AMERICAN JOURNAL OF PHARMTECH RESEARCH
Journal home page: http://www.ajptr.com/

Rapid Determination of Cefazolin Levels in Human Plasma by High Performance Liquid Chromatography

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ABSTRACT

A simple, precise, and rapid high performance liquid chromatography (HPLC) method for the determination of cefazolin level in human plasma using ceftriaxone as an internal standard (IS) was developed and validated. 0.25ml plasma samples containing cefazolin were mixed with 17.5 µg of the IS. After adding 0.3 ml of cold methanol kept in fridge at 4 °C, the mixture was vortexed for two min and then centrifuged for 15 min at 16000 rpm at room temperature. The clear supernatant was transferred into an auto-sampler vial and 100 µl was injected into the HPLC system with a run time of 18.0 min. The compounds of interest were efficiently separated on 4.6 x 150 mm Atlantis dC18-5µm steel column, using a Guard Pak pre-column module with Nova-Pak C_{18}5-µm insert, and detected using Waters 2998 photodiode array detector set at 270 nm. The mobile phase consisted of a mixture of 0.02 M of cetyltriethyl ammonium bromide and 0.01 M dipotassium hydrogen phosphate (pH = 6.5, adjusted with phosphoric acid) and acetonitrile, 60:40 (v:v) for 6 min, 50:50 for 6 min, and 60:40 for 6 min. It was delivered at a flow rate of 1.5 ml/min. No interference in blank plasma or by commonly used drugs was observed; and the detection limit of cefazolin was 0.1 µg/ml. The relationship between cefazolin concentration in plasma and peak area ratio of cefazolin/IS was linear ($r^2 \geq 0.9979$) in the range of 0.2–200 µg/ml. Intra- and inter-day coefficient of variation (CV) was ≤ 7.4% and ≤ 5.7%, with the corresponding bias of ≤ 4.9% and ≤ 5.1%. Mean extraction recovery of cefazolin and IS were 97%, respectively. Using the method, cefazolin was found to be stable in processed samples for 48 hrs at -20°C (≥ 96%), and in unprocessed samples for 8 weeks at -20°C (≥ 98%), Further, the method was successfully used to measure cefazolin level in plasma samples.

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Received 14 November 2016, Accepted 22 November 2016

Please cite this article as: Hammami MM et al., Rapid Determination of Cefazolin Levels in Human Plasma by High Performance Liquid Chromatography. American Journal of PharmTech Research 2016.

INTRODUCTION

Cefazolin sodium (CAS: 27164-46-1), 5-Thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylicacid,3-[[(5-methyl-1,3,4-thiadiazol-2-yl)thio]methyl]-8-oxo-7-[[2-(1H-tetrazol-1-yl)acetyl]amino]-sodium salt[1], is a first-generation cephalosporin antibiotic with broad spectrum activity against both gram-negative and gram-positive bacteria[2]. It is often the preferred drug for surgical prophylaxis[3]. It reaches a plasma concentration of 22.9 to 40.8 µg/ml 6 hrs after a single dose of 2 gm by IV push or 30-minute IV infusion. Following 1 gram IV push, mean peak serum concentration was 185 µg/ml[5]. Following a single 500 mg IM injection, mean peak serum concentration was 34.1 µg/ml and achieved in 1 to 2 hours[6].

Several high-performance liquid chromatography (HPLC) analytical methods have been reported for determination of cefazolin in pharmaceutical preparations[7-9] and human samples[10-14]. Some methods were required longer run time for cafazolin measurement (25 min)[7]and (30 min)[10], some suffered from lack of sensitivity (2.0 µg/ml)[11],and some required large plasma volume (1 ml)[12]. Further, reported information on the stability of cefazolin is inadequate[10].

In this paper, we describe a precise and accurate HPLC method for cefazolin level determination in human plasma. The method uses 0.25 ml plasma and simple extraction method, was fully validated, and was successfully used to determine cefazolin level in human plasma as well as cefazolin stability under some clinical laboratory condition.

MATERIALS AND METHOD

Apparatus

Chromatography was performed on a Waters Alliance HPLC e2695D Separations Module, an 4.6 x 150 mm, Atlantis dC18 (5-µm particle-size) steel column, a Guard Pak pre-column module with Nova-Pak C18, (5-µm) insert, and Waters 2998 photodiode array detector set at 270 nm (Waters Associates Inc., Milford, MA, USA). Data were collected with a Pentium D computer using Empower Chromatography Manager Software (Waters Associates Inc., Milford, MA, USA).

Chemical and reagents

All reagents were of analytical grade unless stated otherwise. Cefazolin sodium salt and ceftriaxone sodium salt standards were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. Cetyltriethyl ammonium bromide and dipotassium hydrogen phosphate were purchased from BDH Chemicals Ltd, Poole, England. Phosphoric acid and acetonitrile (both HPLC grade) were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and was further purified by passing through a Synergy UV (Millipore, Bedford, MA,

USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC) Riyadh, Saudi Arabia.

Chromatographic conditions

The mobile phase consisted of a mixture of 0.02 M of cetyltriethyl ammonium bromide and 0.01 M dipotassium hydrogen phosphate (pH = 6.5, adjusted with phosphoric acid) and acetonitrile and was used in proportion 60:40 (v:v) for 6 min, 50:50 for 6 min, and 60:40 for 6 min labeled run of 18 min, respectively. It was delivered at a flow rate of 1.5 ml/min. The analysis was carried out under gradient condition maintaining column at room temperature. A photodiode array detector set at 270 nm was used for recording chromatograms.

Preparation of standard and quality control samples

Stock solutions (1mg/ml) of cefazolin and ceftriaxone (internal standard, IS) were prepared in water. They were diluted with blank human plasma or water, respectively, to produce working solutions of 200 µg/ml for cefazoline in plasma and 250 µg/µlin water for the IS. Calibration curve standards (ten concentrations) in the range of 0.2–200 µg/ml were prepared in human plasma. Four quality control (QC) samples (0.2, 0.6, 100, and 180 µg/ml) were also prepared in human plasma. 0.25 ml aliquots in 1.5 ml eppendorf micro-centrifuge tubes (Fisher Scientific Co., Fairlawn, NJ, USA) were stored at –20ºC until used.

Sample preparation

Aliquots of 0.25 ml of calibration curve, and QC samples in 1.5 ml eppendorf microcentrifuge tubes were allowed to equilibrate to room temperature. To each tube, 70 µl of the IS working solution (250 µg/ml in water) were added and the mixture was vortexed for 20 seconds. After the addition of 0.3 ml of cold methanol kept in fridge at 4°C, the mixture was vortexed again for 2 min and then centrifuged for 15 min at 16000 rpm at room temperature. The clear supernatant organic layer was carefully transferred into an auto-sampler vial and 100 µl were injected into the HPLC system. The total run time was 18 min.

Stability studies

Three QC samples (concentration 0.2, 0.6, and 180 µg/ml) were used for stability studies: five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed, five aliquots were stored at -20ºC for 8 weeks before being processed and analyzed, and five aliquots were processed and stored at room temperature for 24 hours or -20 ºC for 48 hours before analysis. Fifteen aliquots of each QC sample were stored at -20ºC for 24 hours. They were then taken out of freezer and left to completely thaw unassisted at room temperature. Five

aliquots of each sample were extracted and analyzed and the rest were returned to -20ºC for another 24 hours. The cycle was repeated three times.

Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance[15]. The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Optimal experimental conditions consisted a mobile phase composed of 0.02 M of cetyltriethyl ammonium bromide and 0.01 M dipotassium hydrogen phosphate (pH = 6.5, adjusted with phosphoric acid) and acetonitrile, 60:40 (v:v) for 6 min, 50:50 for 6 min, and 60:40 for 6 min. It was delivered at a flow rate of 1.5 ml/min. Under these conditions cefazolin, ceftriaxone, and components of plasma exhibited a well-defined separation within the18 minute run. The retention times of cefazolin and ceftriaxone were around 4.6 and 10.8 minutes, respectively.

Specificity

Specificity is defined as the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in plasma samples include endogenous components, metabolites, and decomposition products. We screened six batches of blank human plasma and eight frequently used medications (Aspirin, acetaminophen, ranitidine, omeprazole, ibuprofen, nicotinic acid, ascorbic acid, and diclofenac) for potential interference. No interference was found in plasma and none of the drugs co-eluted with cefazolin or the IS. Figure 1 depicts a representative chromatogram of drug free human plasma used in preparation of standard and QC samples.

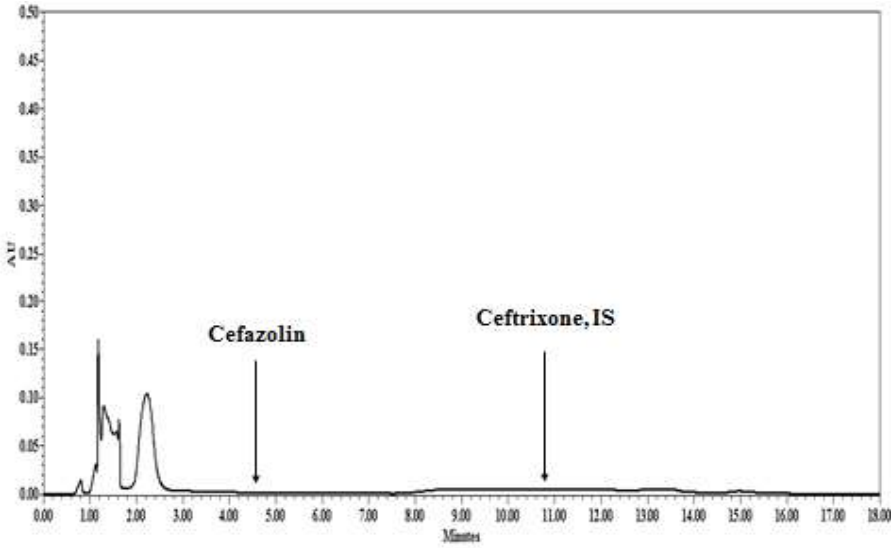

Figure 1: Representative chromatogram of a drug-free human plasma. The arrows indicate retention times of cefazolin and the internal standard(ceftrixone, IS)

Limit of detection & quantification and linearity

The limit of quantification was, defined the lowest concentration on the calibration curve that can be determined with acceptable precision and accuracy (i.e., coefficient of variation and bias ≤15%), was 0.2µg/ml. The limit of detection (signal to noise-ratio ≥ 3) was 0.1µg/ml. Linearity of cefazolin was evaluated by analyzing ten curves of ten standard concentrations (plus zero concentration) prepared in human plasma. Figure 2 depicts an overlay of chromatograms of a typical calibration curve. Mean (SD) of slope, intercept, and coefficient of determination (R^2) of the ten curves were 0.0063 (0.0006), 0.0030 (0.0046), and 0.9979(0.0018), respectively. The suitability of the calibration curves was confirmed by back-calculating the concentration of cefazolin from the calibration curves (Table 1). All calculated concentrations were well within the acceptable limits.

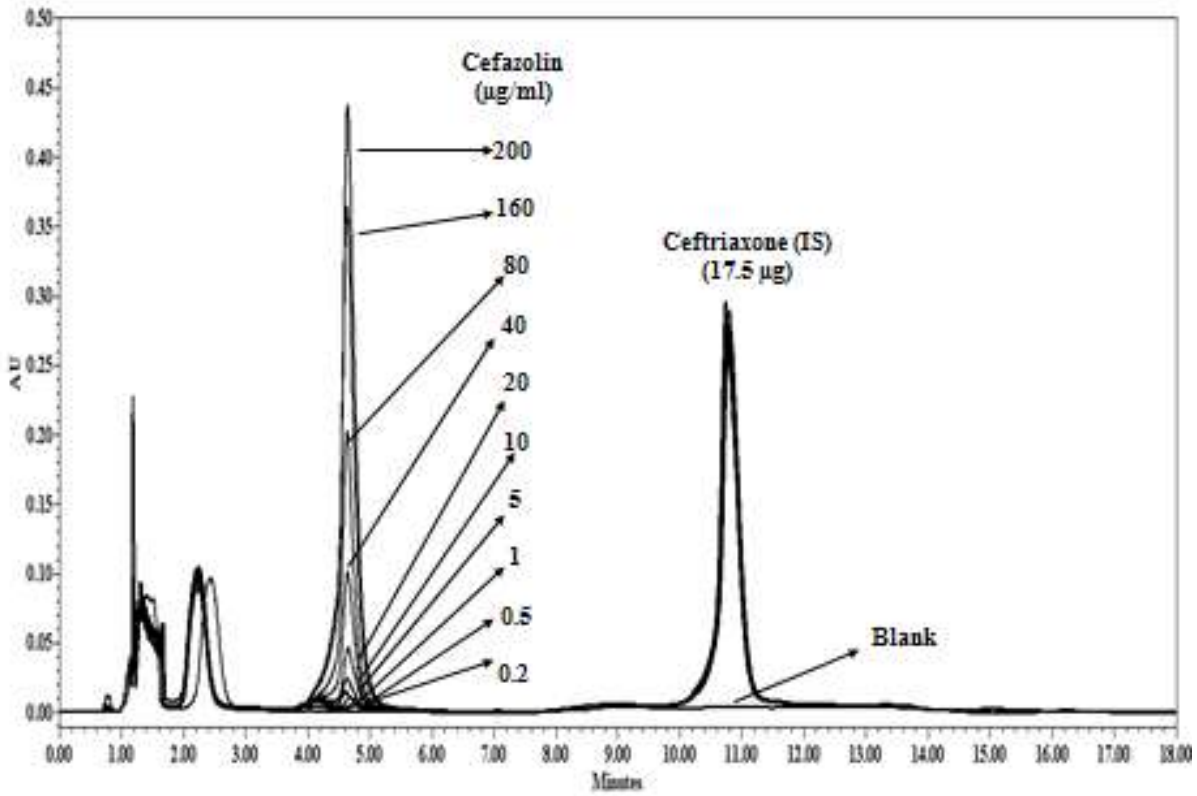

Figure 2: Overlay of chromatograms of extracts of 0.25 ml human plasma spiked with the internal standard (IS) or one of nine concentrations of cefazolin.

Table 1: Back calculated cefazolin concentrations from ten calibration curves

Nominal level (µg/ml)	Measured level (µg/ml) Mean (SD)	CV (%)	Bias (%)
0.2	0.22 (0.02)	7.7	9.7
0.5	0.51 (0.04)	7.7	1.5
1.0	0.97 (0.09)	9.2	-3.3
5.0	4.93 (0.52)	10.6	-1.5
10.0	10.00 (0.88)	8.8	0
20.0	20.58 (1.37)	6.6	2.9
40.0	40.93 (2.45)	6.0	2.3
80.0	83.71 (5.22)	6.2	0.9
160.0	159.47 (5.80)	3.6	0.3
200.0	199.93 (4.71)	2.4	0.5

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100.

Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.

Precision and bias (accuracy)

The intra-day and inter-day precision and bias of the method were evaluated by analyzing four QC concentration (0.2, 0.6, 100, and 180 µg/ml). Intra-day precision and bias (n = 10) ranged from 2.1% to 7.4% and from -4.9% to -0.90%, respectively. Inter-day precision and bias were

determined over three different days (n = 20) and ranged from 3.2% to 5.7% and from –5.1% to 0%, respectively. The results are summarized in Table 2.

Table 2: Intra and inter-day precision and bias of cefazolin assay

Nominal level(µg/ml)	Measured level (µg/ml)	CV (%)	Bias (%)
Intra-day (n= 10)			
0.2	0.19 (0.01)	7.4	-4.9
0.6	0.59 (0.02)	3.9	-0.9
100	95.40 (2.03)	2.1	- 4.6
180	174.05 (5.88)	3.4	-3.3
Inter-day(n= 20)			
0.2	0.19 (0.01)	5.7	-3.9
0.6	0.58 (0.03)	4.3	0
100	94.86 (3.0)	3.2	-5.1
180	171.84 (7.32)	4.3	-4.5

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100.

Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.

Recovery

Extraction recovery of cefazolin was assessed by direct comparison of peak areas from plasma and mobile phase samples, using five replicates for each of QCs (0.2, 0.6, 100, and 180µg/ml). Similarly, the recovery of the IS was determined by comparing peak areas of the IS in five aliquots of human plasma spiked with 70 µl of 250 µg/ml IS solution with the peak areas of equivalent samples prepared in the mobile phase. The results are presented in Table 3.

Table 3: Recovery of cefazolin and the internal standard from 0.25 ml human plasma

Concentration (µg/ml)	Human plasma *Mean (SD)	Mobile phase *Mean (SD)	Recovery (%)
Cefazolin			
0.2	6538 (382)	6607 (231)	99
0.6	17645 (2213)	18947 (330)	93
100	3806508 (17085)	3811195(3402)	100
180	6551088(91261)	6779126(3356)	97
Internal standard			
17.5	6151244 (96657)	6316148(26203)	97

*Mean peak area (standard deviation), n = 5. Recovery is calculated as mean peak area in human plasma divided by mean peak area in mobile phase x 100.

Robustness and Ruggedness

The robustness of a method is a measure of its capacity to remain unaffected by small variations in analysis conditions. The robustness of the current assay was evaluated by slightly altering

proportions of acetonitrile (± 2.0%) in the mobile phase. No significant changes were observed. Ruggedness was tested by conducting split sample test. Two split samples (concentration 0.6 and 100 µg/ml) were analyzed by two blinded technologists on two different instruments. The accuracy of the reported concentrations was within the acceptable limits (bias ≤ 5.0%).

Stability

Stability of analyts in biological matrices is an important pre-analytical variable. It is necessary to perform stability studies of the analyte and IS to determine the range of appropriate conditions and time of storage. Cefazolin and IS stability in processed and unprocessed plasma samples was investigated using three QCs (0.6, 100, and 180 µg/ml). Cefazolin in processed samples was found to be stable for 24 hours at room temperature (≥ 93%) and 48 hours at −20 °C (≥ 96%). Cefazolin in unprocessed samples was stable for at least 24 hours at room temperature (≥ 95%),8 weeks at −20°C (≥ 9 8%), and after three freeze-and thaw cycles (≥ 93%). Table 4 summarizes the results of stability studies.

Table 4: Stability for cefazolin in human plasma

	Stability (%)						
Nominal level (µg/ml)	**Unprocessed**		**Processed**		**Freeze-Thaw Cycle**		
	24 hrs RT	**8wks -20°C**	**24 hrs RT**	**48 hrs -20°C**	**1**	**2**	**3**
0.2	110	98	93	96	100	93	94
0.6	95	105	100	98	101	92	93
180	103	103	110	107	96	102	98

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after 24 hours at room temperature (24 hrs RT), or after freezing at –20ºC for 8 weeks (8wks, –20 ºC), or processed and then analyzed after storing for 24 hours at room temperature (24 hrs, RT) or 48 hours at –20 ºC (48 hrs, -20 ºC). Freeze-thaw (FT), samples were frozen at -20 ºC and thaw at RT.

CONCLUSION

The described HPLC assay is precise, simple, accurate and rapid. It requires only 0.25 ml plasma and utilizes a simple protein precipitation procedure. The assay was successfully applied to monitor stability of cefazolin under various conditions encountered in the clinical laboratories.

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