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Short communication

Alternative method for determination of ceftazidime in plasma by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatography procedure was developed to analyze ceftazidime concentrations in plasma. The procedure consisted of solid phase extraction followed by ion-pairing reverse-phase chromatography. An excellent linear relationship between ceftazidime peak height measurements and concentrations was demonstrated over the concentration range of 1–200 $\mu g\ ml^{-1}$. The advantage of this assay is the elimination of interference at the ceftazidime elution time that has been noted in previous studies and in our experience. Thus, this study describes an alternative, simple methodology that is clinically useful for analyzing ceftazidime in the research setting.

Keywords: Ceftazidime

1. Introduction

Ceftazidime is a third-generation cephalosporin with enhanced bactericidal activity against many Gram-negative organisms, particularly *Pseudomonas aeruginosa* [1]. Therefore, ceftazidime is a widely used and important therapeutic agent for the treatment of many Gram-negative nosocomial infections [2]. The association between antimicrobial activity, clinical outcome and antibiotic concentrations fuels the need to assess the concentration–time profile for a given antibiotic [3].

Several assays have been developed to measure ceftazidime concentrations in serum and plasma using high-performance liquid chromatography (HPLC) [4–8]. However, these assays utilize protein precipitation to prepare samples which is relatively nonselective in segregating compounds [9]. C_{18} columns have been used frequently for the analysis of various cephalosporins in serum or plasma [8,10–12]. These assays often compromise chromatographic conditions so that several cephalosporins can be determined using the same chromatographic system. However, the cephalosporins are quite diverse as to their side chain characteristics. For ceftazidime, at least one of three ionizable groups (pK_a 1.9, 2.7, and 4.1) [13] remains ionized over the recommended pH range for silica based columns. Therefore, ceftazidime is not retained well on a C_{18} column in the partition chromatography mode. This assay was developed secondary to our findings of an interfering substance present at the ceftazidime elution time when employing previously published methodologies [4]. Similar findings have been reported by other investigators [7,14]. This report describes a more specific assay combining solid phase extraction and

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ion-pairing chromatography to analyze plasma ceftazidime concentrations. The utility of the assay for pharmacokinetic studies is shown in the analysis of the ceftazidime concentration–time curve in a trauma intensive care unit patient.

2. Experimental

2.1. Chemicals and reagents

Ceftazidime pentahydrate was supplied by Glaxo Wellcome (Research Triangle Park, NC, USA). Acetonitrile (HPLC grade), methanol (HPLC grade) and sodium phosphate dibasic (Na_2HPO_4) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Potassium phosphate monobasic (KH_2PO_4) was purchased from Mallinckrodt (Paris, KY, USA). Tetrabutyl ammonium hydrogen sulfate (TBAHS) was purchased from Sigma (St. Louis, MO, USA). Ammonium dihydrogen phosphate [$(NH_4)H_2PO_4$] was purchased from Aldrich (Milwaukee, WI, USA). Water used in the preparation of mobile phase, stock solutions, and extraction reagents was produced from a Milli-Q water purification system (Millipore, Bedford, MA, USA). The 47 mm diameter, 0.45 μm cellulose ester membranes (type HA) used to filter the mobile phase were also from Millipore. Stock solutions of ceftazidime were made by dissolving 11.65 mg ceftazidime pentahydrate (equivalent to 10 mg ceftazidime acid) in 10 ml of Millipore water. The standard and control ceftazidime samples were made by spiking blank plasma with freshly prepared ceftazidime stock solution. The plasma samples were stored at −70°C until analysis.

2.2. Apparatus

Analyses were performed on a Shimadzu Scientific Instruments (Columbia, MD, USA) liquid chromatograph system composed of a LC-10 AD reciprocating piston pump and a SPD-10A UV absorbance detector. Samples were injected on column using a Rheodyne (Cotati, CA, USA) model 7125 valve fitted with a 200 μl loop. The analytical column (3.9×300 mm) was packed with 10-μm C_{18} bonded phase silica (μBondapak, Waters, Milford, MA, USA). The analytical column was preceded by a cartridge guard column packed with 5 μm Adsorbosil C_{18} silica (Alltech Associates, Deerfield, IL, USA). Data were processed on a Shimadzu model CR501 integrator.

2.3. Sample preparation

Plasma samples were prepared by solid phase extraction using one ml volume Bond Elut solid phase extraction columns packed with 100 mg of C18 bonded phase silica (Varian Associates, Harbor City, CA, USA) and a Baker-10 Extraction System vacuum manifold (J.T. Baker, Phillipsburg, NJ, USA). The extraction procedure consisted of first wetting the extraction columns with 1 ml of 100 % methanol twice, followed by two 1-ml Millipore water rinses. The plasma sample (500 μl) was then passed through the column followed by two 1-ml rinses of 0.25 *M* $(NH_4)H_2PO_4$ (pH 2.6). Finally, 500 μl of a 50:50 (v/v) mixture of methanol and Millipore water was used to elute the ceftazidime from the column. This sample was collected in a 10×75 mm glass tube and evaporated under nitrogen while immersed in a 30°C water bath. Each sample was reconstituted with 400 μl of mobile phase and vortexed for 15 s. The samples were then centrifuged at 13 400×*g* for 3 min in an Abbott Laboratories (Abbott Park, IL, USA) Model 3531 centrifuge. An 80 μl volume of the sample supernatant was manually injected onto the column using a 100 μl syringe. Prior to each injection, the valve passages were flushed with 200 μl of mobile phase to rinse out any residual sample from the previous injection. Extracted samples were stored at 4°C prior to injection.

2.4. Chromatographic conditions

The mobile phase was composed of 9% acetonitrile and 91% phosphate buffer consisting of 67 m*M* KH_2PO_4 and 67 m*M* Na_2HPO_4. Twenty m*M* TBAHS was then added to the mobile phase and the pH adjusted to 5.5. Prior to use, the mobile phase was filtered through a 0.45 μm Millipore filter (Type HA) in an all-glass apparatus and degassed by sonification. The mobile phase was delivered to the column at a rate of 1 ml min^{-1}. The UV detector wavelength was set at 255 nm.

2.5. Reproducibility

The within-day and between-day precision was assessed by calculating the coefficient(s) of variation (CV). The CV were calculated as the standard deviation of the control concentration divided by the mean control concentration multiplied by 100. The within-day variation was calculated using four sample injections of each control ceftazidime concentration (2, 60, and 150 $\mu g\,ml^{-1}$) that were analyzed on the same day. The between-day variation was calculated using control ceftazidime concentrations (2, 60, and 150 $\mu g\,ml^{-1}$) each analyzed daily for four consecutive days. Accuracy of the assay was assessed using the relative error of the mean.

2.6. Recovery studies

Blank pooled human plasma samples were spiked with ceftazidime at concentrations of 1, 50, and 200 $\mu g\,ml^{-1}$. The recovery of ceftazidime from plasma with the solid phase extraction process was determined by comparing peak heights from the extracted samples with peak heights from the aqueous standards. Three samples of each concentration were assayed.

2.7. Method of calculation

Chromatogram peaks for ceftazidime were identified by their retention times. Ceftazidime samples were prepared at concentrations of 1, 10, 50, 100, and 200 $\mu g\,ml^{-1}$ (standards), and 2, 60, and 150 $\mu g\,ml^{-1}$ (controls) using pooled human plasma and stored at $-70°C$. Standard curves were determined by weighted linear regression (PCNONLIN, Statistical Consultants, Lexington, KY, USA) with each standard peak height weighted to the inverse of the square of its absorbance. The coefficient of determination was used as a measure of goodness-of-fit. This was calculated as the regression sum of squares divided by the total sum of squares.

2.8. Application of assay

The concentration–time profile of ceftazidime was determined in a 34 year-old male trauma patient. The patient received 2 g ceftazidime intravenously over 30 min every 8 h as part of a nosocomial pneumonia study. Blood samples were obtained at 0.5, 2, 4, and 7.0 h after the end of the infusion on the fourth day of therapy. The ceftazidime concentrations were fit to a one-compartment model using ADAPT II (version 3) software. Concurrent medications were vancomycin, heparin, and ranitidine.

3. Results

3.1. Chromatography

Fig. 1 shows a representative chromatogram for blank plasma (a) and blank plasma spiked with ceftazidime (b). The elution time for ceftazidime was 13.1 min and there were no interfering plasma peaks. The nearest plasma peak eluted at 10.4 min and there were no further peaks after ceftazidime elution.

3.2. Reproducibility

A plot of the ceftazidime peak heights against the concentration of drug injected demonstrated a linear response over the concentrations 1 to 200 $\mu g\,ml^{-1}$ (coefficient of determination=0.995). The mean±SD for the slope, intercept, and coefficient of determination for the regression were 1639.7±41.4 (CV 2.5%), 395.6±186.2, 0.995±0.006 (CV 0.6%). The limit of detection for the assay was approximately 1 ng injected onto the column (equivalent to approximately 0.01 $\mu g\,ml^{-1}$) as determined by the lowest detectable signal that is four times greater than the baseline noise. Within-day and between-day CV and relative errors of the mean are shown in Table 1.

3.3. Recovery

The assay demonstrated excellent recovery of ceftazidime. For ceftazidime concentrations of 200, 50, and 1 $\mu g\,ml^{-1}$, the percent recovery was 100 %, 90%, and 96%, respectively. The recovery of ceftazidime implies that ceftazidime degradation during the procedure was minimal. Ceftazidime is noted for its limited stability even under refrigerated conditions [7].

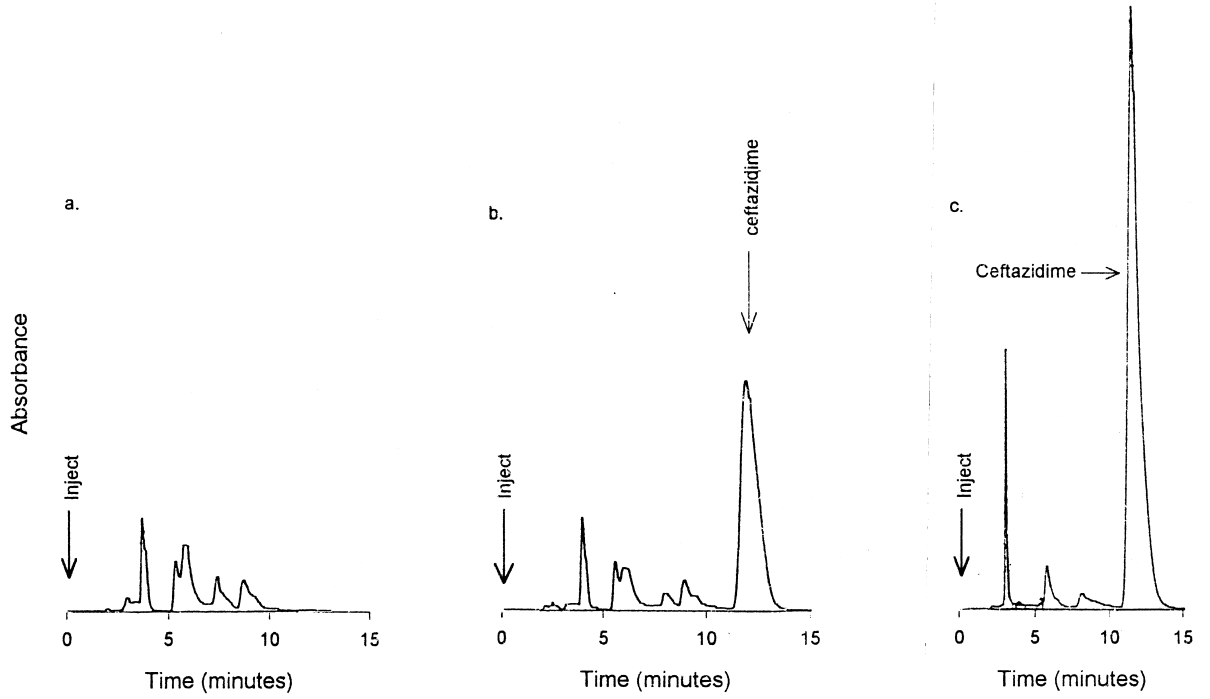

Fig. 1. (a) Representative HPLC chromatogram for blank plasma; (b) chromatogram of plasma spiked with 10 μg ml^{-1} of ceftazidime; (c) chromatogram of plasma sample 2 h post-infusion from a subject receiving 2 g ceftazidime IV over 30 min every 8 h. The measured concentration of ceftazidime is 37.4 μg ml^{-1}.

3.4. Assay application

The chromatogram shown in Fig. 1c shows that there were no endogenous substances that interfered with the ceftazidime peak in an actual patient sample. The clinical usefulness of the assay is represented in Fig. 2.

4. Discussion

The present assay is an alternative method for analyzing ceftazidime concentrations in human plasma. Previously published ceftazidime assays have utilized protein precipitation to prepare samples for injection [4–8]. Protein precipitation is relatively non-specific and can leave behind compounds that may potentially interfere with accurate quantitation. The development of this assay was prompted by our findings of interfering peaks at the ceftazidime elution time when employing the methods described by Leeder et al. [4]. The potential for interfering endogenous substances has been previously reported [7,14]. Although Leeder et al. was able to measure a concentration of 2.92 μg ml^{-1} accurately with a coefficient of variation of 5.2%, we were only able to

Table 1
Assay reproducibility and accuracy

	2 μg ml^{-1}	60 μg ml^{-1}	150 μg ml^{-1}
Within-day CV[a] (%)	0.56	1.63	1.93
Between-day CV (%)	6.20	3.02	2.93
Within-day REM[b] (%)	5.50	−2.50	1.90
Between-day REM (%)	4.0	−2.20	−0.40

[a] CV=coefficient of variation.
[b] REM=relative error of the mean.

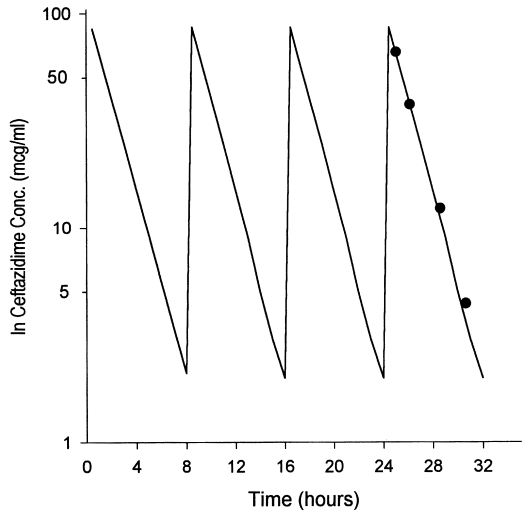

Fig. 2. Simulated ceftazidime concentration-time curve in subject receiving 2 g ceftazidime IV over 30 min every 8 h. (—) indicates simulated concentration–time curve and (●) indicates observed ceftazidime concentrations.

achieve a lower limit of quantitation of 5 $\mu g\,ml^{-1}$ due to the interfering substance. In addition, the authors comment that very low ceftazidime concentrations can be assayed simply by increasing the volume of the injected supernatant. However, we found this did not solve our problem. Increasing the injection volume increased the size of both the ceftazidime and endogenous peak so that there was no benefit to sensitivity. Attempts to adequately separate the endogenous peak from the ceftazidime peak by adjusting mobile phase pH, percent organic modifier, type of organic modifier, and flow rate were unsuccessful. This was due to the apparent low affinity of ceftazidime for the C_{18} column.

Two strategies were employed to improve the analysis of ceftazidime by HPLC. First, a more specific extraction procedure was necessary. Although alternate protein precipitation methods were explored such as acetonitrile precipitation with back-extraction into methylene chloride and liquid–liquid extraction [11], these methods were limited by poor efficiency. This is likely due to the inability to completely unionize ceftazidime. Consequently, a solid-phase extraction procedure was developed. Solid-phase extraction technique has been applied to the analysis of other cephalosporins [12,15,16]. Various other cephalosporins were tested for use as an internal standard. However, none of the tested cephalosporins proved adequate for the assay. Thus, external standardization was employed and resultant coefficients of variation were within acceptable limits (<10%).

The second strategy was to enhance to the resolution of ceftazidime. Ion-pairing chromatography was selected because the greater polarity of the stationary phase allowed more interaction between ceftazidime and the HPLC column. Other cephalosporins have been analyzed using ion-pairing chromatography technique, however ceftazidime was not specifically tested [8,10].

In conclusion, this assay combines solid phase extraction and ion-pairing chromatography to produce a simple alternative assay for the determination of plasma ceftazidime concentrations that can be used in the research setting. The advantage of this assay is that it is more specific and allows more accurate quantitation of low ceftazidime plasma concentrations.

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