Development of HPTLC method for determination of cefpodoxime proxetil and ambroxol hydrochloride in human plasma by liquid–liquid extraction

Abstract

Aim: To develop a simple, sensitive, rapid, and economic high performance thin layer chromatographic method for determination of cefpodoxime proxetil (CEFPO) and ambroxol hydrochloride (AMBRO) in human plasma by liquid–liquid extraction using paracetamol as an internal standard. **Materials and Methods:** The plasma sample was extracted by a mixture of methanol and acetonitrile. A concentration range from 500 to 3500 ng/spot for CEFPO and 1000 to 7000 ng/spot for AMBRO were used for the calibration curve, respectively. This recovery was found to be 74.40 and 94.50 for CEFPO and AMBRO, respectively. The mobile phase used consists of chloroform: methanol (9:1v/v). Densitometric analysis was carried out at a wavelength of 240 nm. **Results:** The Rf values for CEFPO, AMBRO, and paracetamol were found to be 0.69 ± 0.005, 0.49 ± 0.0057, and 0.31 ± 0.0054, respectively. The stability of CEFPO and AMBRO in plasma was confirmed during three freeze–thaw cycles (-20°C), on bench during 24 h and post preparative during 48 h. **Conclusion:** The proposed method was validated statistically and by performing a recovery study for determination of CEFPO and AMBRO in human plasma.

Key words: Cefpodoxime proxetil, ambroxol hydrochloride, human plasma, HPTLC, liquid, liquid extraction

Ambadas R. Rote, Sadhana K. Kande

Department of Pharmaceutical Chemistry, MGV's Pharmacy College, (Pune University) Panchavati, Nashik, India

Address for correspondence:
Mr. Ambadas R. Rote, Department of Pharmaceutical Chemistry, MGV's Pharmacy College (Pune University) Panchavati, Mumbai Agra Road, Nashik, India.
E-mail: roteambadas@gmail.com

INTRODUCTION

Chemically cefpodoxime proxetil (CEFPO) [Figure 1] is (6R,7R)-7-[(2Z)-2-(2-amino-1,3-thiazol-4-yl)-2-(methoxyimino)acetamido]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid. It is an oral third generation cephalosporin antibiotic. It is active against most gram positive and gram negative bacteria. Ambroxol hydrochloride (AMBRO) [*trans*-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol hydrochloride] is a semi-synthetic derivative of vasicine obtained from an Indian shrub "Adhatoda vasica". It is a metabolic product of bromhexine. Ultraviolet-visible spectrophotometric method has been reported for the quantitative determination of CEFPO from pharmaceutical formulation spectrophotometric method…[1] using high performance liquid chromatography (HPLC),[2-4] and high performance thin layer chromatography (HPTLC).[5] A method for the simultaneous determination of AMBRO has not been reported with CEFPO such as UV spectrophotometry,[6] HPTLC[7] and HPLC[8] and in human plasma using LC-MS/MS.[9] No simultaneous estimation method was developed for determination of CEFPO and AMBRO in human plasma. Therefore, a simple, sensitive, rapid, and economic HPTLC method has been developed for the determination of CEFPO and AMBRO in human plasma using paracetamol as an internal standard.
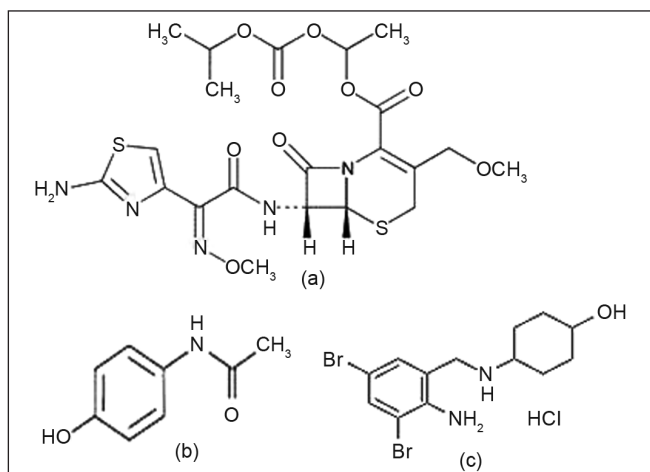
Figure 1: (a) Structure of cefpodoxime proxetil, (b) Paracetamol, (c) (IS), and ambroxol hydrochloride

MATERIALS AND METHODS

Instrumentation
HPTLC Camag with precoated silica gel Plate 60F$_{254}$ (20 cm ×10 cm) 250 μm thicknesses (E. Merck, Darmstadt, Germany) was used as the stationary phase. Sample application was done by using a Camag 100 μl syringe and a Camag Linomat V applicator. The sample was sprayed in the form of narrow bands of 8 mm length at a constant rate 2 μl/s. Linear ascending development was carried out in a 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). The densitometric scanning was performed by using a Camag TLC scanner III supported by win CATS software (V1.4.2.8121 Camag). Chromatogram was evaluated by using a ratio of peak areas of drugs with an internal standard.

Chemicals
CEFPO (Blue Cross Laboratories Ltd., Ambad Nashik, India), AMBRO (Blue Cross Laboratories Ltd., Ambad Nashik, India), and paracetamol (Kirti Pharmachem Ltd., Sinner, Maharashtra, India) were received having 98.80%, 98.70% and 100.1% purity, respectively. They were used as such without checking their purity. The HPLC grade methanol and Analytical reagent grade chloroform were purchased from S D Fine Chem. Ltd., Mumbai, India. Human plasma used for research work was supplied by Arpan Blood Bank, Nashik, Maharashtra, India.

Preparation of stock solution and working standard solution
Stock solutions 1.0 mg/ml each of CEFPO, AMBRO and paracetamol were prepared in methanol.

Preparation of plasma sample
In a 15 ml centrifuge tube 10, 20, 30, 40, 50, and 60 μl of working stock solution of CEFPO were added to drug-free plasma to provide calibration standards of 500, 1000, 1500, 2000, 2500, and 3000 ng/ml and 2, 4, 6, 8, 10, and 12 μl of working stock solution of AMBRO were added to drug-free plasma to provide calibration standards of 1000, 2000, 3000, 4000, 5000, 6000 ng/ml and 1000 ng/ml of paracetamol (internal standard) was kept constant. The quality control (QC) samples were prepared in plasma in the concentration range 1000, 2000, 3000 ng and 2000, 4000, 6000 ng for CEFPO and AMBRO. Protein precipitation and extraction were carried out by using 3 ml of methanol and 0.1 ml of acetonitrile by vigorous vortex mixing using a remi mixer for 2 min and centrifuged at 5000 rpm at 10 min. The organic phase was recovered and evaporated to dryness on a hot plate. The residual mass was reconstituted with 1 ml of methanol. The analysis was carried on HPTLC.

Chromatographic condition
The mobile phase was selected as a mixture of chloroform and methanol (9.0:1.0 v/v) for the development of plates. Time for chamber saturation was optimized to 10 min. The length of the chromatographic development was 70 mm. The densitometric scanning was performed at 240 nm.

Method validation
The method was validated for sensitivity, selectivity, precision, accuracy, linearity, recovery, and stability. The validation of the method was based on FDA guidelines and on the standard bio-analytical method validation recommendations. The selectivity of method was investigated by analyzing six blank plasma samples. Each blank sample was tested for interference using a proposed extraction procedure. Five replicates of three QC sample low, mid, and high were used for the determination of precision and accuracy. Intra-day and inter-day precision were carried out. Precision and accuracies showed 15% relative standard deviation from nominal values, at lower limit of quantitation (LLOQ) these were both 20%. The recovery of CEFPO and AMBRO was calculated by a comparison of the peak areas of low, mid, and high QC sample (1000, 2000, 3000 ng/ml and 2000, 4000. 6000 ng/ml, respectively) prepared in plasma (extracted) with unextracted CEFPO and AMBRO, respectively. Stability studies were performed to detect degradation of CEFPO and AMBRO under certain conditions. Freeze–thaw stability was determined at two QC concentrations

(low, high) after freezing (−20°C) and thawing for three cycles and compared with the nominal value. Bench-top stability was assessed for low and high QC samples by comparing with the nominal value which stored at room temperature for 12 h. The effect of storage within the auto-sampler was assessed by comparing the QC samples injected immediately after preparation with those left in the auto-sampler for 48 h.

RESULTS AND DISCUSSION

Extraction procedure optimization
One of the most difficult tasks during the method development was to achieve a reproducible recovery from the solvent which is used for the extraction of the drug. Different solvents were tried for the extraction of CEFPO and AMBRO from human plasma. Three millilitres of chloroform and 3 ml of ethyl acetate were tried for the precipitation of plasma but the recovery was very less up to 50–60% because of less precipitation of protein from plasma. Finally methanol was tried and 60–80% of recovery was obtained. It was found that the addition of acetonitrile (0.1 ml) increases the recovery which is reproducible as compared to other solvents. Therefore, methanol and acetonitrile (3.0:0.1, v/v) was used as the final solvent for extraction of CEFPO and AMBRO.

Optimization of chromatographic conditions
Initially plane solvents such as methanol, ethyl acetate, chloroform, toluene, and acetone were tried. The spots were developed with chloroform and methanol but no proper resolution was observed between CEFPO and AMBRO. Paracetamol shows the tailing. Then chloroform and acetone (8:1, v/v) were tried but again there was no proper resolution. Lastly upon increasing the concentration of chloroform from 8 ml to 9 ml and adding methanol instead of acetone good resolution with symmetrical peaks of CEFPO, AMBRO and paracetamol was obtained. Finally the mobile phase consisting chloroform: methanol (9:1, v/v) gave good resolution of peaks for CEFPO, AMBRO, and paracetamol. The R_f values for CEFPO, AMBRO, and paracetamol were found to be 0.69 ± 0.005, 0.49 ± 0.0057, and 0.31 ± 0.0054, respectively. Well-defined spots were obtained by an activating plate at 120°C for 20 min. The chamber was saturated with the mobile phase for 10 min at room temperature, which gave reproducible R_f values for cefpodoxime proxeti, AMBRO, and paracetamol, respectively [Figure 2].

Calibration curves
The six-point calibration curve was constructed by plotting the peak response ratio of CEFPO to IS and AMBRO to IS in plasma. Correlation coefficients were found to be 0.9928 and 0.9941 and linearity was found over the range of 10–60 µl/ml and 2–12 µl/ml for CEFPO and AMBRO, respectively. The lower limit of quantification was defined as the lowest concentration in the calibration curve. The CEFPO and AMBRO can be determined at LLOQ.

Recovery
Absolute recoveries were calculated by comparing peak areas obtained from freshly prepared samples extracted with unextracted standard solutions of the same concentration. Recovery data were determined in triplicate at three concentrations (low, mid, and high) as recommended by FDA guidelines. The percent recovery at three concentrations was found to be 65.72, 74.46, and 83.04% for CEFPO and 97.50, 90.89, and 95.11% for AMBRO [Table 1].

Precision and accuracy
Precision of the method was determined by intra-day and inter-day and accuracy for a set of QC samples (low, mid, and high) in replicate (n = 5). In this assay,
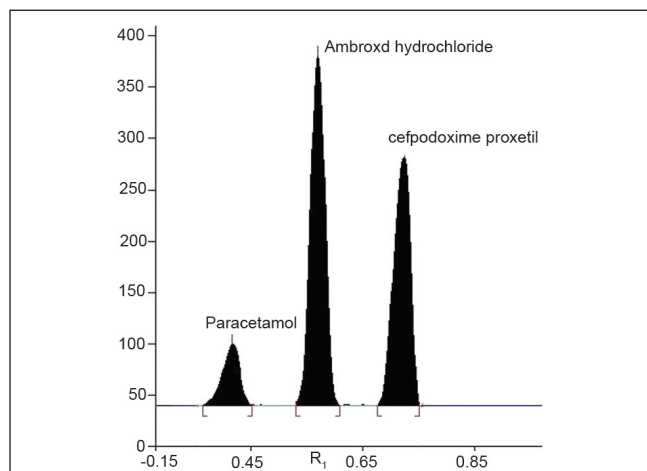

Figure 2: Densitogram of cefpodoxime proxetil (R_f, 0.69 ± 0.005), ambroxol hydrochloride (R_f, 0.49 ± 0.0057), and paracetamol (R_f, 0.31 ± 0.00543.3).

Table 1: Result of recovery of cefpodoxime proxetil (CEFPO) and ambroxol hydrochloride (AMBRO) in human plasma

Concentration		%Recovery		%RSD	
CEFPO	AMBRO	CEFPO	AMBRO	CEFPO	AMBRO
1000	2000	65.72	97.50	1.20	2.30
2000	4000	74.46	90.89	2.25	1.32
3000	6000	83.04	95.11	1.92	2.35

the intra-day precision was found to be in the range of 1.78–2.68% and 6.38–10.77% and the inter-day precision was 6.61–19.73% and 6.38–7.79%. The accuracy was within 1.15–2.50%. The above values were within the acceptable range, it shows that the method is accurate and precise. The low percent relative standard deviation (%RSD) and the percent relative error (%RE) were within the acceptable limit. The results of inter-day, intra-day precision, and accuracy for the CEFPO and AMBRO are shown in Table 2.

Sensitivity and selectivity

Selectivity should be assessed to show that the intended analytes are measured and that their quantitation is not affected by the presence of the biological matrix. For the HPTLC method by LLE as shown in Figure 2, there is no any interference of the biological matrix in the quantitation of CEFPO and AMBRO. Sensitivity of the method is defined as the lowest concentration that can be measured with an acceptable limit of accuracy and precision which is lower than 20%.[10] The accuracy and precision at a lower limit of quantitation (LLOQ) analyzed by using five replicate (*n* = 5) of the sample at the LLOQ concentration. The accuracy is determined by %RE at this LLOQ concentration. The lower limit of quantitation was found to be 500 ng/spot and 1000 ng/spot with %RE = 2.382, 0.198 and

%RSD = 17.28, 5.96 for CEFPO and AMBRO within acceptable limits.

Analysis speed

In case of HPTLC 18 spots can be applied on one plate, so it is less time consuming.

Stability

In bench top stability, the low and high QC samples were thawed and allowed to remain at room temperature for 12 h. A comparison of the results for the QC sample (low and high) with freshly prepared stock solution showed that there was no significant difference between response of freshly prepared solution and a sample of CEFPO and AMBRO after 12 h. Freeze–thaw stability was determined after two freeze–thaw cycles for three replicate of low and high QC samples. The samples were stored at −20°C temperature for 24 h and then thawed at room temperature. No significant difference between the freeze–thaw sample and freshly prepared sample was observed. The result of stability experiments shows that no significant degradation occurred at ambient temperature for 48 h for post preparative stability. Results of stability for the HPTLC method are shown in Table 3.

CONCLUSION

The proposed HPTLC method for the estimation of CEFPO and AMBRO in human plasma is selective and sensitive. The method is economical and faster than earlier published methods. In future, these methods can be used for bioequivalence study.

ACKNOWLEDGMENTS

Table 2: Results of precision of cefpodoxime proxetil and ambroxol hydrochloride in human plasma

Precision	Concentration (ng)		% RSD		% RE	
	CEFPO	AMBRO	CEFPO	AMBRO	CEFPO	AMBRO
Intraday	1000	2000	1.78	6.38	19.31	2.47
	2000	4000	2.80	6.57	6.9	2.07
	3000	6000	2.68	10.77	10.02	3.69
Interday	1000	2000	19.73	6.38	19.40	8.70
	2000	4000	7.38	6.57	16.95	2.49
	3000	6000	6.61	7.79	1.36	6.74

Table 3: Stability study of cefpodoxime proxetil and ambroxol hydrochloride in human plasma

Parameters	Concentration		SD		%RSD	
	CEFPO	AMBRO	CEFPO	AMBRO	CEFPO	AMBRO
Freez–thaw stability						
Low	1000	2000	266.3	179.6	2.93	1.79
High	3000	6000	642.66	269.05	2.63	1.72
Bench top stability						
Low	1000	2000	207.29	263.43	1.81	1.86
High	3000	6000	261.34	362.83	2.0	2.08
Post preparative stability						
Low	1000	2000	455.90	300.74	1.20	2.0
High	3000	6000	214.45	298.18	1.7	1.73

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