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ORIGINAL RESEARCH

Novel HPLC method for quantitative determination of cefazolin sodium in pharmaceutical formulations

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Abstract: This paper reports a validated high-performance liquid chromatography method which is rapid, highly specific, and accurate for determination of cefazolin sodium in injectable pharmaceutical formulations. Separation was carried out using a Hibar® µBondapak® C_{18} column with a mobile phase consisting of an acetonitrile to monobasic sodium phosphate buffer ratio of 17:83 and a flow rate of 1.0 mL per minute, and monitoring at a wavelength of 254 nm. The calibration curve was linear, with a correlation coefficient >0.9995 in the range of 5–100 µg/mL. Drug recovery was 98.35%–100.86%, with a limit of detection of 12.92 ng/mL and a limit of quantification of 43.09 ng/mL. The drug was subjected to stress conditions of hydrolysis (acid, base, oxidation, and thermal degradation), where maximum degradation was observed. Forced degradation studies confirmed stability indicating power of this method, which was validated in accordance with International Conference on Harmonization guidelines and used successfully to quantify the amounts of cefazolin sodium in bulk injectable formulations and in physiological fluid.

Keywords: cefazolin sodium, blood serum, high-performance liquid chromatography

Introduction

Cefazolin sodium is a white powder, and its chemical name is 5-thia-1-azabicyclo[4.2.0] oct-2-ene-2-carboxylic acid, 3-[[(5- methyl-1,3,4-thiadiazol-2-yl)thio]methyl]-8-oxo-7-[[(1H-tetra-zol-1-yl)acetyl]amino]-, monosodium salt (*6R-trans*). It has the molecular formula of $C_{14}H_{14}N_8O_4S_3$ and its structure is shown in Figure 1.[1] Cefazolin sodium is a broad-spectrum cephalosporin antibiotic recommended for the treatment of a wide range of ocular pathogens causing various infections, including conjunctivitis, blepharitis, and keratitis.[2] Being a first-generation bactericidal cephalosporin, it inhibits biosynthesis of the cell wall[3,4] and has the same mode of action as other beta-lactam antibiotics, ie, penicillins. However, it has much wider pharmacologic activity, less toxicity, and better stability than the penicillins because of its distinct structural differences. Cefazolin sodium has been used for the treatment of respiratory tract infections and other infections of the skin, soft tissue, urinary tract, and bone and joints, including septic arthritis and osteomyelitis, and is a drug of choice for both preoperative and postoperative antibacterial therapy.[5–7] Other studies have established the efficacy of cefazolin sodium in cardiovascular indications[8,9] and in gynecology and obstetrics.[10]

Stability studies are a regulatory requirement and establish the quality of a product over time under the influence of environmental conditions, including temperature, humidity, and exposure to radiation. Stability studies help to determine appropriate packaging materials, storage conditions, and the shelf-life for a product.[11,12]

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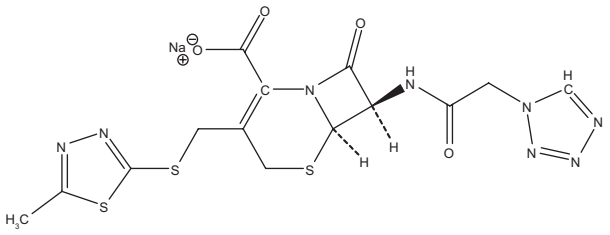

Figure 1 Molecular structure of cefazolin sodium.

Review of the literature shows that considerable attention has been paid to analysis of cefazolin sodium in pharmaceutical preparations,[13,14] urine,[15–17] and serum.[18] Conventional methods used for serum drug assays include gas chromatography,[19] polarography,[20] capillary electrophoresis,[21,22] and ultraviolet-visible spectrophotometry.[23,24] However, these analytical methods do not appear to have widespread utility, especially at the industrial level, where simple, cost-effective, and highly specific methods are needed.

High-performance liquid chromatography (HPLC) is preferred over alternative methods because it does not necessarily require extraction or volatilization for antibiotics, and is widely used for microbiological assays[25] because of its precision and accuracy. A literature search indicates that HPLC has been used to analyze cefazolin sodium alone[26] and in combination with other drugs often coadministered.[27,28] However, there have been few stability studies of cefazolin sodium using HPLC.

Cefazolin sodium contains a β-lactam ring which is very labile to acid and base,[29,30] making it very important to assess this drug under stress conditions for possible degradation products. Methods used to determine the stability of cefazolin sodium include calorimetry, gamma irradiation, and electrophoresis,[31–33] all of which are inferior to HPLC. Therefore, we attempted to develop a sensitive, accurate, and specific HPLC method for assessing the stability of cefazolin sodium using ultraviolet detection. The results were validated in accordance with International Conference on Harmonization guidelines.[34]

Materials and methods

The present method was designed to indicate stability, to be useful for studying pharmacokinetics, and to be easy, sensitive, and rapid to implement. Separation and quantification of cefazolin sodium in pharmaceutical drug formulations and blood serum was achieved by isocratic elution.

Material and reagents

Cefazolin sodium was provided by a national pharmaceutical company. Phosphoric acid was purchased from Merck (Darmstadt, Germany) and acetonitrile (HPLC grade) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Cefazolin sodium injections (Kefzol®, Biozolin®, Cefazol®, labeled as containing 1000 mg or 500 mg cefazolin sodium per vial) were sourced from the local market. Distilled water was obtained by reverse osmosis (Waterman International, Karachi, Pakistan) and further filtration through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA).

Apparatus

An SIL 10A auto injector HPLC system was used, comprising an SCL 10A system controller, SPD 20A prominence ultraviolet-visible detector, and an LC 20 AT pump (Shimadzu, Tokyo, Japan) with LC Solutions software. Separation was performed on a Hibar® μBondapak® C18, 3.5 μm bead size, 4.6 mm × 75 mm column, maintained at 25°C. An ultraviolet-visible 1650 PC spectrophotometer (Shimadzu) with ultraviolet Probe software, an ultrasonic cleaner (Elmasoni E 60 H, Elma Hans Schmidbauer GmbH and Co, KG, Singen, Germany), a Jenway 3240 pH meter (Bibby Scientific Ltd, Staffordshire, UK), and a Sartorius TE2145 analytical balance (Itin Scale Co, Brooklyn, NY, USA) were used in this work.

Chromatographic conditions

The compound was chromatographed isocratically with a mobile phase consisting of acetonitrile (HPLC grade) to monobasic sodium phosphate monohydrate buffer (2.759 g in 1 L of purified water, pH adjusted to 2.5 ± 0.1 with phosphoric acid) ratio of 17:83 (v/v). The mobile phase was filtered through a 0.45 μm membrane filter (Millipore) and degassed. The flow rate was 1.0 mL per minute and the volume injected was 20 μL. The effluent was monitored at 254 nm. All analyses were carried out at 25°C.

Analytical procedure

Standard preparation

Approximately 50 mg of cefazolin sodium was weighed as the reference standard in a 100 mL volumetric flask, dissolved by sonication, and made up to the required volume with mobile phase, giving 500 μg/mL of cefazolin sodium as the stock standard solution used in further processing. For a working standard solution, 10 mL of stock standard solution was diluted in a 100 mL flask with the same diluent. Further dilutions were made to obtain the concentrations required.

Sample preparation

To make a stock sample of cefazolin sodium 500 μg/mL, 10 vials were emptied and the contents were mixed to obtain an

evenly homogenized 50 mg sample, which was added to a 100 mL volumetric flask along with 50 mL of mobile phase. The sample was sonicated for 10 minutes, placed on a stirrer for 10 minutes, and the same diluent was added up to the mark level of the flask. This was used as the stock sample solution, 10 mL of which was diluted further in 100 mL of diluent to prepare a working sample solution. The sample was then filtered through 0.45 µm filter paper and injected into the HPLC system.

Preparation of serum sample for in vitro study

Blood samples were taken from healthy volunteers who were aged 20–30 years, not taking any medications, and not participating in any strenuous activity. All volunteers were informed of the aims and risks of the study, and written informed consent was obtained. Our ethics committee approved the study protocol. Blood was collected into an evacuated glass tube through an indwelling cannula in a forearm vein by a clinical laboratory technician. The blood was shaken and centrifuged at 10,000 rpm for 10 minutes to separate out the plasma. Next, 90 mL acetonitrile was added to 10 mL of plasma, and the mixture was centrifuged at 10,000 rpm for 10 minutes to remove the protein. The supernatant serum obtained was filtered through Whatman 42 filter paper and stored at –20°C. To make a working sample, 5 mL of the stock sample was put into a 50 mL flask and diluted with10 mL of mobile phase, followed by addition of 25 mL of serum. The sample obtained was stirred for 10 minutes and the same diluent was then added up to the mark level of the flask. All samples prepared were filtered through 0.45 µm filter paper and injected in triplicate into the HPLC system. A blank serum sample was made and run to establish the specificity of the method in biological fluid.

Preparation of sample for study of drug degradation

First, 5 mL samples of stock solution were added to four separate 50 mL volumetric flasks. Next, 15 mL of degrading agent was added to each flask individually, with the exception of one flask, to which diluent (mobile phase) only was added. The degrading agents included 0.1 N HCl, 0.1 N NaOH, and 10% H_2O_2, and the same diluent was added to each flask up to the mark level. In one experiment, all four samples were placed in a water bath at 60°C for one hour, and in another experiment, the samples were analyzed without heat. The samples were then filtered through 0.45 µm filter paper and injected into the HPLC system. Force degradation data are shown in Table 1.

Table 1 Results for forced degradation studies

Stress conditions	Heating time (hour)	Assay of active substance	Degradation (%)
Acid hydrolysis (0.1 M HCl)			
Before heat	–	10%	90%
After heat	1	5.40%	94.6%
Base hydrolysis (0.1 M NaOH)			
Before heat	–	33.2%	66.8%
After heat	1	7.2%	92.8%
Oxidative hydrolysis (10% H_2O_2)			
Before heat	–	25%	75%
After heat	1	0.0%	100%
Thermal (60°C)	1	95.3%	4.7%

Stability studies

For the stability studies, commercially available parenteral samples were stored in accelerated conditions of temperature, ie, 40°C with 75% relative humidity, and in ambient conditions of 30°C temperature with 65% relative humidity, in an environmental chamber for 6 months. The stability protocol shown in Table 2 was followed for 6 months.

Validation of methods

International Conference on Harmonization guidelines were used for the method validation studies. Various parameters, including specificity, linearity, range, accuracy, robustness, and intraday and interday precision were evaluated. To study linearity, 20 dilutions were prepared from a stock standard solution with a drug content range of 10%–200%. A standard calibration curve was generated using regression analysis.

Precision was investigated by identifying any intraday and interday variations in the results of the testing method used for cefazolin sodium in the concentration range of 40–60 µg/mL. Precision was determined by analyzing a homogeneous bulk sample against a freshly prepared standard daily three times daily at 8-hour intervals for 3 consecutive days. To determine accuracy, the reference standard for cefazolin sodium was spiked at three different concentration levels into samples for injection and into serum. Samples were prepared in triplicate for each concentration and percent recovery was determined. The limits of detection and quantification for the method were established from a linearity curve with formula (limit of detection, 3 σ/S, and limit of quantification, 10 σ/S) where σ is the standard deviation and S is the slope. This empirical formula was used to obtain the limit of quantification as shown in the chromatogram in Figure 2B.

To study the robustness of the method, injectable cefazolin sodium formulation samples were assayed with intentional variation in the method parameters, including

Table 2 Summary of stability studies

Test (claimed content)	Interval (months)								
	Initial	1	2	3	4	5	6	Mean	%RSD
Accelerated studies (30°C + 65% RH)									
Cefazolin sodium (1000 mg/vial)	100.43	99.84	99.54	99.31	99.22	99.11	98.87	99.474	0.526
Long-term stability studies (40°C + 75% RH)									
Cefazolin sodium (1000 mg mg/vial)	100.43	99.45	99.20	99.10	98.85	98.38	98.01	99.06	0.788

Abbreviations: RH, relative humidity; %RSD, percent relative standard deviation.

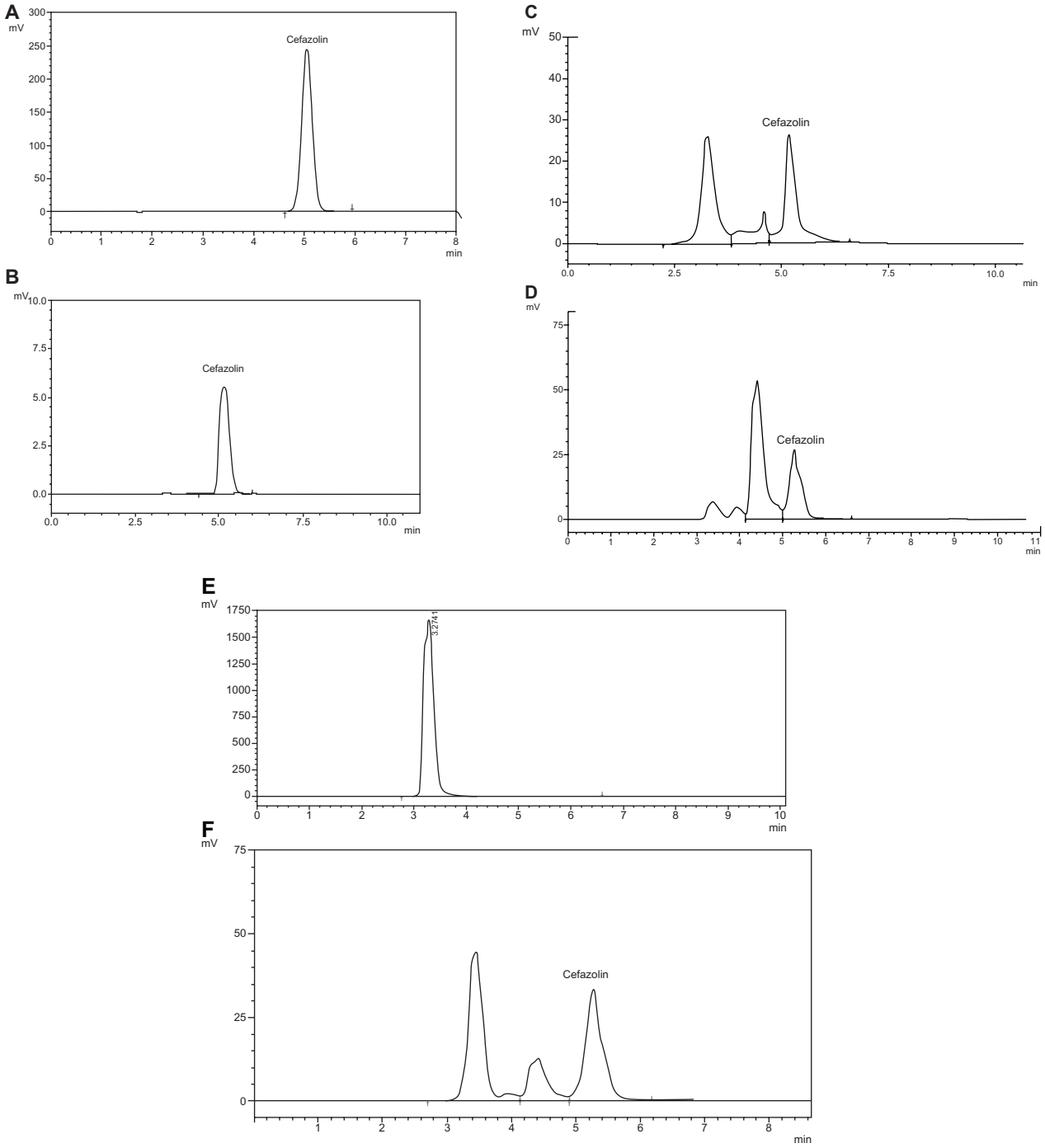

Figure 2 (**A**) Typical chromatogram of sample, (**B**) chromatogram at limit of quantification, (**C**) chromatogram of base-treated sample, (**D**) chromatogram of acid-treated sample, (**E**) chromatogram of H_2O_2 treated sample, and (**F**) chromatogram of heat-treated sample.

chromatographic conditions, mobile phase, flow rate, and temperature. Suitability of the system was established by calculating the percent relative standard deviation of repeat injections and analyzing the symmetry of the peak and theoretical plates of the column.

Results and discussion

Development of an HPLC method and its validation are important issues for any drug entering the marketplace, and is a regulatory requirement for quality assurance. Cefazolin sodium is very vulnerable to hydrolysis, so stability studies have an important role in this context. However, very few methods are available for effective assessment of the stability of cefazolin sodium. Calorimetry,[31] gamma irradiation,[32] and electrophoresis[33] can be used to indicate stability, but are not widely used, especially at an industrial level. Chromatographic methods developed by Mayer and Petsch,[21] Al-Rawithi et al,[26] Arayne et al,[35] Liang and Chow,[36] and Tsai and Chen[37] cannot assess stability. Lalitha et al[38] developed an HPLC method for assessment of stability, but this cannot be used to quantify cefazolin sodium in physiological fluid. However, good results for recovery in studies involving human serum and a low limit of quantification make this method useful for pharmacokinetic studies and routine quality control purposes because it is simple to process, has a short run time, does not require complicated instrumentation, and uses inexpensive reagents.

Development and optimization of methods

Critical parameters, such as wavelength of detection, composition of mobile phase, optimum pH, and concentrations of the standard solutions were studied in detail to develop an effective method for quantification of cefazolin sodium. To obtain the absorbance maxima, solutions containing cefazolin sodium were prepared in various solvents and run through an ultraviolet spectrophotometer in the wavelength range of 190–400 nm. A wavelength of 254 nm was established as the λmax, and was obtained when the solution was scanned in acetonitrile, methanol, and mobile phase. The chromatographic parameters were evaluated using a variety of columns, including C_{18} and C_8, and a µBondapak C_{18} was selected as the most appropriate (note that with the C_8 column the peak is asymmetrical and has a small peak shoulder). Mobile phase was developed using buffer in combination with acetonitrile or methanol. It was found that acetonitrile gave a sharp symmetric peak with a shorter run time than methanol. Similarly, the buffer and acetonitrile ratio was optimized to identify the optimal mobile phase, which had a short run time of 10 minutes without any interference, and was used in the subsequent analyses.

Table 3 Calibration curve data and validation parameters

Parameter	Inference
Linearity range (µg/mL)	5–100
Correlation coefficient (r)	0.999904
Regression equation (y = mx + c) slope (m)	6864.078
Intercept (c)	–4922.01
Limit of detection (ng/mL)	12.92
Limit of quantification (ng/mL)	43.09

Validation studies

Linearity was studied over the range of 5–100 µg/mL. The assay was judged to be linear because the correlation coefficient was >0.999 by the least squares method. A linear correlation was found between the peak areas and concentrations over the given range. The regression analysis data are shown in Table 3 and Figure 3, and the specificity and selectivity of the assay method is demonstrated by chromatography in Figure 2. Sample chromatograms were identical to the standard chromatogram, and no interference peak was observed, as shown in Figure 2A. The peak of interest fulfills all the requirements of a symmetric peak.

The intraday precision of the method was evaluated by running the assay at three drug concentrations, ie, 80%, 100%, and 120%, each in triplicate (n = 3). Interday precision of the method was tested for 3 days using freshly prepared standard calibration curve solutions. The precision and reproducibility of the method was confirmed, given that the interday and intraday precision showed a percent relative standard deviation less than 2% (Table 4).

Accuracy was investigated by spiking reference standards to a mixture of the injection excipients at three different concentration levels, ie, multiple level recovery studies. The recovery obtained (n = 9) was in the range

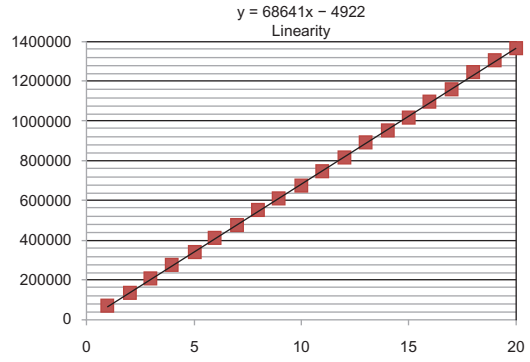

Figure 3 Linearity curve for method used to study cefazolin sodium.

Table 4 Interday and intraday precision and recovery studies

Nominal cefazolin sodium concentration (1000 mg/vial)	80%	100%	120%	Mean	Intraday RSD%
Day 1	99.36	100.67	99.87	99.97	0.661
Day 2	100.86	100.27	98.35	99.83	1.315
Day 3	99.12	99.82	99.92	99.62	0.438
Mean	99.78	100.25	99.38	99.80 overall	0.343 overall
Interday RSD%	0.95	0.42	0.90	mean	RSD%

Abbreviation: RSD%, percent relative standard deviation.

of 98.35%–100.86% (percent relative standard deviation 0.343), demonstrating that the method proposed is highly accurate. Percent recoveries for the marketed products were found to be within acceptable limits, as shown in Table 5.

There was no significant difference between results obtained using the analytical conditions established and those obtained in experiments whereby variations in some parameters were done to establish the robust nature of this novel method (Table 6). The parameters used to test the suitability of the system were symmetry of peaks, the tailing factor, and percent relative standard deviation for replicate injections.

During the degradation studies, cefazolin sodium was investigated under stress conditions, ie, the reactions to base (0.1 M NaOH), acid (0.1 M HCl), and hydrogen peroxide (10%), as well as the effect of heat. It was observed that cefazolin sodium was fairly unstable in almost all these conditions. The chromatograms in Figure 2 show evidence of degradation, which is well described in the literature.[39,40] Accordingly, three degradation products are released on treatment with acid, which correspond to three peaks in the chromatogram (Figure 2D) and two degradation products are released on treatment with base, corresponding to two peaks in the chromatogram (Figure 2C). Similarly, treatment with heat yielded two degradation products (Figure 2F) and oxidation with hydrogen peroxide resulted in complete conversion of cefazolin sodium (Figure 2E). It was found that substantial degradation occurs with oxidation by hydrogen peroxide, followed by base and acid hydrolysis. Further, heat treatment along with exposure to degrading agents increased the extent of degradation, as shown in Table 1.

Table 5 Cefazolin sodium content in three injectable formulations

Sample injection	Cefazolin sodium content (mean ± SD)
Biozolin®	100.02% ± 1.13%
Cefazol®	99.50% ± 0.91%
Kefzol®	99.47% ± 0.87%

Abbreviation: SD, standard deviation.

Table 6 Robustness of the method

Chromatographic conditions	Variation	Cefazolin sodium retention time
Temperature (°C)	25	5.565
	30	5.521
Flow rate (mL per minute)	0.9	5.835
	1.1	4.774
Acetonitrile (%)	17	5.1
	18	4.95

Stability study

Stability testing is an essential aspect of product development. This study shows how environmental conditions, including temperature, humidity, and light, influence the integrity of a drug substance over time, and enable recommendations to be made for appropriate packaging materials, storage conditions, retest periods, and shelf-life. The proposed assay method was used to study the stability of commercially available injections according to International Conference on Harmonization guidelines. Samples were analyzed and the percent contents was measured (Figure 4 and Table 2). Cefazolin sodium was found to be stable at both conditions of temperature and relative humidity, and could be analyzed accurately using the method described here.

Conclusion

This newly developed and validated HPLC method is a simple, universal, convenient, and reproducible approach to identification and quantification of cefazolin sodium in injectable pharmaceutical formulations, and shows good performance. The analytical results are accurate and precise, with good recovery and a low limit of detection. This method is

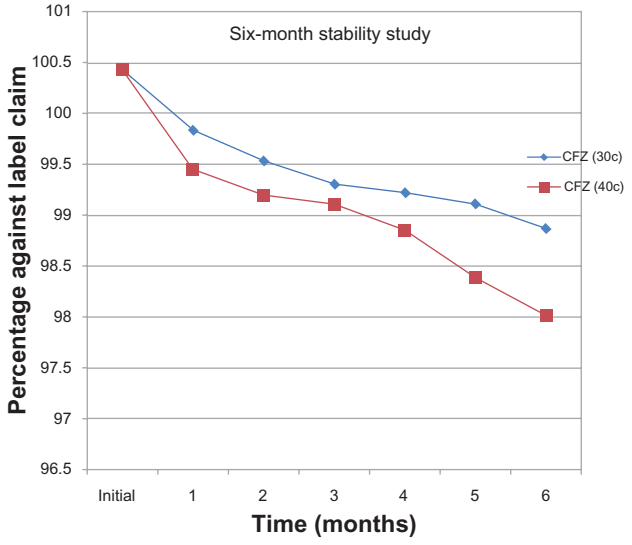

Figure 4 Graph of sample stability under ambient and accelerated conditions.
Abbreviation: CFZ, cefazolin sodium.

simple, sensitive, and robust, requiring only a short chromatographic time to perform, and can be used for routine quality control in the laboratory and for therapeutic monitoring.

Disclosure

The authors report no conflict of interest in this work.

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