Original article

Simultaneous estimation of cefixime and cloxacillin in human plasma by reversed phase-HPLC with UV detection

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Abstract:

An accurate, rapid and simple reversed-phase high performance liquid chromatography (RP-HPLC) method was developed and validated for simultaneous estimation of cefixime, cloxacillin and for chloramphenicol (internal standard) in human plasma. Chromatographic separation was accomplished using CAPCELL PAK C_8 (4.6 mm × 250 mm, 5 μm) analytical column using phosphate buffer, methanol and acetonitrile (40:45:15) as eluent. Detection and quantification were performed by UV/Vis detection at 225 nm. The lower limit of detection and quantification were 0.01 $\mu g\ mL^{-1}$ and 0.25 $\mu g\ mL^{-1}$ for cefixime, 0.03 $\mu g\ mL^{-1}$ and 0.39 $\mu g\ mL^{-1}$ for cloxacillin. The calibration curves were linear over the concentration range 0.5 to 40 $\mu g\ mL^{-1}$ for both cefixime and cloxacillin in human plasma. The method was quantitatively evaluated in terms of linearity, precision, accuracy, recovery, selectivity, and stability. The method is simple, convenient and suitable for the analysis of cefixime and cloxacillin in human plasma.

Keywords: Cefixime; Cloxacillin; Human plasma; High performance liquid chromatography

Introduction

Cefixime, (6R, 7R)-7-[2-(2-amino-4-thiazolyl) glyoxylamido]-8-oxo3-vinyl-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid (CFX; Figure 1a) is a third generation cephalosporin antibiotic. CFX is given by oral rout in the treatment of susceptible infections including gonorrhea, otitis media, pharynsitis, lower respiratory tract infection and urinary tract infection [1]. Cloxacillin monosodium (2S, 5R, 6R)-6-[3-(o-chlorophenyl)-5-methyl-4-isoxazolecarboxamido]-3, 3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylate monohydrate [3.2.0] heptane-2-carboxylate monohydrate (CLX; Figure 1b) is used against staphylococci that produce beta lactamase [2].

Thus quantification methods of the drug in pharmacokinetics studies need to be sensitive and specific for simultaneous estimation. Several analytical methods for analysis of CFX and CLX in biological fluid have been reported for single drug. However, very few methods have been reported for estimation of CFX and CLX in biological samples. Literature survey revealed several analytical methods such as HPLC, LC-MS, LC-MS-MS, have been reported for the determination of CFX [2-9] and CLX [2,10-16] alone or in combination with other antibiotics from dosage forms and biological samples. In continuation with our previous published work [17], the present investigation was taken in the direction to establish a simple and versatile isocratic reversed phase HPLC-UV method for simultaneous measurement of CFX and CLX in human plasma using a simple extraction procedure.

Materials and Methods

Instruments

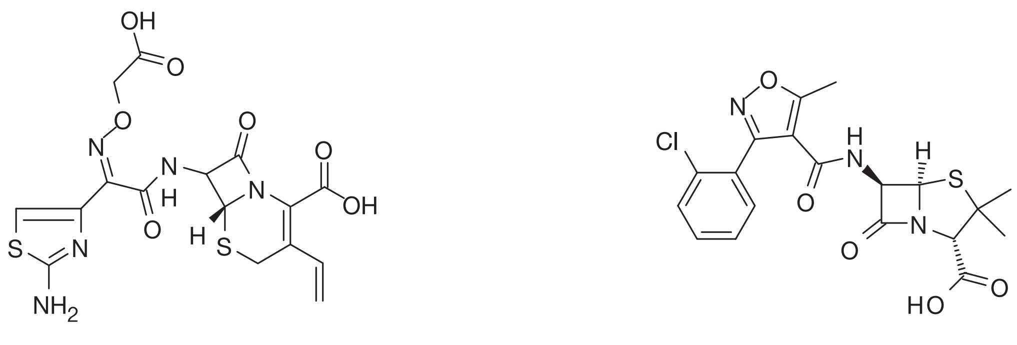
Isocratic high pressure liquid chromatography (Cyberlab-chrom-HPLC V 4.0) with LC-P-100 pump, variable wavelength programmable UV/Vis detector LC-UV 100 and operating software Cyberstore Version no. 4-0512-039 was used. The chromatographic separation carried out by reversed phase capcell pak C_8 DDS5 column (4.6 mm × 250 mm i.d. particle size 5 µm).

Chemicals

Cefixime (Blok Pharma Pvt. Ltd, Kolhapur), Cloxacillin (Blok Pharma Pvt. Ltd, Kolhapur), Chloramphenicol (Smruthi Organics, Solapur), Acetonitrile (Merk Chemicals) and all other chemicals used were of analytical grade. Blank (drug free) plasma was donated from Sangameshwar Teaching Hospital, Gulbarga (India). Doubly distilled water was used for preparing mobile phase solutions.

Preparation of standard solution

Stock solutions containing 1 mg mL^{-1} each of CFX, CLX and chloramphenicol (internal standard, IS) were prepared in methanol:water (5:5). IS was further diluted with methanol:water (5:5) to get final concentration of 10 µg mL^{-1}. Further the stock solutions of CFX and CLX were diluted in methanol:water (5:5) to obtain various concentrations of 0.5, 1, 5, 10, 20, and 40 µg mL^{-1}. Phosphate buffer (25 mM) was prepared in water and adjusted to pH 4.6 with ortho-phosporic acid. Stock solutions were stored in a freezer set to maintain at 20°C.



(a) (b)

Figure 1 Chemical structures of (a) cefixime and (b) cloxacillin

Chromatographic condition

The mobile phase consisting of (25 mM) phosphate buffer-methanol-acetonitrile (40:45:15), (v/v/v) at pH 4.6 was degassed and filtered by using Millipore vacuum filter system equipped with 0.45 μm membrane filter. Chromatography was performed at an ambient temperature by pumping the mobile phase with a flow rate of 1.0 mL min^{-1}. The column effluent was monitored at 225 nm. An injection volume of 10 μL was used. Chloramphenicol was used as an internal standard.

Extraction procedure

Calibration standards were prepared by adding 50 μL of the appropriate CFX and CLX working solutions (0.5-40 μg mL^{-1}) to 500 μL of blank plasma. Calibration standards, samples and controls were processed by adding 10 μL of internal standard working solution (50 μg mL^{-1}) and 1.0 ml of acetonitrile to 0.50 ml of plasma to labeled 13 × 75 mm glass culture tubes. Then this solution was vortex-mixed for 30 s and centrifuged at 5000 rpm for 15 min.

The supernatant was transferred to a clean, similarly labeled 10 ml glass conical centrifuge tube. The acetonitrile was evaporated under a nitrogen steam at 15 psi in a water bath set to temperature of 40°C. The dried extracts were reconstituted in 400 μL mobile phase. All tubes were vortex-mixed and 10 μL aliquots of the extracted solutions were injected into the HPLC system as per literature [18, 19].

Method validation

To develop a precise, accurate and reproducible HPLC method for the estimation of CFX and CLX in human plasma, various mobile phases, stationary phases and sample preparation methods were employed and the proposed chromatographic condition was found to be appropriate for the quantitative determination. After optimization of the analytical conditions, the evaluation of the parameters, such as accuracy, precision, linearity, selectivity, recovery and stability were performed for the method validation [20, 21].

System suitability test

System-suitability tests were performed in accordance to confirm the reproducibility of the equipment adequate for the analysis [22]. The system suitability test was performed before analysis of every batch of sample to ensure the reproducibility of the chromatographic system. The HPLC system suitability test was performed by running six injections of diluted drug and IS in the linear region of the calibration curve and measuring the coefficient of variation in percentage (% CV<2%). Number of theoretical plates, area ratio, tailing factor and resolution were also determined as a means of validation parameter.

Linearity and sensitivity

The linearity was studied using six concentrations viz. 0.5, 1, 5, 10, 20 and 40 μg mL^{-1} of CFX and CLX. Linearity experiment was performed six times to check the detector response to the drug to be linear in function with various concentrations (0.5 to 40 μg mL^{-1}). The working standards were prepared by adding different concentrations of CFX, CLX and fixed concentrations of IS (10 μg mL^{-1}) solution spiked in plasma to obtain the required concentration range. These samples were extracted by suitable procedure and then injected into the HPLC system. The drug/IS peak area ratio was plotted against the concentration of the drug and measured in terms of correlation coefficient (r^2). Limits of detection (LOD) and quantification (LOQ) were determined from signal to noise ratio [23]. LOD was defined as the lowest concentration level resulting in a peak area of three times the baseline noise. LOQ was defined as the lowest concentration level that provided a peak area with signal-to-noise ratio higher than 10 [24].

Precision and accuracy

Precision and accuracy of the developed method was determined by analysis of quality control (QC) samples at three different concentrations covering the low, medium and higher ranges of the calibrations curves. Injecting six samples at each concentration on the same day assessed intraday variation of the assay. Inter day variation was assessed by injecting nine samples of each concentration (on 15 days) over a period of two weeks. The precision of the method was expressed in terms of percent coefficient of variation (%CV), and accuracy was expressed as a percentage of the theoretical concentration (observed concentration × 100/ theoretical concentration) [25].

Recovery

The absolute and relative recoveries for the CFX, CLX and IS were determined by spiking known amounts of CFX, CLX and IS into drug-free human plasma to obtain three different concentrations covering the low, medium and higher ranges of the calibration curves. Absolute recoveries were determined by comparing the peak area of extracted QC samples with the peak area of recovery standards at the same nominal concentrations [25]. Relative recovery study was performed based on intraday studies.

Specificity

The specificity was verified by checking the interference of endogenous compound in human plasma at the retention time of the CFX, CLX and IS by evaluating six lots of plasma.

Stability [8]

Short-term stability study of the stock solution of analytes was evaluated in three ways viz :(1) Human plasma samples were stored over-night at temperature of 15-20°C after the first injection cycle and then it was re-injected on the consecutive next day; (2) Human plasma samples were stored over-night in the freezer at -20°C, collected at room temperature, and injected within 1 h after thawing thoroughly; (3) Standard stock solutions stored for 1 week at -20°C in freezer, brought to room temperature, and injected within 1 h after thawing.

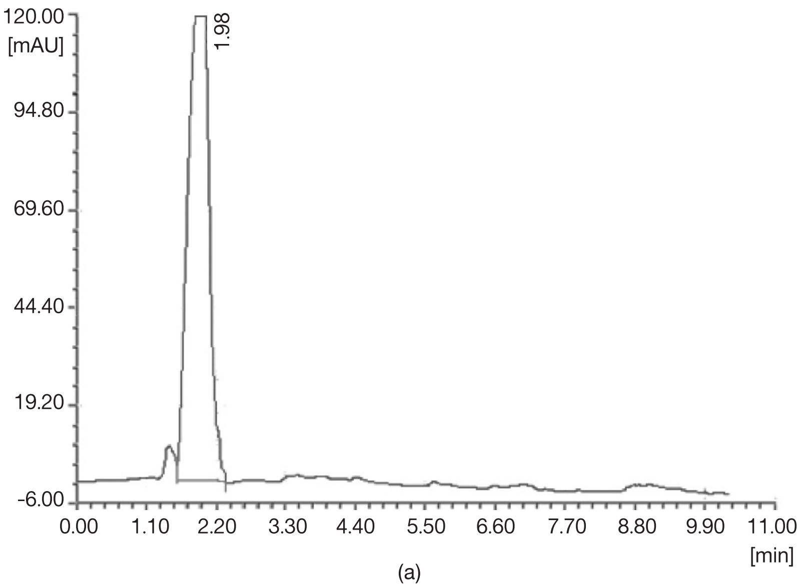
Result and Discussion

The mobile phase was optimized to provide sufficient selectivity towards the drugs. Phosphate buffer contributed high sensitivity and selectivity when compared with other buffers. Methanol and acetonitrile as an organic component resulted in better sensitivity but variation in the amount of methanol and acetonitrile in the mobile phase affected resolution and runtime. Variation of mobile phase pH resulted in bad peak shape and increased interference from the plasma. Therefore pH of buffer was adjusted at 4.6. The optimized mobile phase was phosphate buffer: methanol: acetonitrile (40:45:15) (v/v/v) pH 4.6. Injection volume was optimized to 10 μL and the column temperature was maintained at 25°C (ambient). Retention times of approximately 3.70, 6.52 and 9.19 min were consistently observed for CFX, IS and CLX respectively, throughout all analytical runs. Acetonitrile was the solvent of choice, used to precipitate protein in plasma sample in order to obtain satisfactory values for recovery of CFX and CLX and chromatogram without any interfering peak from the mixture. However, extraction with acetonitrile was optimized as the sample treatment procedure [18, 19].

A typical chromatogram of blank human plasma solution is as shown in Figure 2a. Figure 2b depicts representative chromatogram of blank human plasma and IS (chloramphenicol). Drug free human plasma was screened and interference of endogenous substances was not observed at retention time of CFX, CLX and IS. Figure 2c illustrates chromatogram of human blood plasma spiked with 1 μg mL^{-1} CFX, CLX and chloramphenicol as IS. Chromatograms for 10 μg mL^{-1} and 20 μg mL^{-1} of drugs and IS spiked blood plasma, are shown as Figures 2d and 2e, respectively.

System suitability test

The HPLC system suitability test was performed by running six injections of diluted CFX, CLX and IS in the linear range of the calibration curve and measuring the coefficient of variation in percentage. Number of theoretical plates, area ratio, tailing factor and resolution were also determined as a means of validation parameter. The % CV calculated for the method was found to be less than 2%, which revealed the suitability of the developed method and the optimized chromatographic conditions.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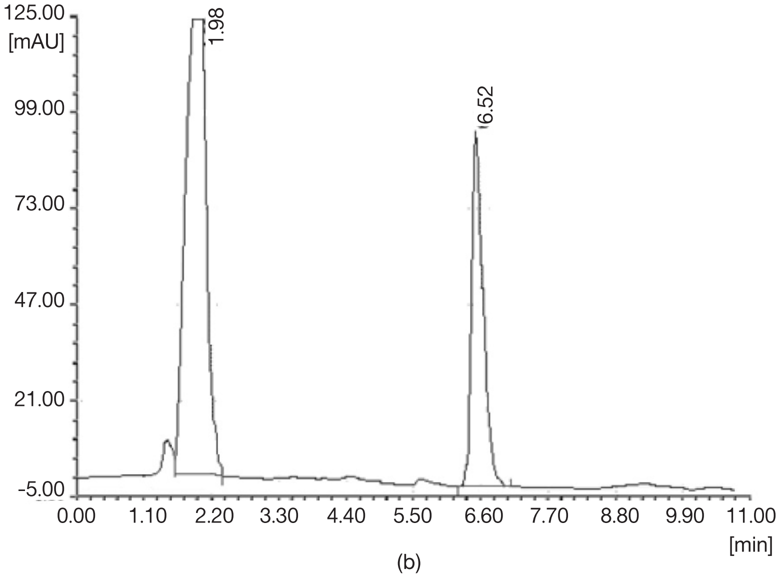
(a)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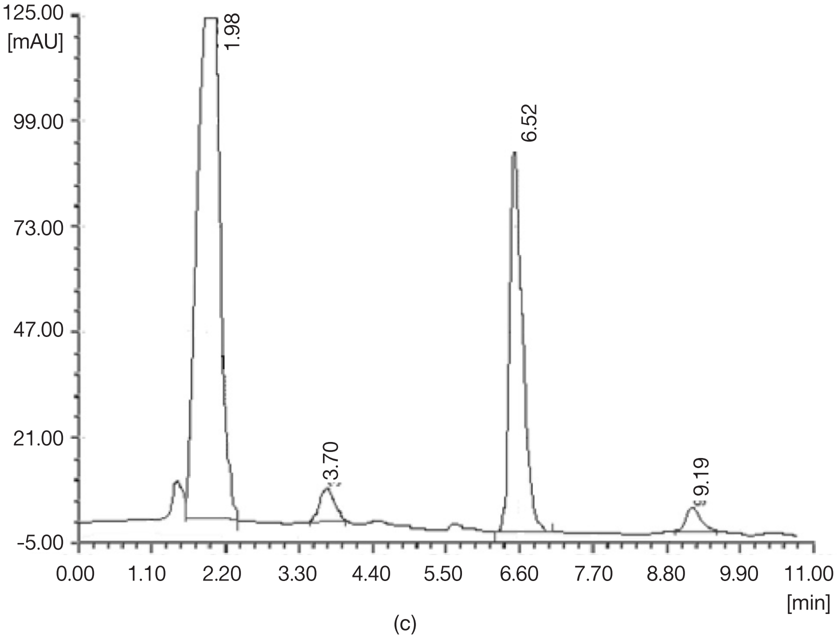
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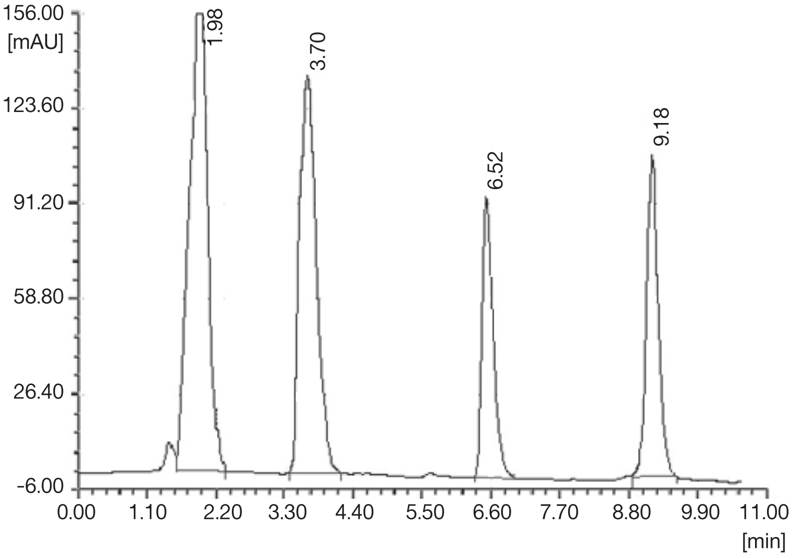
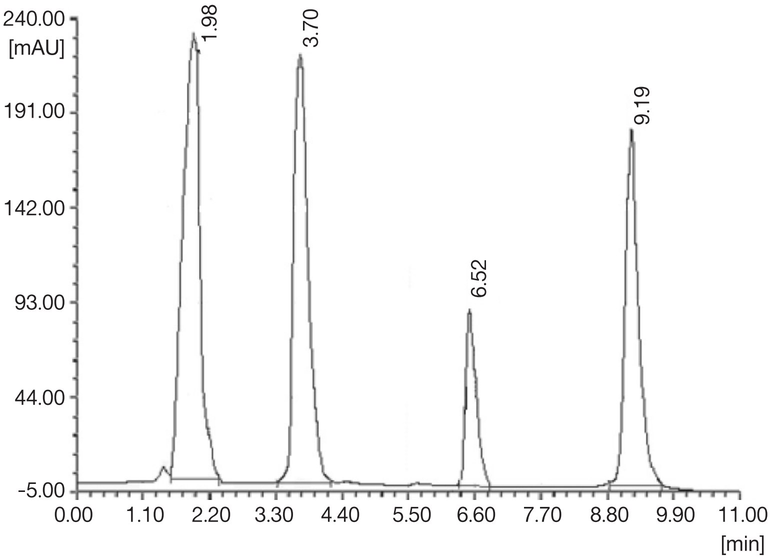


(d)



(e)

Figure 2 Typical chromatogram of human blank plasma (**a**), human blank plasma and chloramphenicol as internal standard (IS) (**b**), human blank plasma spiked with 1 µg mL^{-1} cefixime (CFX) and cloxacillin (CLX) using chloramphinicol as IS (**c**), human blank plasma spiked with 10 µg mL^{-1} CFX and CLX using chloramphenicol as IS (**d**), human blank plasma spiked with 20 µg mL^{-1} CLX and CFX and chloramphenicol as IS (**e**)

Table 1 Linear regression analysis of calibration curves of cefixime (CFX) and cloxacillin (CLX) (mean ± S.D., n = 6)

Drug	**Linearity range (μg mL^{-1})**	**Intercept (Mean ± S.D)**	**Slope (Mean ± S.D)**	**Correlation coefficient (r^2) (Mean ± S.D)**	**LOD (μg mL^{-1})**	**LOQ (μg mL^{-1})**
CFX	0.5-40	6076.500 ± 193.825	15524.670 ± 25.232	0.9996 ± 0.000017	0.01	0.25
CLX	0.5-40	1088.267 ± 362.655	12885.000 ± 137.850	0.9994 ± 0.001065	0.03	0.39

LOD = limit of detection; LOQ = limit of quantification

Table 2 Result from study of intraday and interday precision and accuracy for cefixime (CFX) and cloxacillin (CLX)

Concentration added (μg mL^{-1})	**Intraday[a]**			**Interday[b]**		
	Measured concentration (mean ± S.D.)	**CV (%)**	**Accuracy (%)**	**Measured concentration (mean ± S.D.)**	**CV (%)**	**Accuracy (%)**
CFX						
1	1.010 ± 0.020	2.025	101.08	1.029 ± 0.034	3.335	102.93
5	5.026 ± 0.079	1.571	100.52	5.047 ± 0.087	1.726	100.95
10	10.040 ± 0.106	1.055	100.40	10.039 ± 0.105	1.053	100.39
CLX						
1	1.001 ± 0.025	2.558	100.18	1.026 ± 0.036	3.548	102.60
5	5.056 ± 0.096	1.913	101.12	5.042 ± 0.089	1.768	100.84
10	10.002 ± 0.106	1.065	100.02	10.044 ± 0.109	1.094	100.44

[a] Mean values represent six different plasma samples for each concentration (n = 6).

[b] Interday was determined from nine different runs over two-week period. The concentration of each run was determined from a single calibration curve run on the first day of the study (n = 9). %CV = coefficient of variation.

Table 3 Absolute and relative recovery of cefixime (CFX), cloxacillin (CLX) and internal standard (IS)

Concentration added (μg mL^{-1})	**Absolute recovery[a] (%mean ± S.D.)**	**Relative recovery[a] (%mean ± S.D.)**
CFX		
1	94.79 ± 2.29	101.08 ± 2.04
5	98.04 ± 1.26	100.52 ± 1.59
10	99.20 ± 0.66	100.40 ± 1.06
CLX		
1	93.94 ± 2.54	100.18 ± 2.56
5	97.92 ± 1.18	101.12 ± 1.93
10	98.56 ± 1.20	100.02 ± 1.06
IS (chloramphenicol)	97.16 ± 0.67	

[a] Mean values represent six different plasma samples for each concentration (n = 6).

Linearity and sensitivity

CFX and CLX in human plasma demonstrated linearity in the calibration curves in the concentration range from 0.5-40 μg mL^{-1}. The correlation coefficients (r^2) were 0.9996 for CFX and 0.9994 for CLX. The results of the linearity experiment are listed in Table 1. LOD and LOQ were found to be approximately 0.01 μg mL^{-1} and 0.25 μg mL^{-1} for CFX and 0.03 μg mL^{-1} and 0.39 μg mL^{-1} for CLX respectively, indicative of high sensitivity of the developed method.

Specificity

The data indicate that the assay method is reproducible within the same day and at different days, coefficient of variation (% CV) are less than 15% for both sample types over the concentration ranges assayed. The absence of peak at migration time of CFX, IS and CLX indicates specificity of the developed method.

Precision and accuracy

The precision of the method was measured by the percentage coefficient of variation (%CV) over the concentration range of high, middle and low QC samples respectively of drug during course of validation. Intra-day precision of the method ranged from 1.055 to 2.558 %CV. Inter-day precision of the method was found to be 1.053 to 3.548 %CV. Nominal value (%) for recovery of CFX and CLX from QC samples were tested of intra-day and inter-day. Intra-day accuracy ranged from 100.02 to 101.12%. Inter-day accuracy ranged from 100.39 to 102.93%. Result from determination of intra-day and inter-day, accuracy and precision are listed in Table 2.

Recovery

The absolute recovery for the CFX, CLX and IS were determined by spiking known quantitative of CFX, CLX and IS into drug free human plasma to obtain three different concentrations covering the low, medium and higher ranges of the calibration curve. The samples were then extracted and analyzed as described earlier. The absolute recovery was calculated by comparing the peak areas of the drugs with those obtained from pure standards in methanol: water (5:5) and IS in methanol: water (5:5) at the same concentration. The absolute recovery of CFX and CLX ranges from 93.94 ± 2.54 to 99.20 ± 0.66%, while the absolute recovery for IS was 97.16 ± 0.67% (Table 3). The relative recovery of the CFX and CLX were calculated by comparing the concentration of the drug-spiked plasma with the actual added concentration. Relative recovery of the CLX and CFX ranged from 100.02 ± 1.06 and 101.12 ± 1.93% (Table 3).

Stability

Short-term stability study indicated that spiked human plasma samples remained stable for 24 h even at room temperature (25°C). On the other hand, standard solutions of CFX and CLX remained stable for 8 days when kept frozen conditions.

Conclusion

The developed RP-HPLC method is an accurate, specific and simple method for simultaneous determination of cefixime and cloxacillin in human blood plasma. The method involves simple extraction procedure, separation on a reversed phase column with an internal standard and UV/Vis detector. The validation data demonstrated good precision and accuracy, which proves the reliability of proposed method. Thus the method suits for routine therapeutic drug monitoring (TDM) and pharmacokinetic study of cefixime and cloxacilline in human.

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