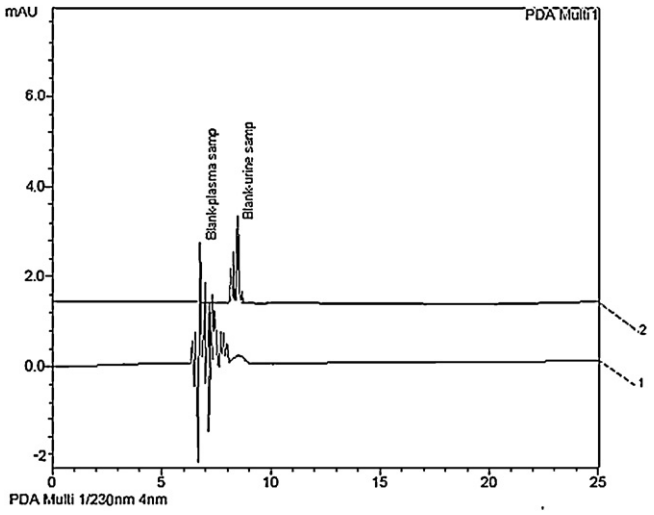

Fig. 7. Overlaid chromatogram of processed plasma and urine.

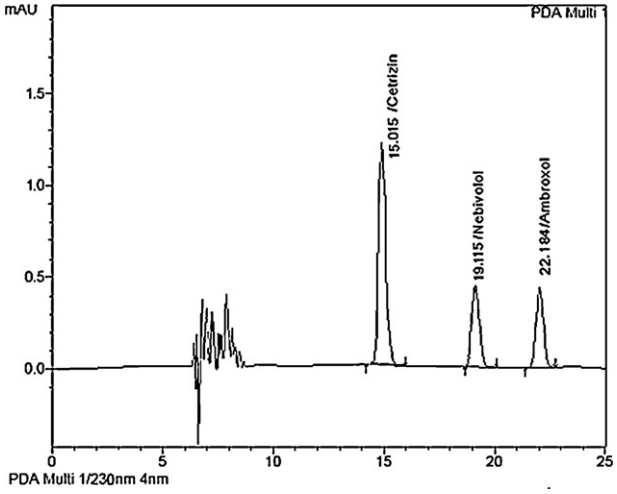

Fig. 8. Chromatogram of LLOQ sample of CTZ, AMB IS in plasma.

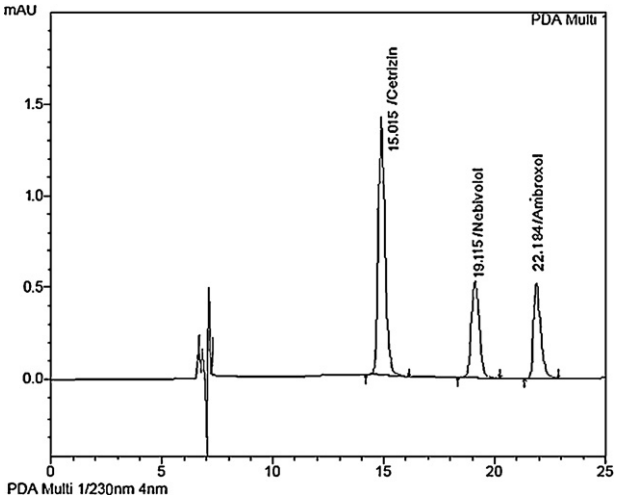

Fig. 9. Chromatogram of LLOQ sample of CTZ, AMB and IS in urine.

3.2. Specificity and sensitivity

Using the above determined column switching HPLC method, typical chromatograms of CTZ, AMB and IS analyzed in human plasma and urine samples were presented. Under this condition, the retention times for CTZ, AMB and IS were 14.8, 20.9 and 21.9 min, respectively, with complete baseline resolution between peaks of CTZ, AMB and IS. Also, no interfering endogenous peaks were detected in chromatogram. The selectivity of the method was further assessed by peak purity curves and chromatograms of processed blank samples of plasma and urine. The peak purity curves for CTZ and AMB are given in Fig. 6. The chromatogram of processed blank plasma and urine are given in Fig. 7.

The lower limit of quantization of the method (LLOQ) was found to be 2 ng/ml for CTZ and 7 ng/ml for AMB in plasma samples. The LLOQ for urine spiked standard solutions were found to be 1 ng/ml for CTZ and 3.5 ng/ml for AMB. The LLOQ was found lower than that obtained by the previous methods [5,10–12,19,21]. Chromatograms recorded for CTZ and AMB at LLOQ concentration are given in Figs. 8 and 9.

Table 1
Precision and accuracy of CTZ and AMB ($n = 4$).

Concentration (ng/ml)	CTZ				Concentration (ng/ml)	AMB			
	Precision (CV%)		Accuracy (%)			Precision (CV%)		Accuracy (%)	
	Inter-day	Intra-day	Inter-day	Intra-day		Inter-day	Intra-day	Inter-day	Intra-day
10	13.5	7.9	89.7 ± 3.9	100.5 ± 0.8	20	8.2	10.2	99.8 ± 4.7	101.1 ± 2.2
20	10.7	13.8	100.5 ± 0.9	99.98 ± 1.2	40	4.8	3.8	99.7 ± 11.2	99.9 ± 5.3
60	12.9	14.2	101.6 ± 2.6	98.9 ± 8.9	80	12.5	12.8	100.1 ± 3.5	99.9 ± 11.1
120	5.7	14.6	99.3 ± 11.2	100.7 ± 4.7	160	5.7	11.1	100.2 ± 5.3	101.2 ± 2.2
240	10.6	4.9	101. ± 5.3	100.1 ± 7.5	240	13.7	6.2	98.6 ± 45	99.7 ± 1.9
450	12.3	12.7	99.77 ± 1.2	98.8 ± 3.9	300	11.9	12.1	100.6 ± 8.9	99.9 ± 5.8

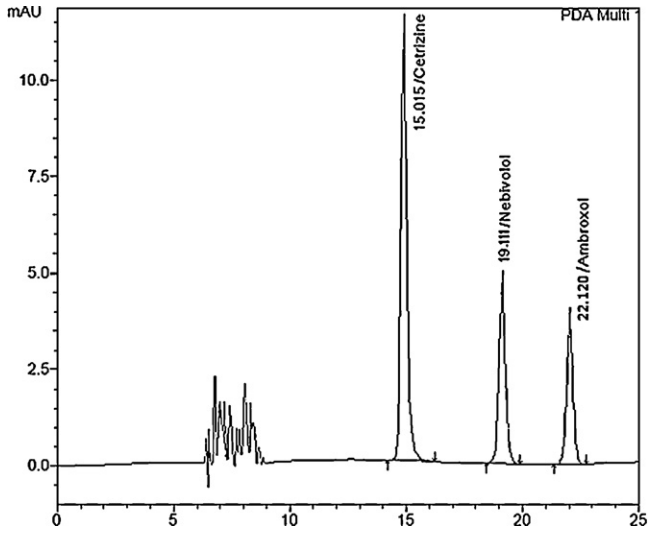

Fig. 10. Chromatogram of CTZ, AMB and spiked IS in plasma of a volunteer (0.5 h sample).

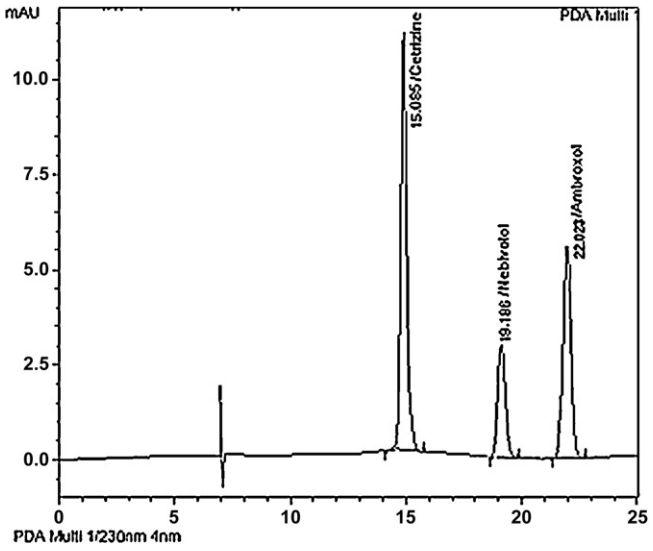

Fig. 11. Chromatogram of CTZ, AMB and spiked IS in urine of a volunteer (0.5 h sample).

3.3. Linearity

The assay was linear for CTZ and AMB over the range of 2–450 ng/ml and 7–300 ng/ml, respectively, with typical calibration curve of $y = 8.975x$ and $y = 19.76x$ (where y is CTZ and MB concentration and x is ratio of peak area) with the correlation coefficient of 0.998 and 0.999.

Spiked standard solutions in urine were linear from 1 to 500 ng/ml for CTZ and 5 to 400 ng/ml for AMB with regression equations $y = 13.20x$ and $y = 19.84x$ for CTZ and AMB, respectively, with correlation coefficient of 0.999 and 0.996.

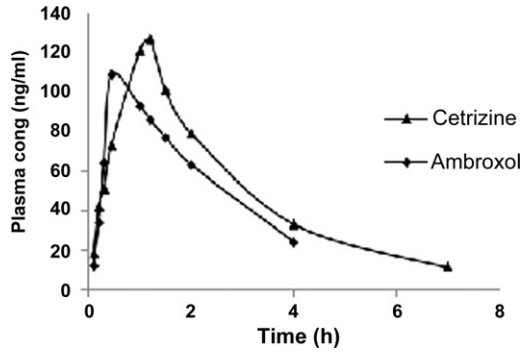

Fig. 12. Mean plasma concentration–time profile of CTZ, AMB in Albino rat plasma.

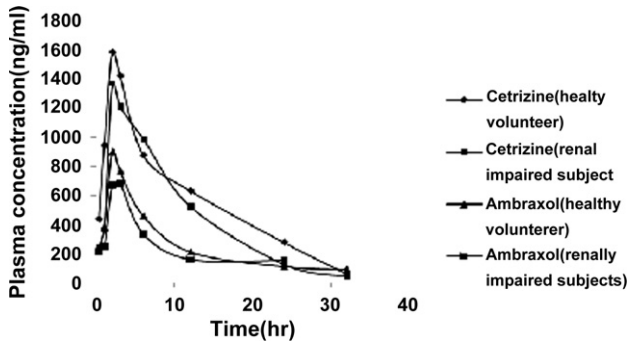

Fig. 13. Mean plasma concentration–time profile of CTZ, AMB in urine samples of healthy volunteer and subjects with renal dysfunction.

3.4. Precision and accuracy

Intra-day and inter-day precision of CTZ and AMB calculated as a coefficient of variation (CV) was always below 15% at LOQ level and accuracy of CTZ and AMB expressed as a percentage of the measured concentration to the theoretical concentration ranged from 94 to 101.6% and 91.1 to 100.2%, respectively, Table 1.

3.5. Recovery

Several sample preparation and extraction methods have been reported for CTZ and AMB in biological fluids. A rapid and efficient liquid–liquid extraction procedure of CTZ and AMB from plasma was achieved by using methylene chloride and diethyl ether

Table 2
Freeze thaw stability of CTZ and AMB in plasma ($n = 5$).

CTZ			AMB		
Sample conc. (ng/ml)	Amt found (mean ± SD) ng/ml	%CV	Sample conc. (ng/ml)	Amt found (mean ± SD) ng/ml	%CV
Short term stability 24 h					
10	11 ± 0.82	8.21	20	18.7 ± 1.21	7.32
60	57 ± 1.1	3.8	80	82 ± .11	6.98
120	123 ± 0.87	7.2	240	234 ± 0.76	4.56
Long term stability 28 days					
10	9 ± 0.72	9.1	20	23.7 ± 1.29	7.32
60	62 ± 0.2	4.3	80	77 ± 0.38	3.98
120	117 ± 0.48	6.9	240	242 ± 0.94	7.48
Freeze thaw stability (3 cycles)					
10	12 ± 1.21	9.32	20	19.1 ± 4.21	9.44
60	64 ± 0.36	7.6	80	80 ± 2.11	7.34
120	116 ± 0.49	9.5	240	232 ± 1.3	5.49

Table 3
Pharmacokinetic parameters of CTZ and AMB in Albino rats.

Parameters	CTZ	AMB
C_{max} (ng/ml)	127	109
T_{max} (h)	1.2	0.45
$AUC_{(0-t)}$ h ng/ml	352.3	240.07
$AUC_{(0-\alpha)}$ h ng/ml	406.11	649.73
$T_{1/2}$ (h)	3.39	11.83

(80:20) mixture as an extraction solvent with recovery of 93% for CTZ and 87% for AMB. Recovery of IS was found to be 84%, which is sufficient to compensate for any loss of analyte during sample preparation and cleanup. The recovery study was carried out at three levels LLOQ, mid and HQC level to prove that the extraction ability is not concentration dependant.

3.6. Stability

CTZ, AMB and IS in plasma showed no tendency of degradation at −20° for 28 days, whereas, urine samples were stable up to 40 days. Plasma and urine samples spiked with CTZ, AMB and NBV showed no loss of analytes at 2 °C for 24 h. The quality control samples were subjected to three freeze–thaw cycles and compared with freshly prepared quality control samples. The mean concentration of CTZ and AMB quality control samples did not change significantly within the time period under the storage conditions. The results of freeze–thaw stability are given in Table 2.

3.7. Assessment of carryover

There was no peak response observed for blank plasma, and urine samples injected immediately after a sample spiked with CTZ, AMB and IS at HQC concentration (240 μg/ml) was injected. This fact confirms that the method developed is immune against carryover effect.

3.8. Pharmacokinetic application

The present study was applied for the analysis of CTZ and AMB in plasma of healthy human volunteer ($n = 3$) after an oral administration of CTZ 5 mg/kg and AMB 30 mg/kg. Chromatograms recorded for plasma and urine samples drawn from human subjects are given in Figs. 10 and 11. The oral pharmacokinetics of both the drugs was calculated in terms of extent of absorption and given in Table 3. The mean concentration versus time graph is given in Figs. 12 and 13 for plasma samples and urine samples. The results showed that the method was simple and selective for the determination of CTZ and AMB in plasma samples. One way ANOVA treatment on the data of urine analysis revealed that subjects with renal impairment excreted CTZ and AMB to significantly lesser extent than healthy humans. From the results, it can be inferred that the column switching HPLC method presently developed can be applied to routine monitoring of CTZ and AMB in human plasma and urine.

4. Conclusions

A specific and sensitive analytical method for the determination of CTZ and AMB in human plasma using column switching HPLC with PDA detection was developed. This method has a major advantage of eliminating the peaks resulting from plasma matrices and concentrating the analyte. The method has been successfully used to probe pharmacokinetic pattern of CTZ and AMB in human plasma and can be used to monitor concentration of these drugs urine samples.

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