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Determination of ceftazidime in dolphin serum by liquid chromatography with ultraviolet–visible detection and confirmation by thermospray liquid chromatography–mass spectrometry

Krystyna L. Tyczkowska* and Shemida S. Seay

Clinical Pharmacology Unit, Department of Anatomy, Physiological Sciences and Radiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606 (USA)

Michael K. Stoskopf

Department of Companion Animals and Special Species Medicine, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606 (USA)

David P. Aucoin

Clinical Pharmacology Unit, Department of Anatomy, Physiological Sciences and Radiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606 (USA)

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ABSTRACT

A simple and sensitive liquid chromatographic method has been developed for the determination of therapeutic levels of ceftazidime in dolphin serum. The method involved an ultrafiltration of diluted serum with an equal amount of acetonitrile–ethanol–water (40:40:20, v/v/v) through a 10 000 daltons molecular mass cut-off filter. Separation of ceftazidime from the other serum components was performed by ion-paired (dodecanesulfonate) liquid chromatography using a reversed-phase column eluted with acetonitrile–water solution. The ultraviolet absorbance of the column effluent was monitored in the 200–340 nm range of a photodiode-array detector or at 258.8 nm on a variable-wavelength ultraviolet–visible detector. Recoveries of ceftazidime from dolphin serum spiked with 20 and 2 μg/ml were 92.9 and 91.1% with coefficients of variation of 5.5 and 5.7%, respectively. A correlation coefficient of 0.9994 occurred with ceftazidime in aqueous solutions ($n = 6$, in duplicates). The limit of detection for this antibiotic was estimated to be approximately 50 ppb (ng/ml). The unbound ceftazidime concentrations in dosed dolphin serum were determined to calculate the protein bindings of this antibiotic which yielded 32 ± 2%. The ceftazidime peak identity in dosed dolphin serum was confirmed by thermospray liquid chromatography–mass spectrometry. The thermospray mass spectrum of ceftazidime exhibited only the fragment ions, involving the opening of the β-lactam ring, at m/z 237, 255 and 315 when positive-ion detection mode was employed and the fragment ions at m/z 235, 253 and 313 when negative-ion detection mode was used.

INTRODUCTION

Ceftazidime belongs to the cephalosporin β-lactam antibiotics, with its structure (Fig. 1) incorporating the 7-aminocephalosporanic acid nucleus. Cephalosporin antibiotics are being used increasingly in veterinary medicine for treating bacterial infections [1]. Ceftazidime has good activity against *Pseudomonas* sp., a genus of disease-causing bacteria which contribute to a large

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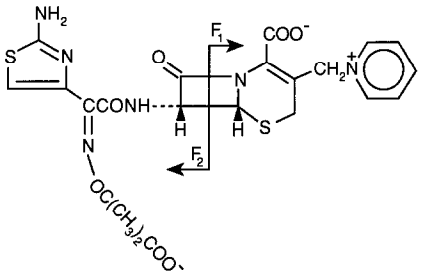

Fig. 1. Structure of ceftazidime and the fragmentation pattern for the mass spectrometric detection.

percentage of deaths of captive and stranded cetaceans (dolphins and whales) [2]. Infection by *Pseudomonas pseudomallei*, endemic in southeast Asia, is particularly serious and causes seasonal fatal septicemias in captive marine mammals characterized by respiratory distress and acute death with multifocal pulmonary and hepatic abscesses [3]. Although, ceftazidime has an excellent penetration into the cerebral spinal fluid in humans and an excellent record of clinical success [4], unfortunately this antibiotic is potentially nephrotoxic, causing renal tubular necrosis in humans receiving high doses [5].

Interest in the use of ceftazidime against bacterial infections in captive killer whales (*Orca orca*) and bottlenose dolphins (*Tursiops* spp.) and the need for precise kinetic data on this drug in these species required the development of an accurate assay for serum levels. Also, the dosing schedule needed to maintain therapeutic concentration of ceftazidime in dolphin and whale sera is not known. Analysis of this antibiotic in marine mammals serum has not been reported. Liquid chromatographic (LC) methods concerning the determination of ceftazidime in human serum, urine and bile are reported [6–11]. Among them only one [11] was simple and employed little labor to prepare the serum sample for determination of ceftazidime.

This paper describes a simple method for determining therapeutic levels of ceftazidime in dolphin serum using ultraviolet–visible (UV–VIS) detectors. A photodiode-array (PDA) detector was used to develop the assay since it produces multi-wavelength chromatograms allowing the evaluation of chromatographic peak homogeneity. A photometric detector was used for quantitative analysis and improved sensitivity. Thermospray liquid chromatography–mass spectrometry (LC–MS) was used to confirm the ceftazidime peak identity in dosed dolphin serum samples.

EXPERIMENTAL

Materials and reagents

The LC solutions were made from highest-purity solvent grade acetonitrile (American Burdick & Jackson, Muskegon, MI, USA). LC-grade water was obtained from Hydro Services and Supplies (Research Triangle Park, NC, USA). Phosphoric acid, acetic acid and ethanol were LC grade (Fisher Scientific, Raleigh, NC, USA). The ion-pairing reagent, dodecanesulfonate (S12) was obtained from Regis (Morton Grove, IL, USA). The microseparation system, Centricon-10, employing a molecular mass cut-off filter of 10 000 daltons, was supplied by Amicon Division of W. R. Grace (Danvers, MA, USA).

Ceftazidime pentahydrate standard was kindly supplied by Glaxo (Research Triangle Park, NC, USA). A 1 mg/ml ceftazidime (calculated as a base) stock solution was prepared in acetonitrile–ethanol–water (20:20:60, v/v/v). The working solution of 10 μg/ml was prepared daily with the same dilutent. All standards were protected from the light with aluminum foil and amber vials.

Dolphin serum samples

Dosed serum samples from dolphins were provided by Ocean Park (Aberdeen, Hong Kong). The dosed serum samples were collected at various periods (10 min to 10 h) after intramuscular injection of ceftazidime pentahydrate. The samples were frozen at −65°C within 30 min from collection. The control serum samples were taken prior to antibiotic injection. These were used for blank analysis or were spiked with ceftazidime for the assay validation.

Sample preparation procedure

A 500-μl aliquot of serum was diluted with an equal volume of acetonitrile–ethanol–water

(40:40:20) in the microseparation system equipped with a 10 000 daltons molecular mass cut-off filter. The sample was vortex-mixed for 10–15 s and centrifuged for approximately 30 min at 4000 *g* with 45° fixed-angle rotor. A 10–60 μl aliquot of colorless ultrafiltrate was injected into an LC system equipped with either a UV–VIS photometric or a PDA detector.

Liquid chromatography with UV–VIS detection

The LC equipment consisted of a Waters Model 600W multi-solvent delivery system with a Waters U6K injector and temperature control accessory set at 40°C. This was coupled to a Model 990 (plus) UV–VIS PDA detector (Waters Chromatography Division, Milford, MA, USA).

For the routine analysis of ceftazidime in dolphin serum, a variable-wavelength UV–VIS detector was used together with a Waters 820 work station, Model 590 LC pump and Model 750 Wisp automatic injector with temperature control accessory at 40°C. The LC separations were performed using a mobile phase consisting of 25% acetonitrile in water (v/v) containing 0.005 *M* dodecanesulfonate and 0.1% phosphoric acid solution. The mobile phase flow-rate was 0.8 ml/min giving a 7–8 min retention time for ceftazidime on a Ultremex 3-μm phenyl column, 150 mm × 4.6 mm I.D. (Phenomenex, Torrance, CA, USA). The column effluent was analyzed in the wavelength range 200–340 nm using the PDA detector or monitored by the variable-wavelength UV–VIS detector at 258.8 nm. Peak-area measurements were computed by the Waters 820 work station.

After comparing the areas of ceftazidime in standard and dolphin serum samples, the quantity of ceftazidime determined by LC–UV–VIS detection was calculated as follows: μg/ml = [ceftazidime (ng) × 2]/[injection volume (μl)]. Usually the injection volume was between 10 and 60 μl. The multiplication by 2 in the equation accounts for the dilution of serum (1:1) with the solution for releasing protein-bound drug.

Protein-binding study

The protein binding of ceftazidime in dolphin serum was determined by the ultrafiltration technique with Centricon-10 microseparators equipped with 10 000 daltons molecular mass cut-off filters. Five dosed dolphin serum samples (13, 20, 30, 40 and 45 min post-dose) were incubated at 37°C for 1 h. After that, the ultrafiltration at 4°C was performed and the unbound concentrations of ceftazidime were determined by LC with UV–VIS detection. The protein binding of ceftazidime in dolphin serum was calculated as follows: percentage ceftazidime protein binding $= [(T - U)/T] \times 100\%$, where T = total ceftazidime concentration and U = unbound ceftazidime concentration.

Liquid chromatography–mass spectrometry

A thermospray mass spectrum of ceftazidime was acquired using a mobile phase of 20% acetonitrile and 1% acetic acid in 0.2 *M* ammonium acetate solution (loop injection). The thermospray interface (Finnigan MAT, San Jose, CA, USA) was operated with the temperature of the source and vaporizer at 300 and 130°C, respectively. A Finnigan MAT 4800 quadrupole mass spectrometer was operated in pulse positive-ion–negative-ion detection mode under full-scan conditions of 200–800 daltons for 1 s. Confirmation of ceftazidime was performed using 10, 15 and 20 μg of analyte standard and ceftazidime samples collected from dolphin serum (30 min post-dose samples from two dolphins were used) under LC–UV–VIS conditions described in the Experimental section. For this purpose a phenyl 3-μm column was used, eluted with acetonitrile in ammonium acetate solution at a 1 ml/min flow-rate. MS confirmation of ceftazidime in dolphin serum was performed in the negative-ion detection mode monitoring ions at *m/z* 235, 253 and 313, with each ion being monitored for 200 ms.

RESULTS AND DISCUSSION

Previous works from this laboratory have shown the usefulness of the ultrafiltration on 10 000 and 30 000 daltons molecular mass cut-off filters for extraction/deproteinization of serum when determining β-lactam antibiotics levels [12–

TABLE I

STATISTICAL SUMMARY OF CEFTAZIDIME RECOVERIES FROM SPIKED DOLPHIN SERUM SAMPLES DETERMINED BY LC–UV–VIS USING A PHOTODIODE–ARRAY DETECTOR

Amount spiked (μg/ml)	*n*	Amount recovered (%)		Coefficient of variation (%)	Mean recovery (%)
		Range	Mean ± S.D.		
20	5	88.2–99.4	92.9 ± 5.1	5.5	92.9
2	5	85.8–99.3	91.1 ± 5.2	5.7	91.1

16]. This requires the use of water-miscible organic solvents such as acetonitrile, methanol, ethanol or propanol-2 to dissociate drug–protein complexes. Ceftazidime exhibits high-polarity properties due to the presence of several ionizable groups in its molecule. Therefore, ion-paired chromatography was performed to separate this antibiotic from the other observable serum components. Based on previous studies with the other β-lactam antibiotics the phenyl column was se-

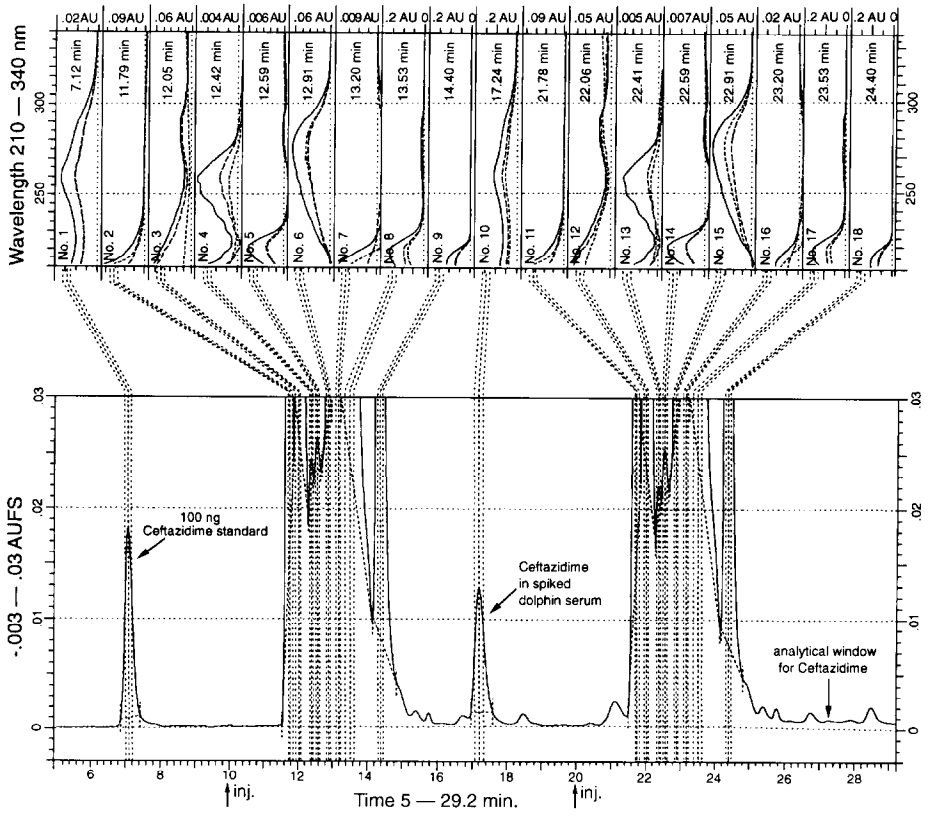

Fig. 2. LC–UV–VIS chromatogram for a ceftazidime standard (0–10 min run time), spiked dolphin serum with 5 μg/ml ceftazidime (10–20 min run time) and blank dolphin serum (20–30 min). The chromatogram was acquired on a PDA detector at the maximum wavelength for all major peaks (bottom) in the 210–340 nm range with the respective UV spectral curves for each major peak (top). Note that spectra No. 1 and No. 10 represent ceftazidime from the standard (100 ng) and a spiked dolphin serum (5 ppm) after a 40-μl injection.

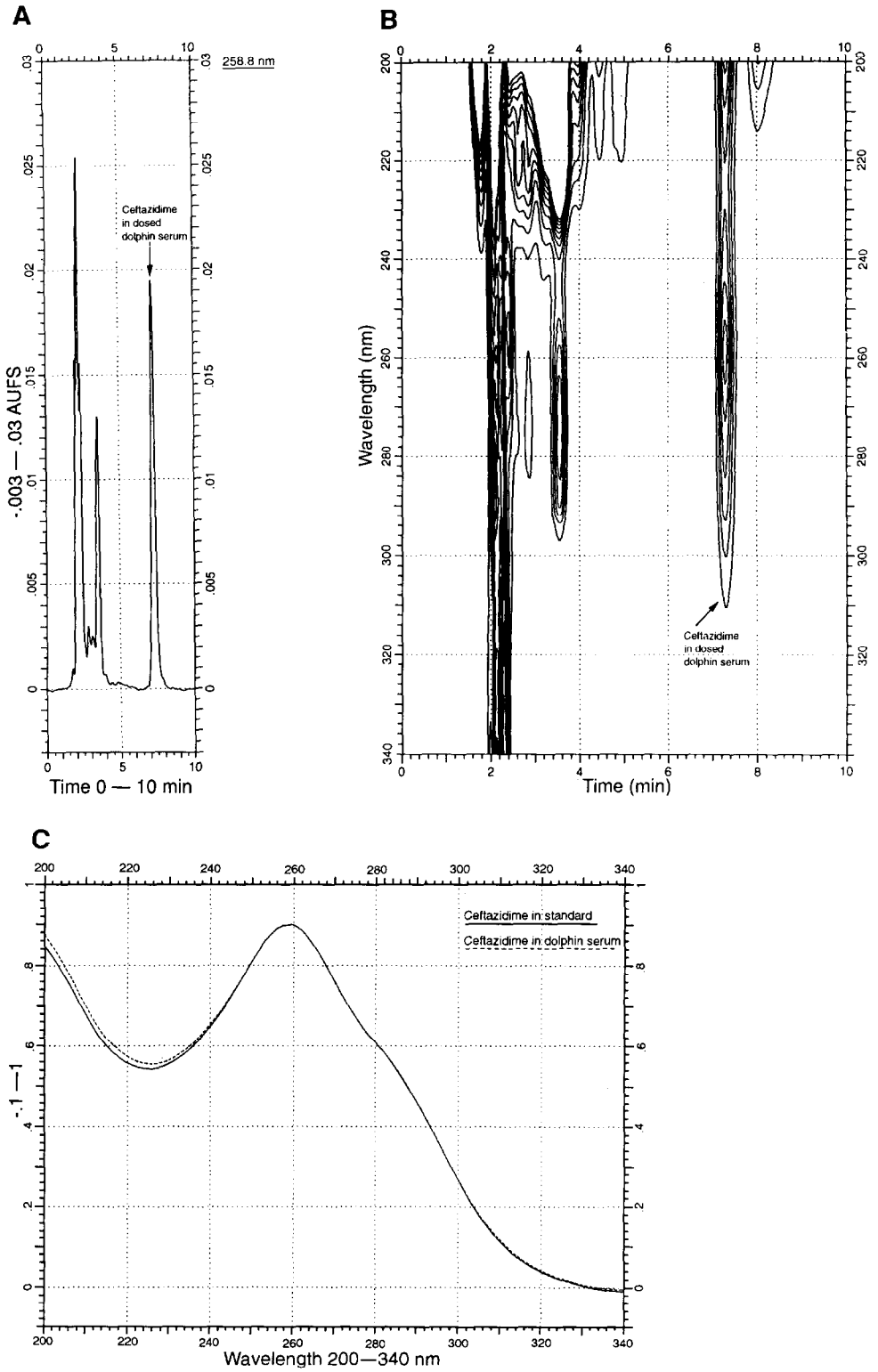

Fig. 3. (A) Typical LC–UV–VIS chromatogram for ceftazidime in dolphin serum acquired at 258.8 nm on the PDA detector. Injection volume was 15 μl of dosed dolphin ultrafiltrate sample collected 10 min after intramuscular injection. (B) UV–VIS absorbance contour plot of the dosed dolphin serum sample introduced in A. (C) UV–VIS spectral curves (200–340 nm) for ceftazidime standard (100 ng) and dolphin serum dosed with this analyte (15.3 μg/ml) introduced in A and B. Spectra were acquired in the auto gain mode of the PDA detector.

lected as a stationary phase [12–16]. An optimal separation of ceftazidime from endogenous constituents present in dolphin serum was obtained with a mobile phase consisting of 25% acetonitrile and 0.1% phosphoric acid in 0.005 *M* dodecanesulfonate solution. Ceftazidime, like many other β-lactam antibiotics, exhibits some binding to serum/plasma proteins. In human plasma, 21 ± 6% of this drug is bound to proteins [6], in dolphin serum we determined protein binding of drug to be 32 ± 2%. Several solutions were tested for their ability to free ceftazidime from the dolphin serum proteins. For our purpose, the ethanol–acetonitrile–water (40:40:20, v/v/v) solution was chosen. This allowed over 90% recovery of ceftazidime from dolphin serum (Table I). The percentage of drug bound to dolphin serum proteins was higher than expected from studies in humans [6]. This higher level of protein binding may result in the need for higher dosages in cetaceans compared to humans to achieve similar therapeutic effects. The mean protein binding of ceftazidime in dolphin serum was 32 ± 2% (n = 5).

A chromatogram of 100 ng ceftazidime standard (0–10 min run time), dolphin serum spiked with 5 μg/ml of this analyte (10–20 min run time) and blank dolphin serum (20–30 min run time) is shown in Fig. 2. The chromatogram depicts the maximum wavelength absorbance for all major peaks found in the 210–340 nm range (bottom) and their respective UV spectral curves (top). The UV spectral curves for ceftazidime in standard solution (No. 1) and dolphin serum ultrafiltrate (No. 10) have similar shapes. The fragment of blank dolphin serum chromatogram (20–30 min run time) showed a good analytical window for ceftazidime.

Fig. 3A shows a typical UV–VIS chromatogram for ceftazidime in dosed dolphin serum ultrafiltrate (collected 10 min after intramuscular injection) acquired at 258.8 nm (maximum wavelength for this β-lactam antibiotic) on a PDA detector. A UV–VIS absorbance contour plot for ceftazidime in dosed dolphin serum is shown in Fig. 3B. This revcaled an excellent analytical window for ceftazidime in the 200–340 nm range. In addition, to prove the homogeneity of the ceftazidime peak in dolphin serum, the spectral curves of this analyte standard (100 ng) and ceftazidime from dosed dolphin serum (115 ng) were acquired in the auto gain mode of the PDA detector and overlaid for comparison. As seen in Fig. 3C except the low UV range of 200–235 nm, there was an excellent conformity between these two curves.

Table I summarizes the statistical data obtained from spiking dolphin serum with 20 and 2 μg/ml ceftazidime. Recoveries of ceftazidime from dolphin serum spiked with 20 and 2 μg/ml were 92.9 and 91.1% with coefficients of variation (C.V.) of 5.5 and 5.7%, respectively. The study on linearity of UV–VIS detector response was performed by injecting a ceftazidime standard containing increasing amounts from 10 to 500 ng. Each level was analyzed twice. The relationship between peak area and concentration of aqueous ceftazidime standard solution was linear within this range with a correlation coefficient of 0.9994 (n = 6). The UV–VIS detection limit was estimated to be 50 ppb (ng/ml) using an injection volume of 60 μl and based on a 3:1 signal-to-noise ratio at 258.8 nm.

Thermospray LC–MS proved to be specific but not sensitive for the detection and confirmation of ceftazidime. New analytical LC conditions were developed due to the incompatibility of the ion-paired LC non-volatile buffer for the sulfonates. The new conditions substituted ammonium acetate buffer for sulfonates. The same phenyl column was used for the separation of ceftazidime from dolphin serum components under LC–MS conditions. The thermospray mass spectrum for ceftazidime exhibited only the fragment ions at m/z 237, 255 and 315 when a positive-ion detection mode was employed (Fig. 4A) and the ions at m/z 235, 253 and 313 when the negative-ion detection mode was used (Fig. 4B). The thermospray LC–MS fragmentation pattern of ceftazidime was similar to that for the other β-lactam antibiotics [16–18].

The negative-ion detection mode was several times more sensitive for ceftazidime than the positive-ion detection mode. Multiple-ion detection,

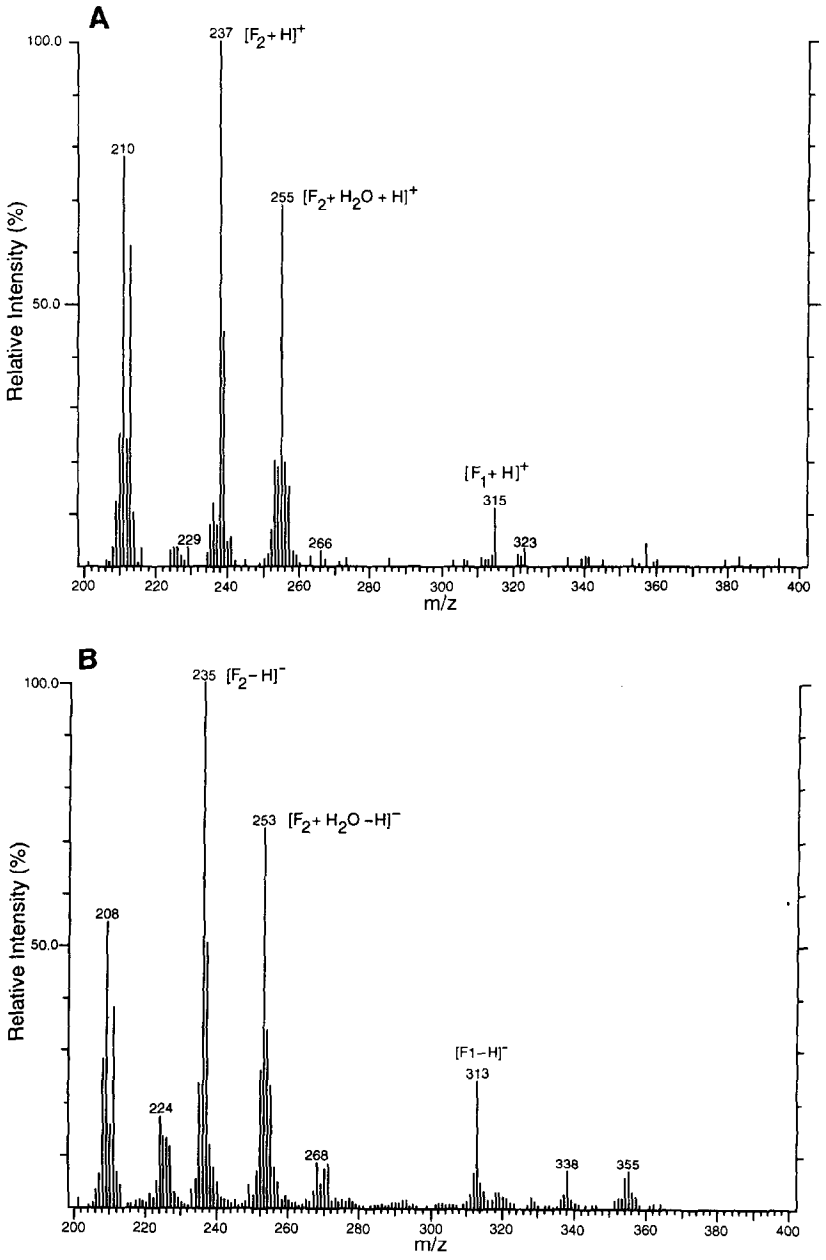

Fig. 4. (A) Thermospray LC–MS spectrum of 50 μg ceftazidime standard acquired using a positive-ion detection mode (200–800 daltons, at 1 s). (B) Thermospray LC–MS spectrum of 50 μg ceftazidime standard acquired using a negative-ion detection mode (200–800 daltons, at 1 s).

monitoring the negative ions at *m/z* 235, 253 and 313, was employed to perform confirmation of ceftazidime in dolphin serum. Fig. 5A shows the mass chromatograms of three standards of ceftazidime used for calibration at 10-, 15- and 20-μg levels. The retention time for this analyte was 1.5 min under conditions described in the Experimental section.

To confirm the presence of ceftazidime in dolphin serum, the concentrated sample (15 μg) col-

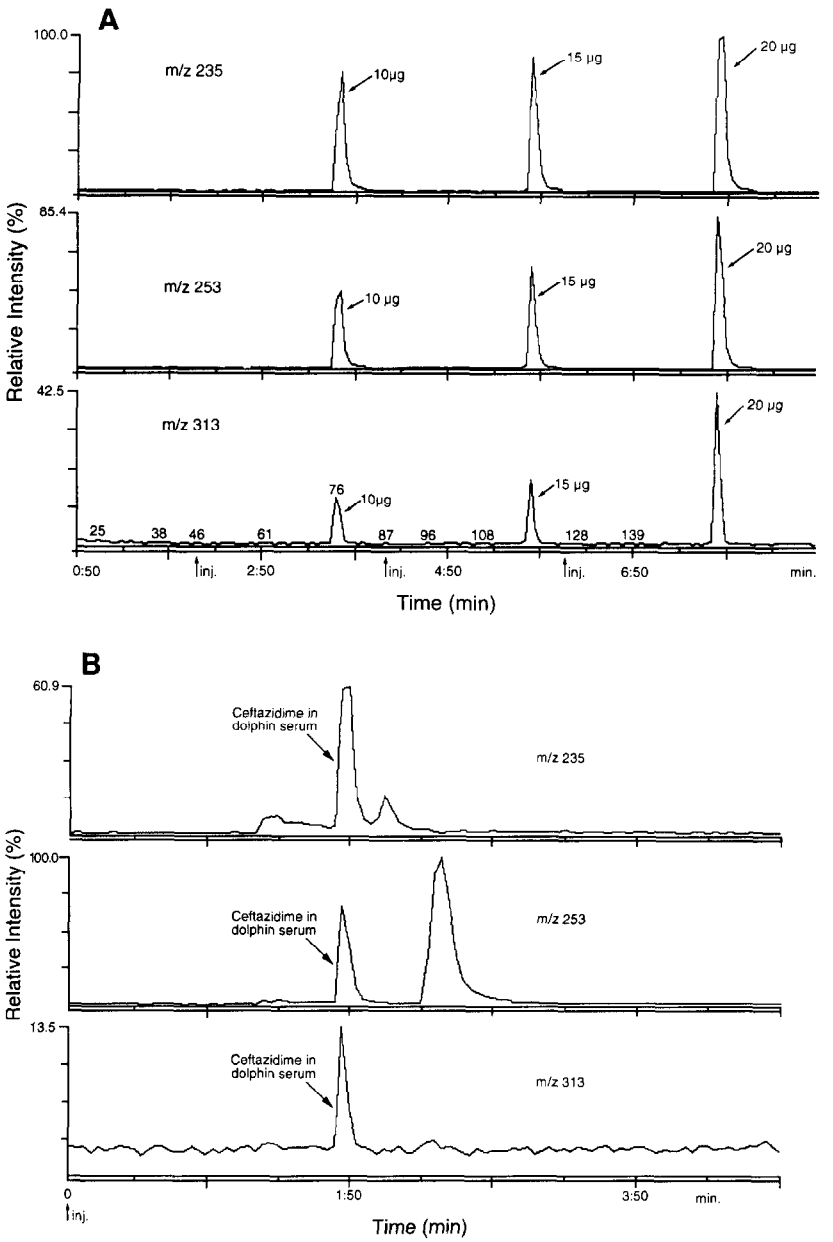

Fig. 5. (A) Thermospray LC–MS chromatograms monitoring negative-ions at *m/z* 235, 253 and 313 for 10, 15 and 20 µg ceftazidime. (B) Thermospray LC–MS chromatograms confirming the presence of ceftazidime in dolphin serum (collected under LC–UV–VIS conditions). The negative ions at *m/z* 235, 253 and 313 maximize at the proper retention times for ceftazidime.

lected under LC–UV–VIS condition was analyzed. The LC–MS profile of dosed dolphin serum (30-min post-dose sample) showed a peak at the proper retention time as well as the co-maximization of the ions at *m/z* 235, 253 and 313 confirming the presence of ceftazidime (Fig. 5B).

CONCLUSIONS

The proposed ion-paired LC method for the determination of ceftazidime levels permits the UV–VIS detection and thermospray LC–MS confirmation of this analyte in dolphin serum.

The method is accurate, selective and simple for performing the routine analysis of ceftazidime in dolphins. Thermospray LC–MS analysis showed no evidence of metabolic conversion of the drug through peak serum concentrations 30 min after intramuscular injection.

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