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Ceftriaxone stability and sensitive analysis by fully validated ion-pair HPLC assay in human plasma

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ABSTRACT

We validated a sensitive, precise, and accurate HPLC assay for ceftriaxone measurement in human plasma and studied the stability of ceftriaxone. After protein precipitation with methanol at 4ºC, ceftriaxone and cefazolin (internal standard) were eluted on XTerra® RP18, 5 µm steel column at room temperature (RT). The mobile phase consisted of 0.02 M cetyltrimethylammonium bromide in 0.01 M dipotassium hydrogen orthophosphate buffer (pH = 6.5, adjusted with phosphoric acid), acetonitrile, and triethylamine (70:30: 0.001, v:v:v) with a run time of 14 min. The analytes were detected using 2998 photodiode array detector set at 272 nm. The response was linear over the range of 0.2–200 µg/ml. Extraction recovery and inter-run bias and precession were = 94% (mean 96%), = 8%, and = 5.7%, respectively. Ceftriaxone was stable in plasma for 24 hours at RT (= 95%), 8 weeks at –200C (= 97%), and after 3 cycles of freeze at –200C and thaw at RT (= 91%). In processed samples, ceftriaxone was stable for 24 hours at RT (= 96%) and 48 hours at –200C (= 91%). Stock solution of ceftriaxone (1 mg/ml in water) was stable for 48 hours at RT (100%) and 8 weeks at –200C (96%).

KEYWORDS

Ceftriaxone;
Cefazolin;
HPLC;
Validation;
Stability.

INTRODUCTION

Ceftriaxone sodium, a 2-aminothiazolyl methoxyimino derivative (CAS number: 73384-59-5), is a semisynthetic, broad-spectrum, third generation cephalosporin, beta-lactam antibiotic. Its bactericidal / antimicrobial activity against both gram positive and negative pathogens results from inhibition of bacterial cell wall synthesis, and is mediated through its binding to one or more penicillin binding proteins (PBPs).[1] It has rapid absorption, wide distribution in tissues and body fluids, and good tolerance after parenteral administration.[2] It is bound extensively to plasma proteins (83% to 96%) in a concentration dependent manner, and has a half-life of 8 hours.[1] Its peak serum levels was 151 µg/ml, 30 minutes after a 1 g iv infusion, and 33 to 38 µg/ml and 68 to 76 µg/ml, 1 to 2 hours after 0.5 g and 1g IM doses, respectively.[1]

Several methods have been described for the determination of ceftriaxone, including microbiological,[3-6] spectrophotometry,[7-10] capillary electrophoresis,[11] High Performance Thin Layer Chromatography (HPTLC),[12] High Performance Liquid Chromatog-

raphy (HPLC),[13-34] and Liquid Chromatography coupled with tandem Mass Spectrometry (LC-MS/MS) methods.[35]

The reported HPLC methods on human plasma[22-34] utilized protein precipitation with acids,[18] water,[24,32,33] mobile phase,[17] ethanol,[34] methanol,[29] or acetonitrile.[22,25,26,30,31] However, we were not able to reproduce a satisfactory chromatogram with any of these reported methods. Further, only limited information on the stability of ceftriaxone has been reported in the literature.[22,25-34]

Therefore, the aims of this study were to: 1) optimize and fully validate a simple, sensitive HPLC assay to measure ceftriaxone level in human plasma with quantitation limits suitable for bioequivalence studies, and 2) determine the stability of ceftriaxone under various clinical laboratory conditions.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of Waters Alliance e2695D Separations Module, an 4.6 x 150 mm, XTerra® RP18, 5 μm (particle-size) steel column, a Guard Pak pre-column module with Nova-Pak C18, 4-μm insert, and Waters 2998 photodiode array detector (Waters Associates Inc., Milford, MA, USA) set at 272 nm. Data were collected with a Pentium D computer using Empower Chromatography Manager Software (Waters Associates Inc., Milford, MA, USA).

Chemicals and reagents

Ceftriaxone sodium standard (Figure 1-a) and the internal standard (IS), cefazolin sodium (CAS **n**umber**;** 25953-19-9) (Figure 1-b), were purchased from Sigma-Aldrich, St. Louis, MO, USA. Cetyltriethylammonium bromide and dipotassium hydrogen orthophosphate were purchased from BDH Chemicals Ltd, Poole, England. Phosphoric acid, triethylamine, and acetonitrile (all 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).

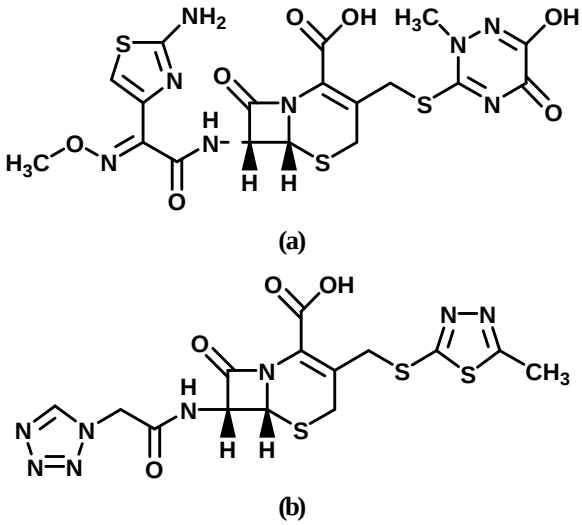

Figure 1 : Chemical structures of ceftriaxone (a) and cefazolin (b).

Chromatographic conditions

The mobile phase consisted of 0.02 M of cetyltrimethylammonium bromide in 0.01 M dipotassium hydrogen orthophosphate buffer (pH = 6.5 adjusted with phosphoric acid), acetonitrile, and triethylamine (70:30: 0.001, v:v:v). It was filtered through a 0.45 μm size membrane filter (Millipore Co., Bedford, MA, USA), degassed, and delivered according to the following flow rate program: 1.5 ml/min for 5 min, 2 ml/min for 7 min, and 1.5 ml/min for 2 min (total run time of 14 min).

Preparation of stock and working solutions

Ceftriaxone stock solution (1 mg/ml) was prepared in water and used for stability studies and to prepare a working solution (200 μg/ml) in plasma. The working solution was prepared weekly to construct calibration curve and quality control (QC) samples. The internal standard (cefazolin) working solution (250 μg/ml) was prepared weekly in water from a stock solution (1 mg/ml) in water.

Calibration standard / quality control samples

Calibration standards were prepared by mixing appropriate volumes of ceftriaxone working solutions with blank human plasma to produce final concentrations of blank, zero (blank plasma spiked with IS only), 0.2, 0.4, 1.0, 5, 10, 20, 40, 80, 160 and 200 μg/ml. QC samples were prepared by mixing appropriate volumes of ceftriaxone working solution with blank human

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plasma to produce final concentrations of 0.2, 0.6, 100, and 180 µg/ml. Samples were vortexed for 20 s, and 0.25 ml aliquots from each flask were transferred into 1.5 ml eppendorf microcentrifuge tubes (Fisher Scientific Co., Fairlawn, NJ, USA) and stored at –20ºC.

Sample preparation

Aliquots of 0.25 ml of calibration standard or QC samples in 1.5 ml eppendorf microcentrifuge tubes were allowed to equilibrate to room temperature. To each tube, 50 µl of 250 µg/ml internal standard (IS) working solution was added and vortexed for 10 s. After the addition of 0.35 ml of methanol at 4ºC, the mixture was vortexed again for 2 min and then centrifuged for 15 min at 16000 rpm at room temperature. The supernatant was transferred into 1.5 ml eppendorf microcentrifuge tubes, and centrifuged again for more 5 min at 16000 rpm at room temperature. The clear supernatant was transferred into the auto-sampler vials and 100 µl were injected into the HPLC system. The run time was 14 min.

Stability studies

Stability of ceftriaxone in plasma: adequate numbers of aliquots of three QC samples (0.2, 0.6, and 180 µg/ml) were prepared. Aliquots were analyzed in 5 replicates immediately (baseline), after being processed and stored at room temperature for 24 h or at –200C for 48 h (auto-sampler stability), after being allowed to stand on the bench-top for 8 or 24 h at room temperature before processing (counter stability), after being stored at –200C for 8 weeks before processing (long term freezer stability), or after being stored at –200C for 24 h and then left to completely thaw unassisted at room temperature before processing (with the cycle repeated three times, freeze-thaw stability).

Stock solutions stability: five aliquots of the stock solutions of ceftriaxone and the IS were analyzed (after dilution to 100 µg/ml in water) at baseline, after storage for 48 h at room temperature, or after storage at –200C for 8 weeks. Stability of the working solutions of ceftriaxone and the IS, were evaluated up to 2 weeks at –200C.

Assay validation method

The method was validated according to FDA guidelines[36] in terms of intra-run and inter-run precision and accuracy, linearity, specificity, recovery, and stability.

RESULTS

Optimization of chromatographic conditions

Different combinations of the components of the mobile phase, and pH were investigated to optimize the separation of ceftriaxone and the IS. Since ceftriaxone is a highly polar compound, we added cetyltrimethylammonium bromide, an ion-pairing agent, to the mobile phase. Further, in order to shorten the run time, it was necessary to program the flow rate as follows: 1.5 ml/min for 5 min, 2 ml/min for 7 min, and 1.5 ml/min for 2 min. This change in flow rate did not result in any appreciable drift of the chromatographic base line or either in peak area or shape.

To improve specificity and detection limit, and minimize interference from plasma and solvent system that may occur at lower wavelengths, we scanned for the best absorbance wavelength using photodiode array extracted spectra (Figure 2), and chose 272 nm. wavelength. We used a Guard Pak pre-column module with Nova-Pak C18, 4-µm insert to reduce damage to the analytical column. The chromatographic resolution and peak responses were stable over about 450 injections of processed plasma samples using one column.

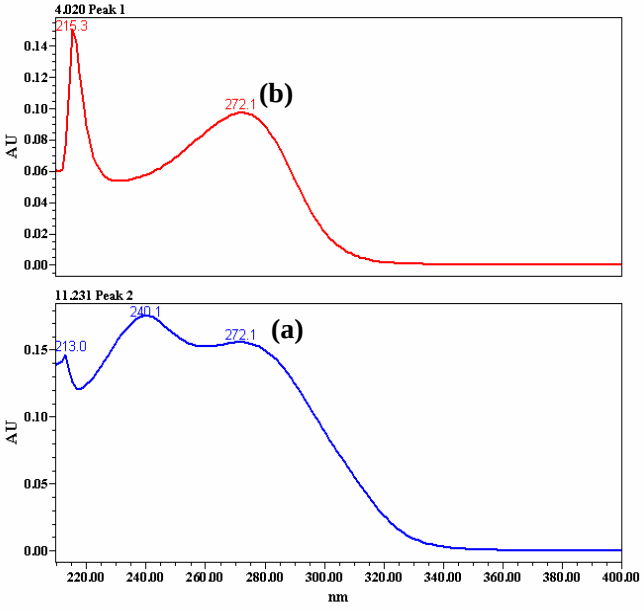

Figure 2 : PDA extracted ultraviolet spectra of ceftriaxone (a) and cefazolin (b).

Under the described conditions, the IS and ceftriaxone were resolved within a total run time of 14 min, with a retention time of 4.1 and 10.5 min, respectively.

Linearity

Linearity was determined in the range of 0.2 – 200 µg/ml using ten calibration curves. The data were analyzed by using linear regression equation: *Conc. = a + b (PAR)*, where *Conc*. is the concentration of ceftriaxone, *a* is the intercept, *b* is the slope, and *PAR* is the peak area ratio of ceftriaxone divided by the peak area of the IS. The concentrations of the calibration standards of the ten calibration curves were back-calculated using the individual regression lines. Linearity studies (n=10) showed mean (SD) for R2 of 0.9984 (0.0009), slope of 0.0219 (0.0056), and intercept of 0.0065 (0.0286). Figure 3 shows an overlay of chromatograms of a representative standard curve.

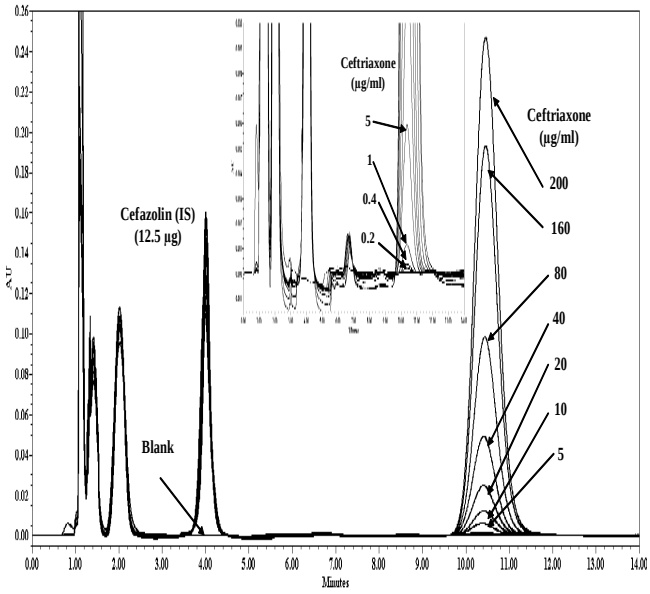

Figure 3 : Overlay of ceftriaxone calibration curve chromatograms spiked with ceftriaxone. The insert is a blow up of the lower concentrations.

Limit of detection

The limit of detection (LOD), defined as three times the baseline noise, was 0.1 µg/ml.

Specificity

To evaluate specificity, we screened eight frequently used medications and six different batches of human plasma. All batches of blank plasma were free from interfering components. None of eight commonly used drugs co-eluted with ceftriaxone or the IS (TABLE 1).

TABLE 1 : Specificity of ceftriaxone assay

Drug Name	Retention Time (minutes)
Ceftriaxone	10.5
Cefazolin	4.1
Ibuprofen	ND
Acetaminophen	1.4
Ranitidine	0.9
Nicotinic Acid*	2.6
Ascorbic Acid*	2.1
Caffeine*	1.2
Diclofenac	ND
Omeprazole	3.1

Drug solutions (1 mg/ml) in methanol or water * were diluted to (10 µg/ml) in mobile phase and 100 µl were injected.

Recovery

The extraction recovery of ceftriaxone was determined by dividing mean peak areas of five replicates of four QC samples (0.2, 0.6, 100, and 180 µg/ml) prepared in plasma (as described under sample preparation), by mean peak areas of five replicates of equivalent concentrations prepared in the water. The recovery of the IS was determined similarly at a concentration of 50 µg/ml. The results of the extraction recovery studies of ceftriaxone and the IS are presented in TABLE 2. Recovery was = 94% (mean 96%) for ceftriaxone and 97% for the IS.

TABLE 2 : Extraction recovery of ceftriaxone and cefazolin

Nominal Concentration (µg/ml)	Plasma		Water		** Recovery (%)
	*Mean Peak Area	SD	*Mean Peak Area	SD	
Ceftriaxone					
0.2	7834	367	8146	571	96
0.6	26129	924	27340	1018	96
100	5166134	5775	5355566	3260	96
180	9119290	69846	9729504	46981	94
Cefazolin (IS)					
12.5	1676554	17202	1720320	43001	97

*** Mean peak area of 5 replicates. ** Mean peak area of spiked plasma sample divided by mean peak area of spiked water sample x 100. SD, Standard Deviation**

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Precision and bias

Precision was calculated as coefficient of variation (standard deviation divided by mean measured concentration x 100), and bias as the absolute value of (1 minus mean measured concentration divided by nominal concentration) x 100. The intra-run and inter-run precision and bias of ceftriaxone were determined by analyzing four QC samples: 0.2, 0.6, 100, and 180 μg/ml over three days (TABLE 3). Intra-run precision and bias (n = 10) ranged from 1.9% to 4.8% and from <1% to 10%, respectively. The inter-run precision and bias (n = 20) ranged from 4.6% to 5.7% and from 1 % to 8 %, respectively.

Stability

The stability of ceftriaxone in plasma and extracted samples under usual laboratory storage conditions was investigated. The results are presented in TABLE 4. The data indicate that ceftriaxone is stable: 1) in plasma for at least 24 h at room temperature or 8 weeks at –200C, 2) in processed samples for at least 24 h at room temperature or 48 h at –200C, 3) in plasma after at least three cycles of freeze at –200C and thaw at room temperature, and 4) in water (1 mg/ml) is stable for at least 48 h at room temperature or 8 weeks at –200C. The IS stock solution (1 mg/ml in water) was also stable under the same conditions (100% and 99%, respectively). Further, the working solutions of ceftriaxone (200 μg/ml in plasma) and the IS (250 μg/ml in water) were stable for at least 2 weeks at –200C (104% and 99%, respectively).

TABLE 3 : Intra-run and inter-run accuracy and precision of ceftriaxone assay

Nominal Concentration (μg/ml)	Intra-run (n=10)				Inter-run (n=20)			
	Mean Measured Concentration (μg/ml)	SD	Precision (CV*, %)	** Bias (%)	Mean Measured Concentration (μg/ml)	SD	Precision (CV*, %)	** Bias (%)
0.2	0.20	0.010	4.8	<1	0.20	0.011	5.7	2
0.6	0.60	0.021	3.9	<1	0.61	0.032	5.3	1
100	110.13	3.302	2.6	10	108.06	4.941	4.6	8
180	193.77	3.223	1.9	8	185.58	8.971	4.8	3

*** Coefficient of variation (CV) = Standard Deviation (SD) divided by mean measured concentration x 100. **Bias = absolute value of 1 minus mean measured concentration, divided by nominal concentration x 100.**

TABLE 4 : Stability of ceftriaxone in plasma samples and stock solution

Nominal Concentration (μg/ml)	*Plasma Samples								**Stock solution	
	Unprocessed			Processed		Freeze-thaw				
	8 h RT	24 h RT	8 wks –200C	24 h RT	48 h –200C	One cycle	Two cycles	Three cycles	48 h RT	8 wks –200C
0.2	100	100	98	96	96	97	100	99	100	96
0.6	90	95	97	100	100	95	95	94		
180	99	99	97	100	91	95	92	91		

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 8 or 24 hours at room temperature (8 h RT and 24 h RT), after 8 weeks at – 200C (8 wks –200C), or processed and analyzed after 24 hours at room temperature (24 h RT) or 48 hours at –200C (48 h -200C); or after 1 to 3 cycles of freezing at –200C and thawing at room temperature (freeze-thaw). ** Ceftriaxone, 1 mg/ml in water.

DISCUSSION

We optimized previously published HPLC assays[24,29] for the determination of therapeutic levels of ceftriaxone in human plasma and applied the optimized assay to study the stability of ceftriaxone under various laboratory conditions.

Some of the previously reported ceftriaxone HPLC assays required long sample processing time because of liquid-liquid extraction with consecutive evaporation,[20,27,28] solid-phase extraction,[20,23] a very high pH

for the mobile phase which may accelerate the analytical column damage,[30] or a long chromatographic run time.[23] Further, some of these methods lacked adequate sensitivity, with lower limit of detections of 0.5 μg/ml,[24,25,28,32,34] 1 μg/ml,[22,30,35] 5 μg/ml,[26,29] and 10 μg/ml,[27] used equipment that may not be available in many pharmaceutical laboratories or need highly trained persons,[11,35] suffered from a low recovery (87%),[18] or were applied to non human plasma (rabbit,[17] goat,[18,19] camel,[20] or rat[21] plasma).

The majority of the previously reported ceftriaxone HPLC-UV assays used protein precipitation with (acids,[18] water,[24,32,33] mobile phase,[17] ethanol,[34] methanol,[29] or acetonitrile.[22,25,26,30,31]). When we attempted to adopt these methods in our laboratory we were not able to obtain a satisfactory chromatogram. We reasoned this may be due to interference of the highly polar compound ceftriaxone with the precipitating agents. This problem was eliminated by using methanol at 4 ºC (1:1.4, v:v) as a protein precipitant agent together with two steps of centrifugation.

Limited data on the stability of or ceftriaxone during the analytical process,[22,25-34] a requirement for analyzing a large number of samples in bioequivalence studies, have been reported. We obtained complete data on stability of ceftriaxone under various laboratory conditions, including freeze thaw cycle, 8 weeks in plasma or water, and after processing; as well as the stability of ceftriaxone and the IS in stock and working solutions. The new optimized current assay has the following overall advantages: simple sample preparation with one-step protein precipitation, using simple HPLC equipment, high sensitivity (0.1 μg/ml), and high extraction recovery (96%). The data indicate that the current assay is suitable for therapeutic drug monitoring and bioequivalence studies in humans.

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