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LC determination of cephalosporins in *in vitro* rat intestinal sac absorption model

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Abstract

Cefotaxime sodium (CX) and Ceftazidime pentahydrate (CZ) are peptidomimetic cephalosporins (CPS) which exist as zwitterionic compounds at physiological pH and because of this reason they are not absorbed appreciably on peroral administration. The permeability of these compounds can be increased transiently by altering membrane characteristics of absorptive epithelium by use of sorption promoters (SPs). In present work a simple validated HPLC method utilizing isocratic mobile phase and having short retention times for CX and CZ is developed which can be used to monitor their concentrations in Kreb's Ringer Bicarbonate (KRB) solution in in vitro intestinal sac absorption model. The same was utilized to determine apparent permeability coefficients and absorption profiles of CPS by modified Wilson–Wiseman method. The CPS were analysed by the reverse phase HPLC method using Shim-pack C18 column. The mobile phase used was of isocratic composition with phosphate buffer (pH 7.0, 3.5 g/l of KH_2PO_4 dissolved in 0.03 M $Na_2HPO_4 \cdot 2H_2O$) and methanol in proportion 85:15 for CZ and 70:30 for CX. The flow rate was 1ml/min and quantitative determinations were carried out at 254 nm at 25 °C. The method was found specific because none of the proposed SPs, components of KRB and intestinal sac artefacts interfered with the drug peaks. The drug concentration versus area under peak relationship was found to be linear in concentration range of 0.25–20.0 μg/ml. The recovery studies, intraday variation, interday variation and interanalyst variation were within statistical limits. The limit of detection (LOD) was 95.0 and 100.0 ng/ml for CZ and CX, respectively. The limit of Quantitation (LOQ) was 240.0 and 250.0 ng/ml for CZ and CX, respectively. The proposed method was found to be rapid and selective and hence applied for continuous monitoring of CPS in in vitro intestinal sac absorption studies.
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Keywords: Reversed phase chromatography; Cefotaxime sodium; Ceftazidime petahydrate; Apparent permeability coefficient

1. Introduction

Cephalosporins are semi-synthetic antibiotics of β-lactam family. Ceftazidime pentahydrate (CZ)

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and Cefotaxime sodium (CX) are third generation peptidomimetic cephalosporins which, because of their low lipophilicities and zwitterionic character at physiological pH [1], exhibit low intrinsic membrane permeabilities.

CZ (usual dose 1–6 g daily administered at 8–12 h intervals) has been shown to be effective in wide range of infections which are usually of moderate or greater severity, including lower respiratory tract infections, urinary tract infections, septicaemia/bacteraemia, skin, soft tissue, bone and joint infections, intra-abdominal, obstetric and gynecological infections, meningitis, infections in febrile neutropenic patients [2]. On administration of 1 g of CZ intramuscularly and intravenously, the plasma concentrations of 40 and 70 μg/ml are obtained, respectively. It is 80–90% eliminated in urine with biological half-life of 2 h [3].

CX is more active against multiple drug resistant gram-negative bacilli than are moxalactum, CZ and cefoperazone. It is indicated in genitourinary infections, gynecological, cutaneous, intra-abdominal, bone and joint infections, septicemia and in surgical prophylaxis [3]. When 2 g of CX is administered intravenously, it produces plasma levels of 80–90 μg/ml. About 20–36% of the administered drug is eliminated unchanged in the urine [4]. Half-life is 1–1.3 h but increases to 3–12 h in renal failure. It is metabolised up to extent of 30–50% to give an active β-lactamase stable metabolite.

Both, CX and CZ, are peptidomimetic drugs (water soluble and poorly permeable through biological membranes) and fall into the category of class III drugs according to BCS (Biopharmaceutics Classification System) [5]. They exhibit poor oral bioavailabilities because of their incapability to permeate gastrointestinal tract mucosa. Due to their similarity in structural and biopharmaceutic profile with proteins and peptides they can be used as model drugs for characterising absorption of peptidomimetics in in vitro everted rat intestinal sac model.

This necessitates the need for analytical tool to monitor cephalosporins in in vitro everted rat intestinal sac absorption model. Although, there are several methods for determination of cephalosporins in simple solutions and biological fluids [6,7], none of them qualify for absorption studies. Among these are microbiological [8,9], colorimetric [10], high performance liquid chromatography [11–14], spectroscopic [15], fluorescence ELISA [16], fluorimetric, enzymatic and electrometric methods [17]. The method should be specific enough to detect drug in presence of components of Kreb's Ringer Bicarbonate (KRB) solution, intestinal sac artifacts and absorption enhancers (pharmaceutical excipients added in medium to increase absorption of cephalosporins). Additionally, it should be able to analyze drug content in large number of samples with minimum cost, time and labour. In the present work a simple validated HPLC method utilizing isocratic mobile phase and with short retention times for the CX and CZ is developed to monitor their concentrations in in vitro intestinal sac absorption model. The same is utilized to determine apparent permeability coefficients and absorption profiles of these drugs in in vitro conditions.

2. Experimental

2.1. Materials

CZ and CX were gift samples generously supplied by Lupin Labs, India. All the solvents used were of HPLC grade (J.T. Baker) and reagents were of analytical grade (Ranbaxy Labs Ltd, India and Loba Chemie Pvt, India). The sorption promoters used are designated as SP-1 and SP-2 and were purchased from Sigma Chemicals. Purified water obtained by reverse osmosis (USF ELGA) filtered through 0.45 μm membrane filter was used throughout the study.

2.2. Chromatographic system and instrumentation

Shimadzu HPLC system equipped with LC-10AT VP pump, DGU-14AM on-line degasser, SIL-10AD VP refrigerated autosampler, CTO-10AS VP column oven and SPD-10AVP UV-VIS detector was used. Shimadzu CLASS-VP software

was used for data acquisition and mathematical computations. In addition, Mettler Toledo AG 245 electronic balance, Branson 3210 sonicator, Millipore filtration assembly and filters (HVLP and HVHP), Minisart® NML (0.45 μm) Sartorius filters for samples, Nichipet Nichiryo (10–100 and 100–1000 μl) micropipette, Hypodermic syringes (2 ml), Microlitre syringes from Hamilton, Remi Magnetic stirrers with thermostatic controls (1 MLH), Beckman UV–VIS spectrophotometer 640i and USF ELGA for preparation of reverse osmosis water were used in the study.

The chromatographic conditions used in analysis are outlined in Table 1.

2.3. Preparation of standard stock solutions

Standard stock solutions of CZ and CX were prepared in KRB solution to obtain concentration of 1 mg/ml. The KRB composition was: NaCl: 7.0 g; KCl: 0.35 g; $CaCl_2$: 0.28 g; $MgSO_4$: 0.28 g; $NaHCO_3$: 2.1 g; KH_2PO_4: 0.16 g; Glucose: 5.05 g and water prepared by reverse osmosis: 1000 ml. The 100 μl of standard stock solution was diluted to 10.0 ml to obtain secondary stock solution (10 μg/ml). This solution was finally diluted to obtain working standards in the concentration range of 0.25–10 μg/ml. In all preparations KRB was freshly prepared, sonicated and filtered through 0.45 μm and all solutions were stored at 4 °C.

2.4. Assay characteristics

The LOD and LOQ were determined using aliquots of secondary stock solutions and KRB as diluent. The test solutions of five different concentrations were prepared from secondary stock solutions to determine recovery studies in KRB. The intraday and interday variations were determined by analysis of the same calibration working standards prepared within same day and between the different days by the same analyst. In case of interanaylst variation the calibration standards were prepared by different analysts within the same day and analysed by HPLC.

2.5. Stability studies in KRB

Drug solutions in KRB of three different concentrations (0.1, 0.5 and 1 mM) were selected for experimentation to observe stability of drug in experimental conditions of absorption model. Drug solutions in KRB were kept at 37 °C with constant aeration for 120 min in in vitro permeation assembly. Aliquots of 1 ml sample solution were withdrawn in triplicate from the assembly at 0, 15 and 120 min, and filtered through Minisart® NML (0.45 μm) Sartorius filters using 2 ml hypodermic syringe assembly. For assay by HPLC 300 μl of this solution was used to determine the drug content and 700 μl was used to obtain UV spectra of sample solutions. Triplicate of each concentra-

Table 1
HPLC parameters for determination of CZ and CX in KRB solution

Parameter	CZ	CX
Method	Reversed phase high performance liquid chromatography	Reversed phase high performance liquid chromatography
Mobile phase	Isocratic composition, phosphate buffer[a]: Methanol 85: 15 (v/v)	Isocratic composition, phosphate buffer[a]: Methanol 70: 30 (v/v)
Column	C_{18} Shim-Pack (CLC-ODS-M) 4.6 mm ID × 250 mm and 5 μm particle size	C_{18} Shim-Pack (CLC-ODS-M) 4.6 mm ID × 250 mm and 5 μm particle size
Flow rate	1 ml/min	1 ml/min
Detection	UV detector, 254 nm	UV detector, 254 nm
Column temperature	25 °C	25 °C
Injection volume	50 μl	50 μl

[a] Phosphate buffer (pH 7.0) composition: 3.5 g/l of KH_2PO_4 dissolved in 0.03M $Na_2HPO_4 \cdot 2H_2O$.

tion at each time interval was assessed for stability. The samples were kept at 4 °C in refrigerated autosampler during the sequence run of samples to quench the probable degradation of the drug. Additionally, the stability of drugs in KRB at 4 °C in refrigerated autosampler (SIL-10AD VP), after subjecting them to experimental conditions of in vitro absorption studies of 2 h, was also assessed by injecting samples of drug solutions at 0, 8, 12 and 24-h intervals.

2.6. In vitro everted intestinal sac absorption studies

2.6.1. Surgical excision of intestinal segments

All the animal studies were done according to the guidelines of the local Institutional Animal Ethical Care committee (IAEC). In vitro absorption studies were performed using everted rat jejunal segments [18]. Male-Sprague Dawley rats were fasted for 16–24 h. Water was allowed *ad libitum*. The animals were sacrificed by excessive ether inhalation. The intestine was rapidly removed from the anaesthetized rat and put into beaker with KRB solution on ice, which was continuously aerated. The intestine was divided into the duodenum (below the pylorus), jejunum (5 cm away from ligament of Treitz), ileum (above the caecum) and colon below the caecum. The jejunal segments ($\sim$6 cm) are used in present studies ($n = 3$–6).

2.6.2. Eversion of small intestine

The flattened end of the everting smooth glass rod was carefully inserted in the intestine, which was affixed to the rod with a piece of silk thread. The intestine was gently rolled over itself until the tied end emerges at the other end of the intestinal segment. The intestinal segments of 5 cm length when stretched by an 8 g weight are then cut from jejunal region of intestine. The distal end of the segment was tied and attached to a 2 g weight and the proximal end was attached to the cannula. The segment was suspended in the predetermined volume (40 ml) of mucosal solution containing the drug (0.5 mM). The cannula was adjusted to immerse the sac completely in mucosal solution and then 3 ml of drug free KRB was then placed into the serosal compartment using polyethylene tube attached to the needle of 5 ml glass syringe. The mucosal solution was continually aerated. The 1.6 ml of serosal solution was removed from the sac at each time interval and equal volume of drug free KRB was replaced by sampling syringe with polyethylene tube attached to its needle. The removed serosal solution was filtered via Minisart® NML (0.45 μm) Sartorius filters and 50 μl of filtered solution was injected in HPLC to estimate drug content. The data acquisition was done on Schimadzu CLASS-VP software and analysed using Microsoft Excel. The cumulative amount of drug permeated in μg through the sac was plotted against time (min). The slope of linear portion of the graph was taken as permeation flux (F, μg min^{-1}) [19]. The Apparent Permeability Coefficient (APC) was calculated using following formula:

$$[N1]APC = \frac{F}{SA \times IDC} \text{ cm min}^{-1}, \quad (1)$$

where: SA, surface area of barrier membrane (cm^2); and IDC, initial drug content in mucosal solution (μg)

3. Results and discussion

3.1. Range and linearity

The calibration curve of CZ and CX were constructed in KRB solution. It was used as solvent medium because this method will be used to monitor concentrations of these drugs in in vitro rat intestinal sac absorption experiments. The linearity was observed in the concentration range of 0.25–20 μg/ml. The regression statistics are shown in Table 2. This concentration range was selected on the basis of anticipated drug concentrations in in vitro absorption studies. The goodness of fit (r^2) in both cases was found to be > 0.97 indicating functional linear relationship between concentration of analyte and the area under peak. The mean values of slope and intercept in case of CX are 8.5295×10^{-6} (RSD = 4.43) and 0.2264 (RSD = 3.65) whereas in case of CZ are 7.8301×10^{-6} (RSD = 5.16) and 0.2783 (RSD = 0.92), respectively.

Table 2
Regression statistics of CZ and CX

Drug	Analyst	Day	Number of replicates (n)	Goodness of fit (R^2)	Slope	Intercept
CZ	Analyst 1	Day 1	6	0.9952	8.2880×10^{-6}	0.2754
		Day 2	3	0.9932	7.5254×10^{-6}	0.2801
	Analyst 2	Day 1	6	0.9960	7.6771×10^{-6}	0.2795
CX	Analyst 1	Day 1	6	0.9948	8.1538×10^{-6}	0.229
		Day 2	3	0.9969	8.7949×10^{-6}	0.2172
	Analyst 2	Day 1	6	0.9978	8.8289×10^{-6}	0.2331

Table 3
LOD and LOQ of CZ and CX

Drug	Method parameter	Parameter value (ng/ml)	No. samples	%RSD
CZ	LOD	95	5	0.95
	LOQ	240	3	0.69
CX	LOD	100	3	1.95
	LOQ	250	6	1.47

3.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection is defined as the lowest concentration of the analyte in a sample that can be detected by the method of analysis. It is expressed as a concentration at a specified signal to noise ratio, usually two or three to one [20]. The lower limits of detection of CZ and CX were found to be 95 and 100 ng/ml, respectively (Table 3). The limit of quantitation is defined as the lowest concentration of the analyte in sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The signal to noise ratio of 10:1 can be taken as LOQ of the method [20]. The method was sensitive enough to quantitate 240 ng/ml of CZ and 250 ng/ml of CX solutions in KRB.

3.3. Accuracy

Accuracy is the exactness of the analytical method or the closeness of the agreement between the value (which is accepted either as a conventional true value or an accepted reference value) and the value found. It is measured as the percent of the analyte recovered by assay or by spiking samples in a blind study. The percentage recoveries of CZ and CX were in the range of 96.98 to 104.13 and 95.67 to 101.72, respectively. The RSD values in all the cases were $<2\%$, which implies the accuracy of the method. To confirm the accuracy of the proposed method, recovery experiments were carried out by standard addition technique in which quality control samples were prepared by spiking the KRB with known amount of drug to obtain 5 different concentration levels within the calibration curve range. Each level was determined in replicates ($n=6$) and the amount of drug was found by the assay method. The results are reported in the Table 4. The results show that the method is accurate.

3.4. Precision

Precision is the degree of repeatability of an analytical method under operation and is usually expressed as the percent relative standard deviation for a statistically significant number of samples. To determine intra- and interday precision of the assay, replicates ($n=3$–6) sets of calibration samples were analysed within the same day and between different days by the same analyst. The

relative standard deviation of the assay results was determined and the results are shown in Table 5. The variation in intraday estimations of drug content in KRB is least followed by interday variation and interanalyst variation. Since the drug solutions are not very stable in aqueous solutions, the stock solutions were prepared everyday. This accounts for greater interday variation compared to intraday variation. The stock solutions prepared by different analysts on different days will tend to be even more susceptible to variation due to different working habits in unit operations like weighing, dilutions etc. Further, the percentage variation observed for CX is more than CZ indicating that method is more sensitive to CZ than CX.

3.5. Specificity

Specificity according to USP is the ability to measure accurately and specifically the analyte of interest in the presence of the other components that might be expected to be present in the sample matrix. The mean retention time of CZ was found to be 7.96 (1.73% RSD). The drug peak was not interfered by any of the components of KRB and absorption enhancers, which will be used in in vitro absorption studies. The representative chromatograms of CX and CZ with (0.25%w/v) SP-1 & (0.25 w/v) SP-2 are shown in Figs. 1 and 2. The method is specific to detect the peak of drugs in presence of the intestinal sac artifacts as evident from chromatograms in Figs. 1 and 2. The mean retention time of CX was found to be 7.52 (0.27% RSD) and similar to CZ, peak of CX was also not interfered by sorption promoters and intestinal sac artifacts.

3.6. Stability of the drugs in in vitro absorption model

It is absolutely essential to check whether the drug degrade in the KRB under the stated conditions of temperature and aeration in in vitro intestinal sac absorption studies. The three different concentrations of both the drugs, viz, 0.1, 0.5 and 1.0 mM were studied for their stability in conditions of in vitro absorption model. CZ was found to be stable in conditions of in vitro absorption model experimentation as indicated in Table 6 within the limits of experimental error of developed HPLC method. This data was qualitatively supplemented by UV spectra obtained at different intervals of time. It was observed that characteristic λ_{max} of CZ was unaltered and spectral shape was retained till 120 min. Mason et al [21] had observed that CZ and tobramycin were stable up to 16 h at room temperature when combined in a dextrose containing dialysis solution, and for a further 8 h at 37 °C. CZ concentration remained $>90\%$ of the initial concentration for 3 days in parenteral nutrition mixture at 4 °C and for 12 h at 22 °C in admixtures containing 6 mg/ml of CZ [22]. Previous reports indicate that reconstituted 1 g vials of CZ for injection added to 50 ml minibags of sodium chloride injection (0.9%) were found to be stable for 97 days when stored at -20 °C. A frozen shelf life of 42 days was suggested, to allow for a refrigeration life of 4 days followed by 24 h at room temperature [23]. The stability data for CX is depicted in Table 6. The results are consistent with previous work done by Rivers et al. [24] according to which the CX remained stable for 72 h at 8 °C in i.v. admixture containing metronida-

Table 4
Percentage recovery of CZ and CX in KRB solutions

Concentration (μg/ml)	CZ% recovery	% RSD	CX% recovery	% RSD
0.4	104.13	1.83	98.66	0.89
0.8	96.98	0.97	98.59	1.69
2.5	98.03	0.44	95.67	1.87
7.5	99.45	0.44	95.79	0.85
18.0	99.42	0.54	101.72	1.32

Table 5
Precision of the method

Concentration (μg/ml)	CZ			CX		
	Intraday variation (%RSD)	Interday variation (%RSD)	Interanalyst variation (%RSD)	Intraday variation (%RSD)	Interday variation (%RSD)	Interanalyst variation (%RSD)
0.25	1.32	1.15	1.06	0.86	1.53	1.14
0.5	0.79	0.83	1.55	1.14	0.52	1.40
0.75	1.63	0.90	1.45	0.50	1.65	0.66
1.0	0.35	1.98	1.07	0.18	1.99	1.41
5.0	0.28	0.53	0.80	0.38	1.60	1.85
10.0	0.31	0.62	1.23	0.10	1.30	1.45
15.0	1.03	0.84	0.97	1.06	1.63	1.25
20.0	1.03	0.52	1.60	0.62	0.76	1.60

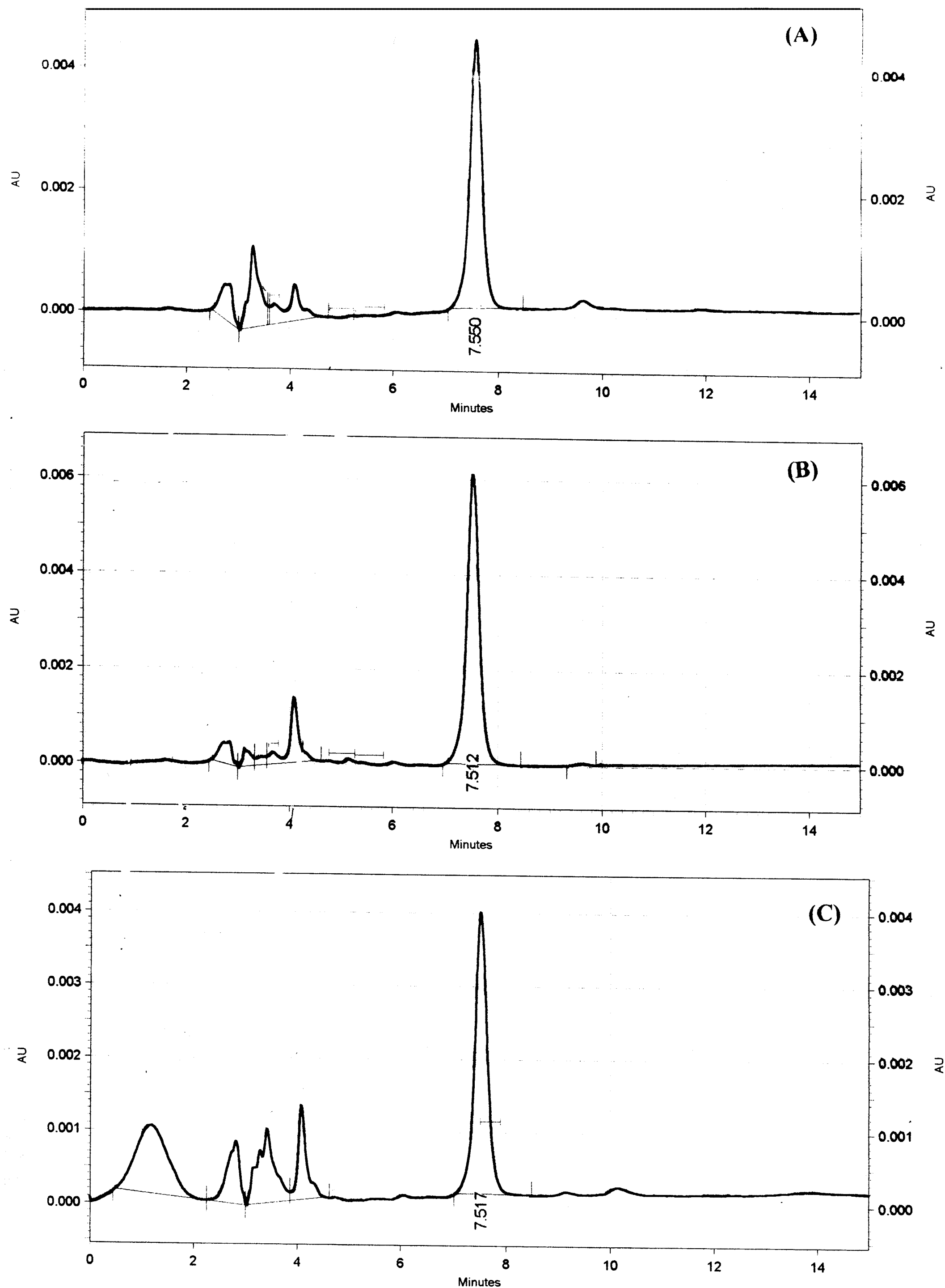

Fig. 1. Representative chromatograms of CX with: (A) SP-1 (0.25%); (B) SP-2 (0.25%); and (C) along with the intestinal sac artifacts.

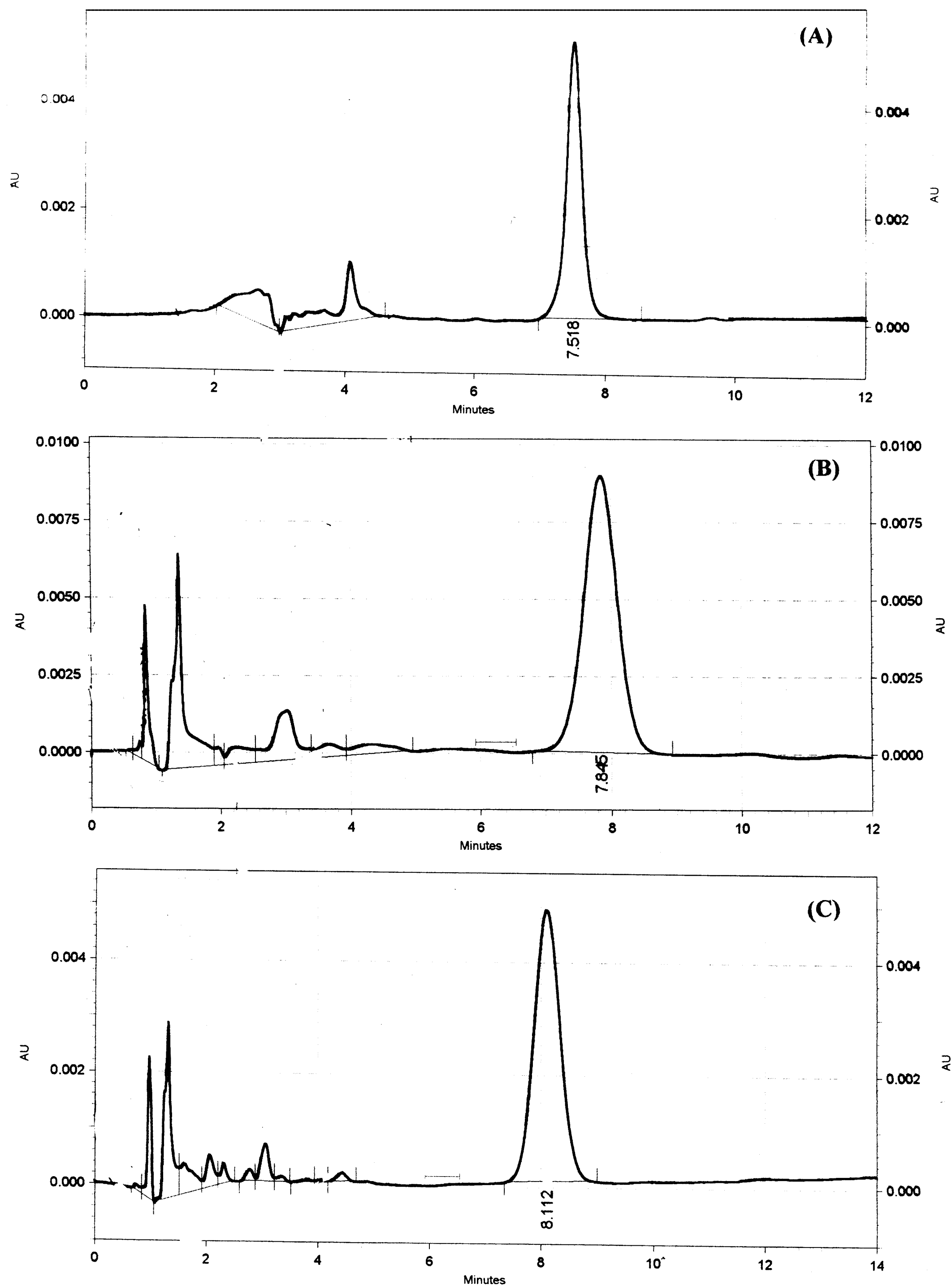

Fig. 2. Representative chromatograms of CZ with: (A) SP-1 (0.25%); (B) SP-2 (0.25%); and (C) along with the intestinal sac artifacts.

Table 6
Stability of CX and CZ in KRB at 4 °C in refrigerated autosampler (SIL-10 AD VP) for 24 h ($n = 3$)

Concentration (mM)	Time (min)	Percentage of drug remaining as compared to '0' min	
		CZ	CX
0.1	0	100.00 (0.14)	100.00 (0.94)
	30	100.30 (0.70)	99.72 (0.56)
	60	99.76 (0.04)	99.10 (0.56)
	120	99.34 (0.61)	99.22 (0.63)
0.5	0	100.00 (0.27)	100.00 (0.06)
	30	99.63 (0.44)	99.07 (0.12)
	60	99.67 (0.24)	99.85 (.072)
	120	99.37 (0.14)	100.37 (0.51)
1.0	0	100.00 (0.18)	100.00 (0.06)
	30	99.92 (0.80)	99.86 (0.05)
	60	100.1 (0.51)	100.03 (0.09)
	120	99.60 (0.43)	99.39 (0.15)

S.D. is indicated in parentheses.

Table 7
Stability of CZ and CX in KRB at 4 °C in refrigerated autosampler (SIL-10 AD VP) for 24 h ($n = 3$)

Concentration (mM)	Time (h)	Percentage of drug remaining as compared to '0' min	
		CZ	CX
0.1	0	100.00 (0.07)	100.00 (0.24)
	8	100.69 (0.21)	100.72 (0.06)
	12	98.27 (0.18)	100.24 (0.29)
	24	98.05 (0.77)	98.24 (0.20)
0.5	0	100.00 (0.17)	100.00 (0.36)
	8	100.04 (0.73)	100.46 (0.72)
	12	99.22 (0.26)	99.18 (0.39)
	24	99.16 (0.16)	98.40 (0.41)
1.0	0	100.00 (0.18)	100.00 (0.08)
	8	100.83 (0.17)	99.63 (0.25)
	12	99.74 (0.84)	98.52 (0.14)
	24	97.66 (0.25)	97.80 (0.06)

S.D. is indicated in parentheses.

zole. Thus, it is possible to use in vitro everted sac absorption model to predict the absorption profiles of cephalosporins. Raeissi et al. [1] had used cephalosporins in concentration of 1 mM to observe the role of an α- amino group on H^+-dependent transepithelial transport of cephalosporins in CaCo-2 cells with drug solutions of pH 6.0 and 7.4 at 37 °C wherein transportation was monitored for 120 min. The concentration of 0.5 mM is arbitrarily selected for further in vitro absorption studies of both drugs. Additionally, both of the drugs were analysed for chemical stability in KRB at 4 °C in HPLC autosampler for 24 h. This was necessary to insure that drug do not degrade while the solutions were analysed in sequence run using the autosampler. The data is shown in Table 7. Although, solutions of both the drugs are stable up to 12 h, they show sign of some degradation as evident from decrease in percentage drug content at 24 h. Thus, it would be better to analyse the drug solutions in KRB within 12 h of collection from in vitro absorption experiments.

3.7. In vitro everted intestinal sac absorption studies

The cumulative amount of drug permeated in μg through the sac was plotted against time (min). The slope of linear portion of the graph was taken as permeation flux (F, μg min^{-1}) [17]. The APC was calculated using Eq. (1). The amount of drug absorbed in μg per unit area of sac is plotted against time (min) to obtain absorption profiles of CX and CZ (Fig. 3). The APC of CZ and CX are 1.17 $(\pm 0.10) \times 10^{-5}$ cm/min and 1.20 $(\pm 0.11) \times 10^{-5}$ cm/min, respectively.

4. Conclusions

The proposed HPLC method is simple and has short retention times so that single analysis can be completed within 9 min for both the drugs. Hence, this method can be used to process large number of samples and detect low concentrations in in vitro samples (CZ = 240.0 ng/ml and CX = 250.0 ng/ml). It was specifically optimized for monitoring of cephalosporin concentrations in permeation studies through rat intestine in everted sac absorption model. The data generated from studies was analyzed to determine drug flux through the rat intestine and apparent permeability coefficient.

The peak area versus concentration curve was linear over the concentration range of 0.25–20 μg/ml. ($R^2 > 0.99$). The drug peak is not interfered by any of absorption enhancers and intestinal sac artifacts. The method is accurate and precise as evident from low % RSD values in recovery studies, interday and intraday variation.

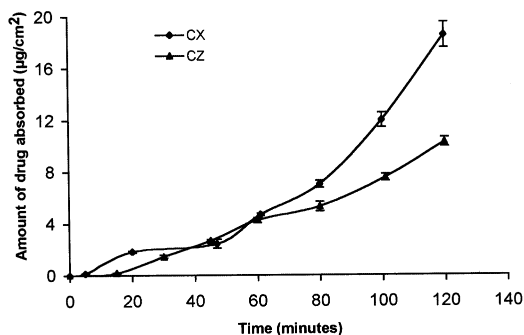

Fig. 3. In vitro absorption profile of CX and CZ in rat everted intestinal sac model.

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