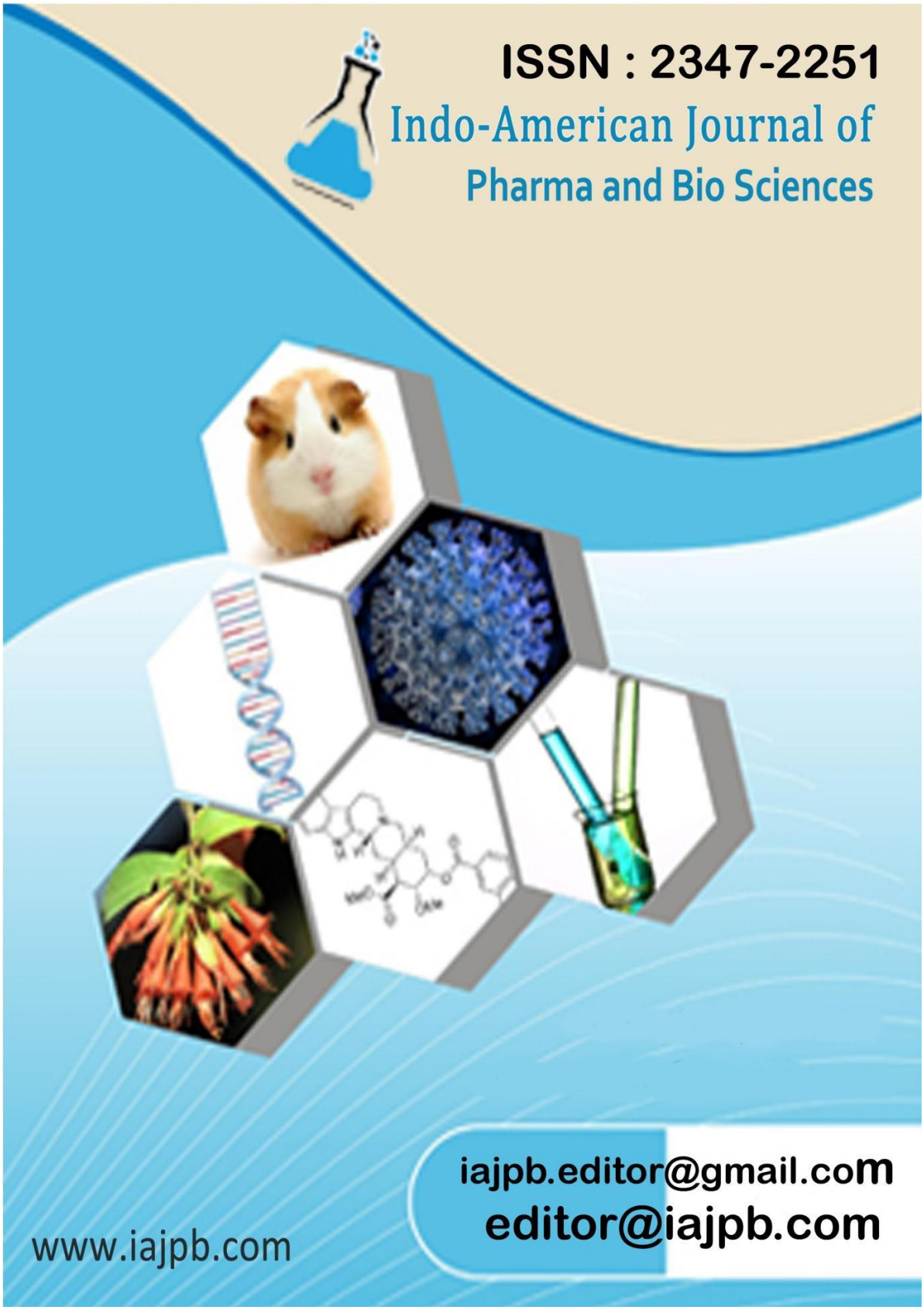
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Cefaclor in Biological Fluid: A Stability-Indicating High Performance Liquid Chromatographic (HPLC) Assay

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SUMMARY. Cefaclor may be broken down by oxidation, hydrolysis, and racemization when found in biological fluid. Moreover, a variety of factors, including pH, temperature, carbon dioxide, oxygen, light, humidity, and storage, might speed up this deterioration process. These steps are thus essential for determining the amount of cefaclor in biological fluids and for slowing down or stopping the degradation process when assaying is required. Samples were analyzed using the described technique either without pretreatment or after being treated with perchloric acid and protein precipitation. As an internal standard, sulphamethoxazole was used. Using a lichrospher RP-18 column and a mobile phase 80:20 v/v (potassium dihydrogen phosphate buffer (0.067M): methanol, pH 4.5), chromatographic separation was performed. Hexane-1-sulphonic acid sodium salt (0.002 M) was used as an ion pair, and the flow rate was 1.3 mL/min. A UV detector set at 265 nm was used to monitor cefaclor. It was discovered that pre-sample treatment with plasma protein precipitant and perchloric acid as an acidifying agent improved cefaclor stability. The limit of quantitation was calculated to be 0.25 µg/mL, and the calibration curve was shown to be linear in the 0.25–20 µg/mL range (r2 = 0.999). In conclusion, a reversed phase HPLC method for determining cefaclor in biological fluids was developed that is stability-indicating, accurate, precise, simple, and highly sensitive. It can be effectively utilized in bioequivalence studies and other pharmacokinetic evaluations.

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INTRODUCTION

Cefaclor is a water-soluble, semi-synthetic, second-generation cephalosporin antibiotic 1, 2. Cefaclor comes in both sustained-release and immediate-release formulations. It possesses a wide range of antibacterial activity against both gram-positive and gram-negative bacteria, including Klebsiella species 3, 4, and Haemophilus influenzae. Cefaclor formed a complex with plasma proteins in plasma. 5. Cefa-clor has been identified in biological fluids using techniques including microbiology and thin-layer chromatography. 6.7, HPLC–UV, or high performance liquid chromatography 8.9, tandem mass spectrometry–liquid chromatography (LC-MS-MS) 10. The primary sample preparation technique for determining the amount of cefaclor in human plasma was protein precipitation, which was extensively used. 11, 12. In order to remove cefaclor from the plasma, ion pairs were used. 13, 14. Numerous approaches that use a range of complex equipment and techniques to determine the presence of cefaclor and other cephalosporins in plasma are described in the literature. 15, 16. However, oxidation,

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hydrolysis, and racemization may cause it to deteriorate throughout the cefaclor determination process. Furthermore, the pH, temperature, carbon dioxide, oxygen, light, humidity, and storage conditions may all speed up this degradation process. Therefore, these steps are essential for determining cefaclor in biological fluids and for minimizing or preventing degradation when assaying is required 17–19. Thus, the development and validation of an ion-pair re- versed phase HPLC technique for determining cefaclor stability in plasma was described in the present study. Parameters including the mobile phase composition, pH, and ion pair concentration were all considered in the creation and improvement of the chromatographic conditions.

KEY WORDS; Sulphamethoxazole, HPLC, Cefaclor, biological fluid, and stability.

and kind of detector. Since stability assessment is regarded as a crucial parameter for the

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validation of bio-analytical methods, it also offers information regarding the stability of cefaclor in plasma and during sample processing. This is obviously advantageous for determining a large number of plasma samples for pharmacokinetic and bioequivalence in vivo studies. Therefore, the purpose of this work is to develop an analytical technique for cefaclor stability in biological fluids in accordance with ICH guidelines, which will be used to assess the pharmacokinetic profile of cefaclor in volunteers.

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MATERIAL AND METHODS

Chemicals and reagents

Cefaclor 99.5% (Fig. 1a) was acquired from Amman, Jordan's United Pharmaceutical Company. Acros (USA) supplied the following HPLC grades: sulphamethoxazole (Fig. 1b), hexane-1-sulphonic acid sodium salt, potassium dihydrogene phosphate, perchloric acid, phosphoric acid 85%, sodium hydroxide, acetonitrile, and methanol. Blank (drug-free) human plasma and deionized water (Nanopure) were acquired from Islamic Hospital in Amman, Jordan.

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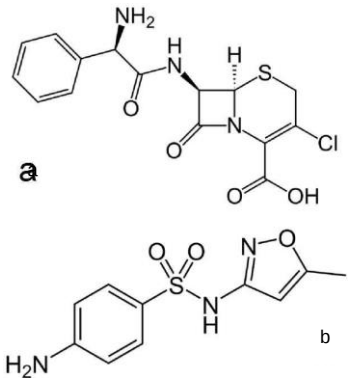


Figure 1. Chemical structures for cefaclor (**a**) and sul- phamethoxazole (**b**).

Instrumentation

A pump L-7100 Lachrom, a UV detector L-7400 Lachrom, an autosampler L-7200 Lachrom, and an interface D-7000 made up the Merck Hitachi HPLC system. A Varian 4400 integrator, Spectra 100 variable wavelength detectors, and an SP 8810 precision isocratic pump made up the Spectra Physics HPLC system. A P580 pump, an ASI-100 auto-mated sample injector, and a light diode array detector made up the Dionex HPLC system.

Chromatographic conditions

A Lichrosphere® 100 RP-18 (12.5 × 4.6 mm, 5 µm) column, 5.0 µg/mL sulphamethoxazole (IS) in 5% perchloric acid, a mobile phase 80:20 v/v (potassium dihydrogen phosphate buffer (0.067 M): methanol, pH 4.5), and 0.002 M of hexane-1-sulphonic acid sodium salt as an ion pair were used for the chromatographic analysis, which was carried out at a flow rate of 1.3 mL/min. Prior to analysis, the mobile phase was ultrasonically degassed and filtered through a 0.22 µm filter (Sartorius, USA). The wavelength of detection was 265 nm, and the analysis was carried out at room temperature.

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Chromatographic variables

Four distinct protein precipitants—acetonitrile, 3% trichloroacetic acid, 20% HCl, and 5% per-chloric acid—four distinct ion pair concentrations (0.002, 0.01, 0.015, and 0.020 M), four mobile phase pH values (3.0, 4.5, 6.0, and 7.0), and two detector types—photo diode array (PDA) detector and ultra violet (UV) detector—were tested for their effects on cefaclor and (IS) resolution.

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Preparation of calibration Standards and Quality control Samples

For samples of spiked plasma (plasma containing pharmaceuticals), 50 µL of 20% perchloric acid/mL of plasma was added to bring the pH of the plasma down to 4.5. Separately, 12.5 mg of IS and cefaclor were dissolved in 500 mL of 5% perchloric acid to provide 500 µg/mL stock solutions (A, A1) for IS and cefaclor, respectively. Cefaclor concentrations of 40 µg/mL (stock solution B) and 5.0 µg/mL of (IS) working solution (B1) were obtained by properly diluting other solutions from (A and A1) with 5% percloric acid. Seven final concentrations (0.5, 1.0, 3.0, 5.0, 8.0, 15.0, and 20.0 µg/mL) and three QC concentrations (1.5, 10.0, and 18.0 µg/mL) as low, medium, and high concentrations, respectively, were obtained from stock solution (B) by serial dilution of calibration standards in

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plasma. Eppendorf tubes containing 500.0 µL of each concentration were kept at -20 °C.

Sample Preparation

In a 2 mL centrifuge tube, 250.0 µL of cefaclor plasma concentrations were mixed with 250.0 µL of a 5% perchloric acid solution (containing 5.0 µg/mL of IS). After 15 seconds of vortex mixing, the mixture was centrifuged for 10 minutes at 4000 rpm. Twenty microliters of the supernatant were extracted and added to the column.

Method Validation

The ratio of the peak area of cefaclor to that of I.S. was used as the assay parameter to quantify plasma samples. Cefaclor concentrations were computed using weighted least squares linear regression, and peak area ratios were shown versus concentrations. Recovery, linearity of response, accuracy, and precision were all part of the validation process; sensitivity, stability, and specificity were all examined. Three QC concentrations and a seven-point standard line were used for the validation, which was carried out on three different days. Plasma samples representing three full standards and QC sample lines, together with five replicates of each standard sample and ten repetitions of each QC sample, were included in each validation day.

Stability

Cefaclor stability was assessed using plasma QC samples with low, medium, and high cefaclor concentrations of 1.5, 10.0, and 18.0 µg/mL, respectively, under the analytical conditions of the test technique. QC samples (n = 3) were left at room temperature for eight hours in order to assess their short-term stability. Three freeze-thaw cycles were used to determine the samples' freeze-thaw stability. After being kept at -20 °C, the three QC concentrations were thawed for two to three hours at room temperature. Testing was done on the samples at 0, 24, 48, and 72 hours. It was also evaluated for long-term stability at -20°C and -70°C for 30 days. For a month, samples were injected once a week. Additionally, the stability of standard solutions was examined for four hours at ambient temperature and for thirty days after being refrigerated at four degrees Celsius. In a single analytical batch, six duplicates were examined for every concentration and storage condition. The starting concentration of cefa-clor was correlated with the concentration at the end of each storage period. Additionally, the stability of cefa-clor in plasma was examined and contrasted with plasma that was not acidified, as well as the impact of adding 20% perchloric acid.

RESULTS AND DISCUSSION

Method development

Because cefaclor is not very stable in biological fluids, it was difficult to develop an HPLC technique for quantifying it (20, 21). The following chromatographic conditions were used to produce good separation, resolution, and symmetrical peaks for the determination of cefaclor in plasma: a potassium dihydrogen phosphate buffer (0.067 M) 80:20 v/v mobile phase mixture: pH 4.5, methanol, and 0.002 M hexane-1-sulf

Lichrosphere® 100 RP-18 as a column (5 µm) (12.5 × 4.6 mm id), phonic acid sodium salt as an ion pair, and sulphamethoxazole as an internal standard (IS). The retention durations for IS and ce-flor were 7.2 and 4.3 minutes, respectively, and the run time was 8 minutes under the current chromatographic conditions. Cefaclor's wavelength of maximum absorbance was determined to be 265 nm.

Plasma Sample Preparation

The literature has documented a number of biological sample preparation techniques, such as liquid-liquid extraction (LLE), solid-phase phase extraction (SPE), and protein precipitation (PPT) 22–24. It is challenging to extract cefaclor, an amphoteric polar molecule, from plasma using organic solvents 25, 26. The straightforward and universal PPT technique of blank plasma samples was used in the present investigation as a first step in the assay method's development. Acetonitrile, methanol, 3% trichloroacetic acid, 20% HCl, and 5% perchloric acid were the five solvents that were

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evaluated. Using 5% perchloric acid produced a suitable resolution with acceptable separation (Fig. 2). Perchloric acid is a potent protein precipitant that effectively solubilizes medications. 27, 28

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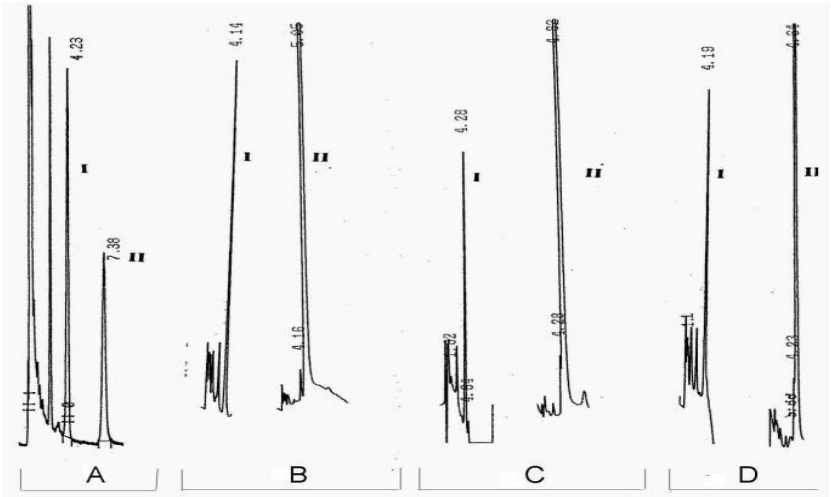


Figure 2. Chromatograms of precipitant effect on Ce- faclor (I) and Sulphamethoxazole (II): **A**. 5% percloric acid, **B**. acetonitrile, **C**. methanol, **D**. 3% trichloro- acetic acid and **E**. 20% HCl.

The impact of buffer pH Under normal conditions, cefaclor partially forms complexes 27–29 with plasma proteins. Therefore, in order to facilitate the decision-making process, Cefaclor has to be hired from the complex.

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pH	Retention time of cefaclor (min)	Retention time Value (min)	sulphamethxazole
3.00	5.74	7.43	
4.50	4.14	7.24	
6.00	4.39	5.26	
7.00	5.16	2.24	

Table 1. Effect of mobile phase pH on the retention time of cefaclor and sulphamethoxazole (IS).

country with the highest concentration of drugs. bringing the plasma samples' pH down to 2 in order to boost cefaclor recovery. Maximum resolution and separation between the medication and IS was achieved at pH 4.5 (Fig. 3) after buffer pH optimization was examined in this investigation (Table 1).

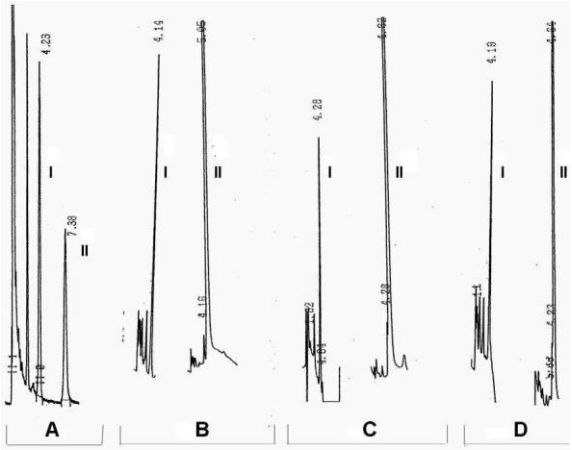


Figure 4. Effect of ion pair concentration, (**A**) 0.002M, (**B**) 0.01M, (**C**) 0.015M, and (**D**) 0.02M.

Effect of ion pair concentration

The ion pair moved the IS from 4.0 to 7.38 minutes and the cefaclor peaks from 2.7 to around 4.23 minutes away from the garbage of the plasma peaks, which showed up between 2 and 3.5 minutes, as shown in Fig. 4. Table 2 indicates that although the retention time of the IS decreased with increasing ion pair concentration, the retention time of cefaclor did not vary appreciably. Since it provides a decent and improved resolution and separation between the medication and IS, 0.002 M was determined to be the most appropriate ion pair concentration among those evaluated.

Ion Pair (M)	Retention time (min)	Retention time of Concentration (min)	of cefaclor	sulphamethoxazole
0.00 M	2.67	4.00		
0.002 M	4.23	7.38		
0.010 M	4.14	5.05		
0.015 M	4.28	4.82		
0.020 M	4.19	4.64		

Table 2. Effect of different concentrations of the ion pair on the retention times of cefaclor and Sul- phamethoxazole (IS).

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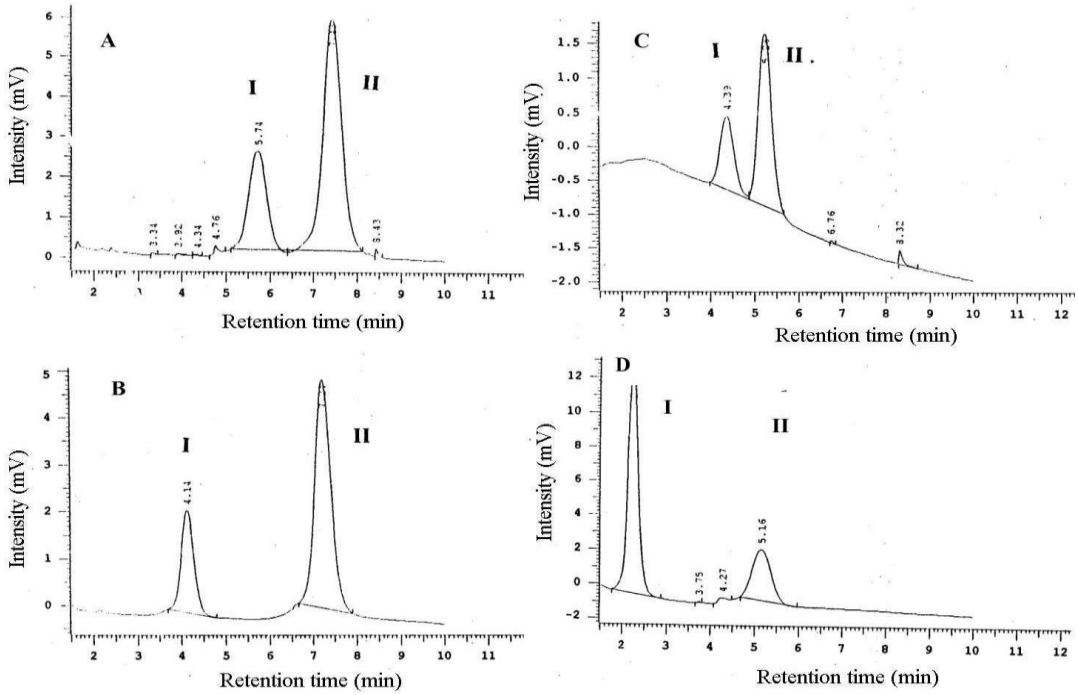


Figure 3. Chromatograms of mobile phase pH effect on cefaclor (I) and sulphamethoxazole (II): **A**) pH 3, **B**) pH 4.5, **C**) pH 6, **D**) pH 7.

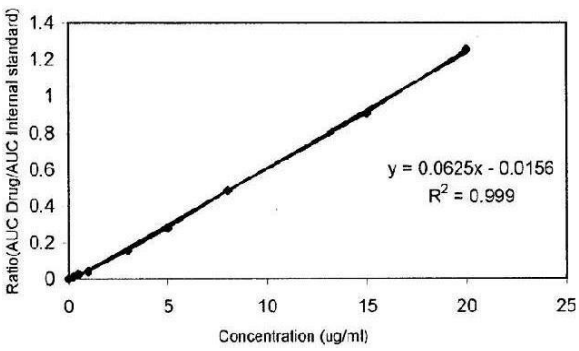
Type of detectors

UV and photo-diode array (PDA) detectors were the two kinds of detectors that were tested. With linearity up to 20.0 µg/mL and correlation coefficients (r2) of 0.999 and 0.994, respectively, the UV detector displayed a lower limit of quantization (LOQ) of 0.25 µg/mL in contrast to 0.50 µg/mL utilizing a PDA detector. Furthermore, the cefaclor area ratios over IS regions measured with a UV detector were greater than those measured with a PDA detector. These findings led to the employment of the UV detector in the current investigation.

Method validation

Specificity Four distinct individuals' blank plasma samples were used to assess the method's specificity. Cefa-clor IS and six common medications—nicotine, paracetamol, caffeine, salicylic acid, metronidazole, and ibuprofen—were added to plasma samples. There were no interferences seen between the cefaclor and IS peaks, which were well separated. Lower Limit of Quantitation (LOQ) and Linearity Plotting the peak area ratios (cefaclor/IS) against cefaclor concentrations produced the calibration curves (Fig. 5). Over a concentration range of 0.25 to 0.00 µg/mL, linearity was established (y = 0.062/C–1./6×10–2, r 2 = 0.999), where y is the ratio of the analyte's peak area to that of the IS and C is the concentration.

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Figure 5. Standard curve of cefaclor in spiked plasma on HPLC (Merck) using UV detector.

Cefaclor concentration in µg/mL. The lower limit of quantification (LLOQ) for the present technique was 0.25 µg/mL.

.Accuracy, precision and absolute recovery

As shown in Table 3, QC samples were determined at three concentration levels on three separate validation days in order to assess accuracy, precision, and recovery. [(mean observed concentration)/(spiked concentration) × 100%] was used to express accuracy, while relative standard deviation (RSD) was used to express precision. Three QC samples with concentrations of 1.5, 10.0, and 18.0 µg/mL were used to assess intra- and inter-day precisions. The accuracy and precision of the approach were excellent. According to measurements, the intra- and inter-assay precisions were less than 8 and 10%, respectively. The range of the inter-assay accuracy was 1.2 to 2.9%.

Stability

One of the fundamental parameters for the validation of the bio-analytical approach is stability evaluation. At ambient temperature and at -20°C, cefaclor in plasma shown significant deterioration; however, in frozen plasma, cefaclor degradation is slowed down by acidification of the plasma (pH < 4.5). Plasma samples used in our experiment were acidified with 20% perchloric acid (50 µL/mL) and kept between -20 and -70 degrees Celsius until they were analyzed. Cefaclor remained stable for a month at -20 and -70 °C when plasma was acidified with 20% perchloric acid, as shown in Figs. 6a and 6b. In contrast, cefaclor QC sample concentrations decreased in non-acidified plasma (Figs. 6c and 6d). Furthermore, compared to the acidified plasma (Fig. 6f) at ambient temperature, significant deterioration was observed (Fig. 6e).

CONCLUSIONS

In terms of specificity, time savings, and minimal detection limit, the current approach offers clear benefits over previous approaches now in use. Furthermore, by using perchloric acid as a precipitant and acidifying agent, the suggested approach increased the stability of cefaclor in plasma.

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Added Conc. (µg/mL)	Found Conc. (µg/mL)	Inter-day R.S.D (%)	Intra-dayR.S.D (%)	Relative error (%)
15.0	15.56	6.5	4.8	2.9
10.0	10.16	7.9	5.9	2.2
18.0	18.18	4.2	9.5	1.2

Table 3. Accuracy, recovery and precision for the analysis of cefaclor in Samples.

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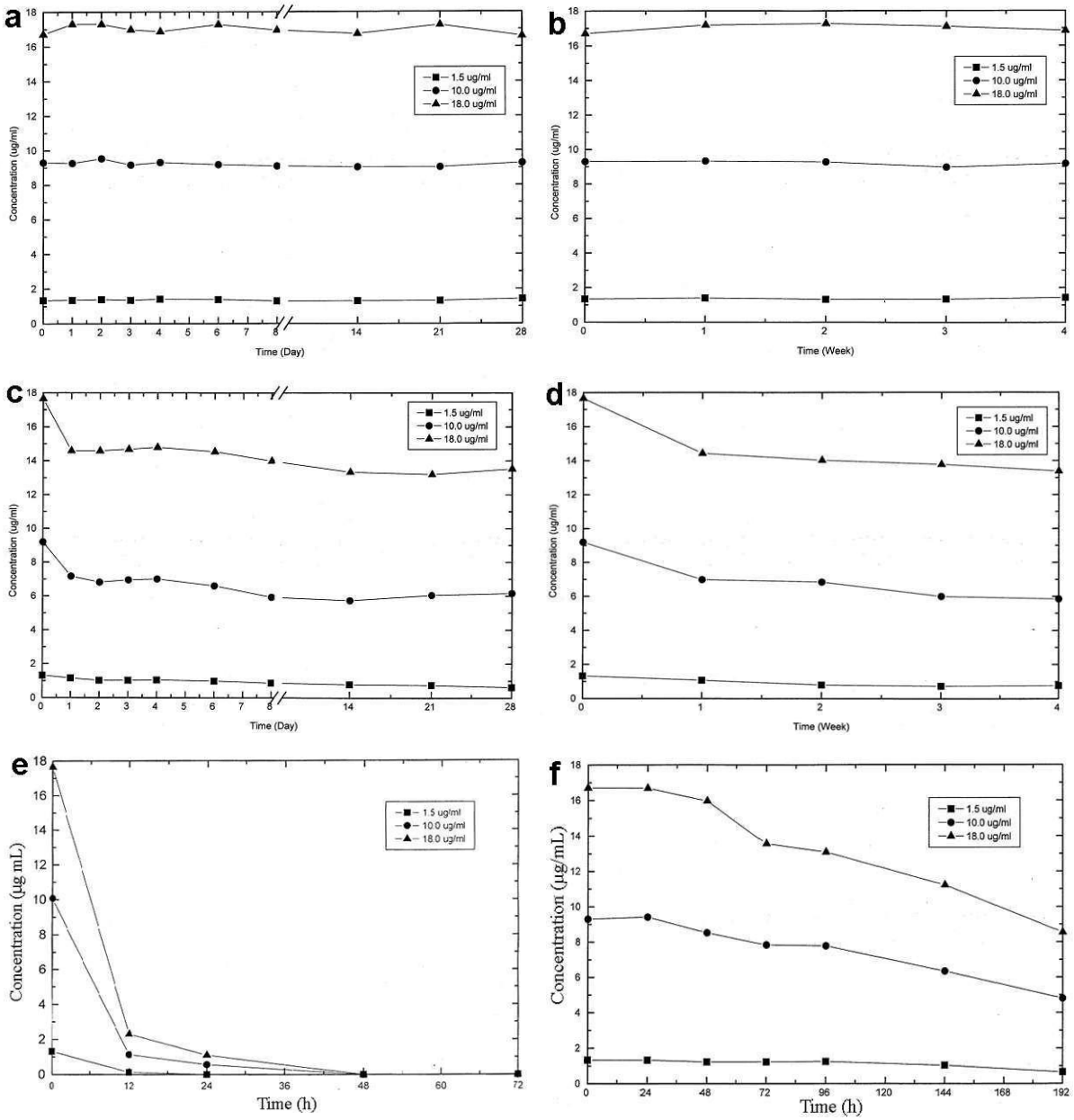


Figure 6. Cefaclor quality control samples in plasma were stable at the following temperatures: (a) -20 °C after 20% perchloric acid was added, (b) -70 °C after 20% perchloric acid was added, (c) -20 °C without 20% perchloric acid, (d) at -70 °C without 20% perchloric acid, (e) at room temperature without 20% perchloric acid, and (f) at room temperature after 20% perchloric acid was added.

Because stability assessment is regarded as a fundamental criterion for the validation of bio-analytical methods for evaluating a large number of plasma samples, bioequivalence studies and other pharmacokinetic evaluations may successfully use this approach.

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