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A Validated Reversed Phase HPLC Assay for the Determination of Cefuroxime in Human Plasma

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Original Research Article

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Abstract: A simple and precise reversed-phase high performance liquid chromatography (HPLC) method for the determination of cefuroxime in human plasma was developed and validated. Using cefazolin as an internal standard (IS), 0.25 ml plasma samples were deproteinized with 90 µl of 3% trichloroacetic acid in methanol, the supernatant was extracted with 150 µl acetonitrile, and 100 µl of the second supernatant were injected into the HPLC system. Separation was achieved on Atlantis dC18 column with a mobile phase composed of 0.01 M cetyltriethylammonium bromide, 0.01 M dipotassium hydrogen phosphate (pH 6.5), and acetonitrile (30:30:40, v:v:v). The mobile phase was spiked with triethylamine (10 µl/L) and delivered at 1.0 ml/minute. The eluent was monitored spectrophotometrically at 278 nm. No interference with cefuroxime and IS peaks by extracted blank plasma components or commonly used drug was observed. The relationship between cefuroxime concentration and peak height ratio of cefuroxime to the IS was linear over the range of 0.25-14.0 µg/ml. Coefficient of variation and bias were ≤ 12.6% and ≤ 11.0%, respectively. Mean extraction recovery of cefuroxime and the IS was 99% and 95%, respectively. The method was applied to assess the stability of cefuroxime under various conditions encountered in the clinical laboratory. Cefuroxime stability in processed samples stored at room temperature for 24 hours or 48 hours at -20 °C, and in unprocessed samples for 24 hours at room temperature or 14 weeks at -20 °C was ≥ 96% and ≥ 83%, respectively.
Keywords: Cefuroxime, Cefazolin, Human plasma, HPLC.

INTRODUCTION

Cefuroxime (CAS: 55268-75-2) is a second generation broad spectrum, semi synthetic cephalosporin antibiotic [1-2]. Its oral bioavailability is about 52% with a mean (SD) peak plasma concentration of 4.86 ± 1.33 µg/ml within 2 hours after ingestion of a 500 mg therapeutic dosage [3]. Figure-1 depicts the chemical structures of cefuroxime and the internal standard (IS), cefazolin.

Several analytical methods have been reported for the determination of cefuroxime in pharmaceutical formulations and biological samples [4-15], using microbiological techniques [4-6] or high performance liquid chromatography (HPLC) coupled with ultraviolet (UV) [7-12] or mass spectrometric detectors [13-15]. Cefuroxime level in human serum/plasma has been mainly determined by HPLC with UV detector [7-10]. Most of the reported methods involved liquid-liquid [7-8] or solid-liquid [9-10] extraction procedures and used ≥ 0.50 ml human plasma [12]. Few methods were based on protein precipitation [11, 13].

In the present study, we describe a simple, precise, and rapid cefuroxime HPLC assay that requires 0.25 ml human plasma. The assay is based on protein precipitation followed by UV detection. It was fully validated and successfully applied to assess the stability of cefuroxime under various laboratory conditions.

MATERIAL AND METHODS

Apparatus

Chromatography was performed on a Waters Alliance HPLC 2695 system (Waters Associates Inc., Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, and photodiode array detector. We used a reversed-phase Atlantis dC-18 (4.6 x 150 mm, 5µm) column protected by guard column Symmetry C18 (3.9 x 20 mm, 5µm). Data were collected with a Pentium IV computer, using Empower Chromatography Software.

Chemical and reagents

All reagents were of analytical-reagent grade unless stated otherwise. Cefuroxime, cefazolin and dipotassium hydrogen phosphate were purchased from Sigma-Aldrich Co, Steinheim, Germany. Acetonitrile,

methanol (both HPLC grade), trichloroacetic acid, and triethylamine were purchased from Fisher Scientific, Fairlawn, NJ, USA. Cetyltriethylammonium bromide was purchased from Aldrich chemical company Inc., Milwaukee, WI, USA. HPLC grade water was prepared by reverse osmosis and further purified by passing through a Synergy Water Purification System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre, Riyadh, Saudi Arabia.

Chromatographic conditions

The mobile phase was composed of 0.01 M cetyltriethylammonium bromide, 0.01 M dipotassium hydrogen phosphate, (pH= 6.5, adjusted with phosphoric acid), and acetonitrile (30:30:40, v:v:v). It was spiked with triethylamine (10 µl/L) in order to improve peaks' symmetry and delivered at a flow rate of 1.0 ml/minute at ambient temperature with a run time of 9.0 minutes. A photodiode array detector set at 278 nm was used.

Preparation of standard and quality control samples

Stock solution of cefuroxime and cefazolin (1.0 mg/ml) were prepared in methanol. They were further diluted with methanol to produce working solutions of 100 µg/ml. Ten calibration standards in the range of 0.25 – 14.0 µg/ml and four quality control (QC) samples (0.25, 0.75, 7.0, and 12.6 µg/ml) were prepared in human plasma. Calibration standards and QC samples were vortexed for one minute and 0.25 ml aliquots were transferred into 1.5 ml Eppendorf microcentrifuge tubes and stored at –20 °C until used.

Sample preparation

Aliquots of 0.25 ml of calibration curve samples or QC samples were allowed to equilibrate to room temperature. To each tube, 40 µl of the IS working solution (100 µg/ml in methanol) were added and the mixture was vortexed for 10 seconds. After the addition of 90 µl of 3% tricholoroacetic acid in methanol, the mixture was vortexed again for 2 minutes and then centrifuged for 10 min at 4200 rpm. The supernatant was carefully collected into a clean tube and 150 µl of acetonitrile was added, vortexed, and centrifuged for 5 minutes. The second supernatant was transferred into an auto-sampler vial and 100 µl were injected into the HPLC system.

Stability studies

A total of 40 aliquots of each QC sample (0.25, 0.75, and 12.6 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 (counter stability, 24 hours at room temperature), five aliquots were stored at –20 °C for fourteen weeks before being processed and analyzed (long term freezer storage stability), and five aliquots were processed and stored at room temperature for 24 hours or 48 hours at –20 °C before analysis (autosampler stability). Finally, fifteen aliquots of each QC sample were stored at –20 °C for 24 hours. They were then left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest returned to –20 °C for another 24 hours. The cycle was repeated three times (freeze-thaw stability).

Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance [16]. The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

A mobile phase composed of 0.01 M cetyltriethylammonium bromide, 0.01 M dipotassium hydrogen phosphate, (pH= 6.5, adjusted with phosphoric acid), and acetonitrile (30:30:40, v:v:v), spiked with 10 µl/L triethylamine, and delivered at flow rate of 1.0 ml/minute provided optimal experimental conditions with a well-defined separation within 9 minutes run time. The retention times of the IS and cefuroxime were around 6.0 and 7.1 minutes, respectively.

Specificity

Specificity is a measure of the ability of the analytical method to differentiate and quantify the analytes of interest in the presence of other components in the sample. No endogenous component from extracted human plasma co-eluted with cefuroxime or the IS. Figure-2 depicts a representative chromatogram of drug free human plasma used in preparation of calibration standards and QC samples.

Linearity, Accuracy and Precision

Linearity of cefuroxime was evaluated by analyzing ten curves of ten calibration standard concentrations over the range (0.25-14.0 µg/ml) prepared in human plasma. Figure-3 represents an overlay of chromatograms of extracts of 0.25 ml human plasma spiked with the IS and one of ten concentrations of cefuroxime. The peak height ratios were subjected to regression analysis. The suitability of the calibration curves was confirmed by back-calculating cefuroxime concentration from the calibration curves (Table-1). All back-calculated concentrations were well within the acceptable limits. Precision and bias were also determined for four QCs (0.25, 0.75, 7.0, and 12.6 µg/ml). The intra-day (n=10) and inter-day (n=20, over three consecutive days) precision was ≤ 12.6% and ≤ 8.8%, respectively. The intra-day and inter-day bias ranged from was in the range of +0.2 to +11.0% and

from -2.8 to +9.0%, respectively. The results are summarized in Table-2.

Table-1: Back-calculated cefuroxime concentrations from ten calibration curves

Nominal level (µg/ml)	Calculated level (µg/ml)		CV (%)*	Bias** (%)
	Mean	SD		
0.25	0.2672	0.0199	7.5	+6.9
0.50	0.5361	0.0305	5.7	+7.2
1.0	1.0578	0.0505	4.8	+5.8
2.0	2.1562	0.0907	4.2	+7.8
4.0	4.1913	0.2346	5.6	+4.8
6.0	6.2211	0.3496	5.6	+3.7
8.0	8.0166	0.3371	4.2	+0.2
10.0	9.5264	0.4927	5.2	-4.7
12.0	11.9880	0.5095	4.2	-0.1
14.0	14.4359	0.4574	3.2	+3.1

*Coefficient of variation (CV) = standard deviation (SD) divided by mean measured concentration x 100. **Bias = measured level – nominal level divided by nominal level x 100.

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

Nominal level (µg/ml)	Measured level (µg/ml)		CV (%)*	Bias (%)**
	Mean	SD		
Intra-day (n=10)				
0.25	0.2775	0.0309	11.1	11.0
0.75	0.7650	0.0275	3.6	2.0
7.00	7.0169	0.8875	12.6	0.2
12.6	12.6706	0.5183	4.1	0.6
Inter-day (n=20)				
0.25	0.2713	0.0238	8.8	9.0
0.75	0.7577	0.0489	6.5	1.0
7.00	6.8022	0.5633	8.3	-2.8
12.6	13.4302	0.6445	4.8	6.6

SD, standard deviation. *CV, coefficient of variation (CV) = standard deviation divided by mean measured concentration x100.
**Bias = measured level - nominal level divided by nominal level x 100.

Recovery

Cefuroxime recovery was assessed by direct comparison of its peak height in plasma and in methanol samples, using five replicates for each of four QCs (0.25, 0.75, 7.0, and 12.6 µg/ml). Similarly, the recovery of the IS was determined by comparing its peak height (at 16.0 µg/ml) in plasma and in methanol samples. The results are presented in Table-3. Recovery of cefuroxime and the IS was ≥ 96% and 95%, respectively.

Table-3: Recovery of cefuroxime and internal standard (IS) from 0.25 ml human plasma

Concentration (µg/ml)	Mean peak height (SD)*		Recovery** (%)
	Human Plasma	Methanol	
Cefuroxime 0.25	22244 (365)	22078 (270)	101
0.75	19965 (1398)	20782 (153)	96
7.0	11290 (2725)	11364 (2608)	99
12.6	10345 (3837)	10444 (1524)	99
Internal standard 16.0	34586 (529)	36414 (382)	95

*Mean peak height (SD), n = 5. **Recovery = peak height in human plasma divided by peak height in methanol x 100.

Table-4: Stability of cefuroxime under various clinical laboratory conditions

Nominal level (µg/ml)	Unprocessed		Processed		Freeze-Thaw Cycle		
	24 hrs RT	14 wks -20 °C	24 hrs RT	48 hrs -20 °C	1	2	3
0.25	101	83	107	96	98	99	86
0.75	98	91	98	100	101	103	104
12.6	92	96	105	110	110	101	88

Data represent stability (%) calculated as 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 –20 ºC for 14 weeks (14 wks, –20 ºC), or were 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) or after 1 to 3 cycles of freezing at –20 ºC and thawing at room temperature (freeze-thaw).

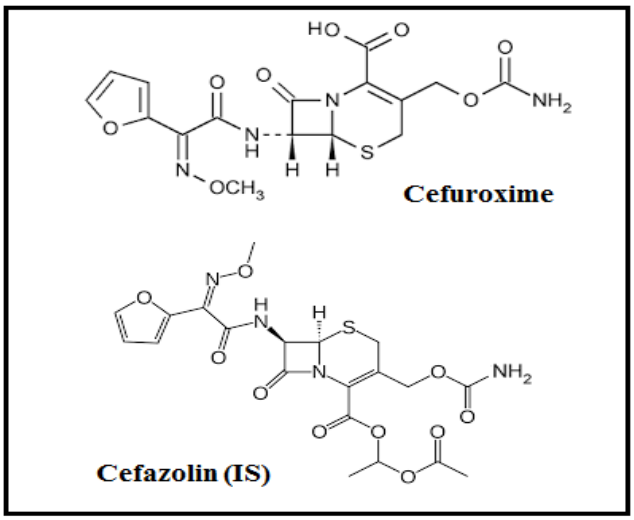

Fig-1: Chemical structures of cefuroxime and cefazolin (IS)

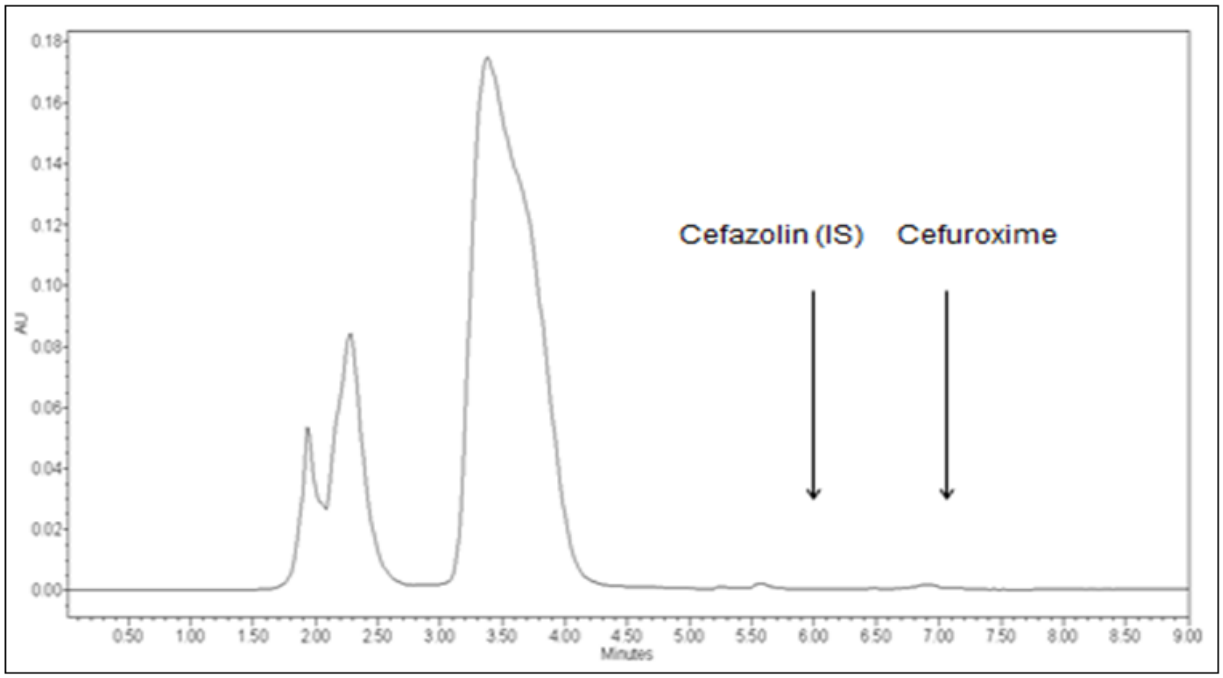

Fig-2: Representative chromatogram of blank human plasma

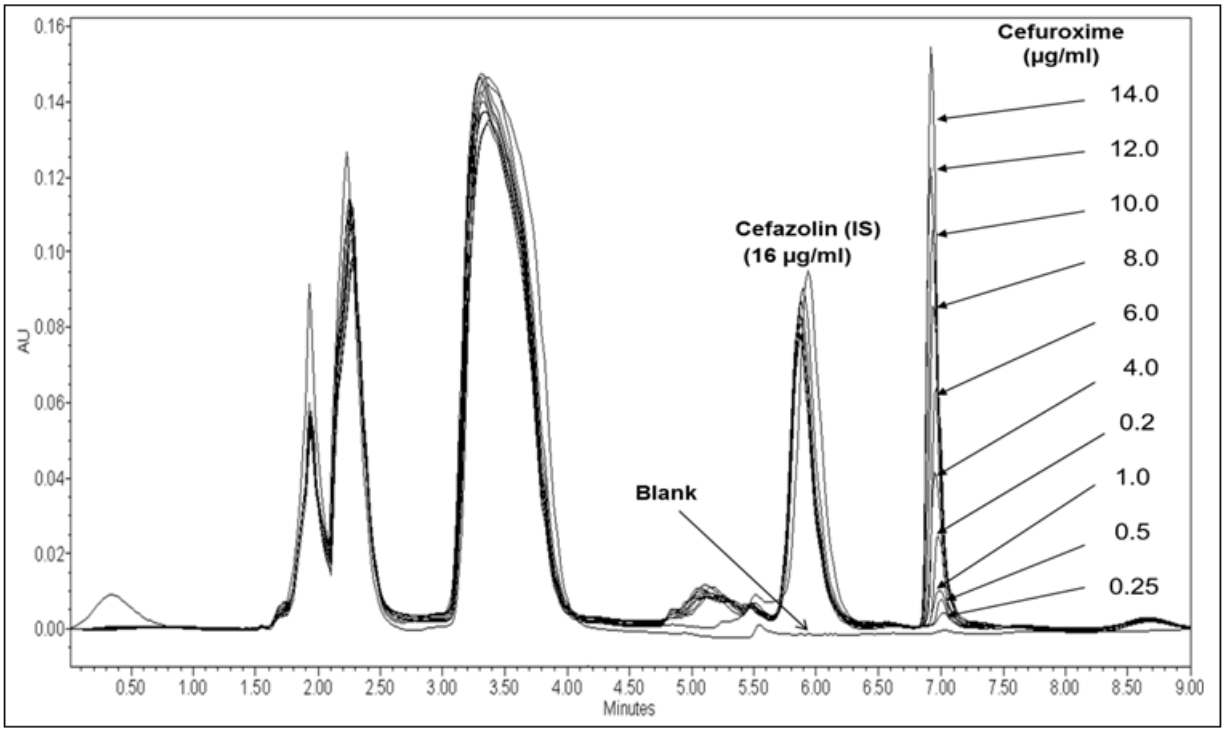

Fig-3: Overlay of chromatograms of extracts of 0.25 ml human plasma spiked with the internal standard (IS) alone or with cefuroxime at one of ten concentrations

Stability

Cefuroxime stability in processed and unprocessed QC samples (0.25, 0.75 and 12.6 µg/ml) was investigated. Cefuroxime was stable in processed samples for at least 24 hours at room temperature (≥ 98%) or 48 hours at -20 °C (≥ 96%). Cefuroxime in unprocessed samples was stable for at least 24 hours at room temperature (≥ 92%), fourteen weeks at -20 °C (≥ 83%), or after three freeze-and thaw cycles (≥ 86%).

CONCLUSION

The described HPLC assay is accurate, precise, and rapid. It requires only 0.25 ml plasma and utilizes a simple and convenient precipitation method for sample preparation. The assay was applied to monitor stability of cefuroxime under various conditions generally encountered in the clinical laboratories.

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