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Determination of ceftiofur and its metabolite desfuroylceftiofur in bovine serum and milk by ion-paired liquid chromatography

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ABSTRACT

A simple and sensitive liquid chromatographic method has been developed for the simultaneous determination of ceftiofur and its metabolite desfuroylceftiofur in bovine serum and milk. The method involved an ultrafiltration of diluted serum/milk with an equal volume of 50% acetonitrile through a 10 000 dalton molecular mass cut-off filter. Separation of ceftiofur and desfuroylceftiofur from the other serum/milk components was performed by ion-paired (octane and dodecanesulfonate) liquid chromatography using a reversed-phase column eluted with acetonitrile–water solution. The ultraviolet–visible absorbance of the column effluent was monitored in 200–350 nm range of a photodiode-array detector or at λ_{max} 289.6 nm for ceftiofur, λ_{max} 265.8 nm for desfuroylceftiofur and λ_{max} 271.4 nm for dimer of desfuroylceftiofur. Recoveries of ceftiofur from bovine milk spiked with 1 and 10 μg/ml were 95.9 and 97.0% with coefficients of variation of 3.69 and 2.51%, respectively. Recovery of ceftiofur from bovine serum spiked with 10 μg/ml was 90.4% with a coefficient of variation of 5.29%. A correlation coefficient of 0.9992 occurred with ceftiofur in aqueous solutions ($n = 5$, in duplicates). The limit of detection was estimated to be approximately 50 ppb (ng/ml). Additionally, this paper documents the presence of a ceftiofur metabolite in bovine serum under *in vitro* and *in vivo* conditions. The metabolite was identified as desfuroylceftiofur together with its dimer 3,3'-desfuroylceftiofur disulfide by thermospray liquid chromatography–mass spectrometry.

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INTRODUCTION

Ceftiofur, a cephalosporin β-lactam antibiotic, is produced semisynthetically with its primary structure (Fig. 1A) based on the 7-aminocephalosporanic acid nucleus. Cephalosporin antibiotics are being used increasingly in veterinary medicine for treating bacterial infections [1]. The occurrence of metabolites have been reported for cephapirin [2,3] and ceftiofur [4–8] in bovine milk and serum. A liquid chromatographic (LC) method for the determination of desfuroylceftiofur in plasma of cattle, rats, horses, pigs an dogs was reported [4]. The method utilized laborious sample preparation and derivatization of the analyte with iodoacetamide.

This paper describes the development of an LC method with ultraviolet photodiode array (UV PDA) detection for the simultaneous determination of ceftiofur and its metabolite in bovine serum and milk without derivatization. The identification of desfuroylceftiofur (Fig. 1B) and its dimer (Fig. 1C) was performed using LC–UV and LC–mass spectrometric (MS) techniques. Also, the presence of a ceftiofur metabolite, desfuroylceftiofur, and its dimer in bovine serum both *in vivo* and *in vitro* is documented.

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 aid and triethylamine were LC grade (Fisher Scientific, Raleigh, NC, USA). The ion-pairing reagents, dodecanesulfonate (S_{12}) and octanesulfonate (S_8) were 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).

Ceftiofur hydrochloride standard was kindly supplied by Upjohn (Kalamazoo, MI, USA). A 1 mg/ml ceftiofur stock solution (calculated as a

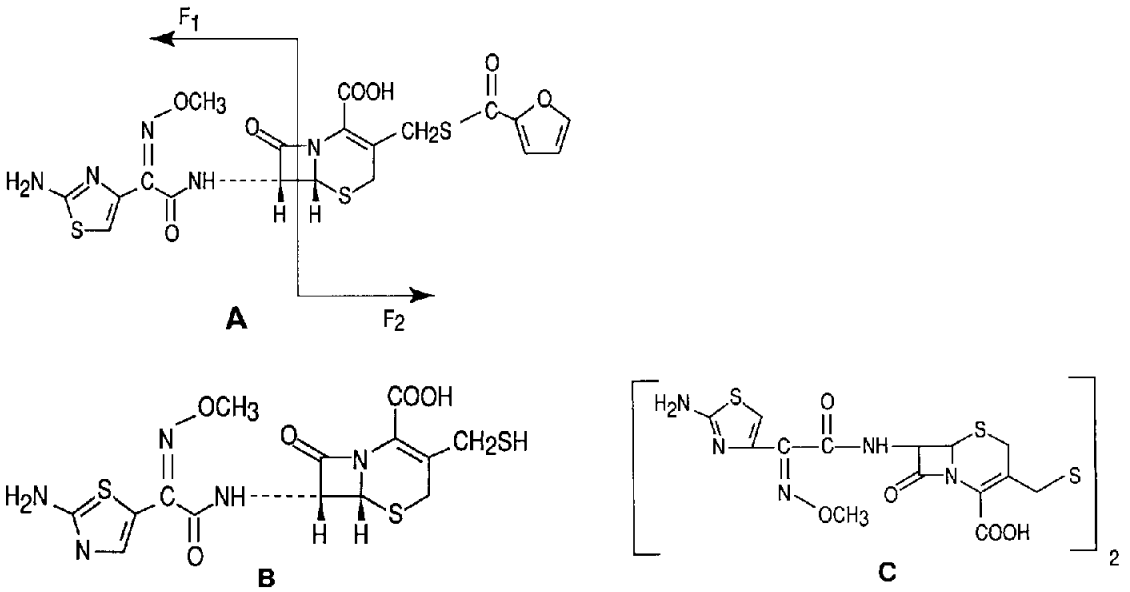

Fig. 1. (A) Chemical structure of ceftiofur and fragmentation pattern for the thermospray mass spectrometric detection. (B) Chemical structure of desfuroylceftiofur. (C) Chemical structure of 3,3′-desfuroylceftiofur disulfide (dimer of desfuroylceftiofur).

free acid) was prepared in acetonitrile–water (50:50, v/v, and 1 mg/ml). Working standard solutions of 1 and 10 μg/ml were prepared daily with the same diluent. All standards were protected form light with aluminum foil and amber vials.

Bovine milk and serum samples

Bovine milk and serum samples from lactating dairy cows treated with ceftiofur were obtained from the College of Veterinary Medicine, North Carolina State University (Raleigh, NC, USA). Samples from dosed cows were collected at 15, 30, 45, 60 min and 24 h after intravenous injection of ceftiofur sodium at 2.2 and 11 mg/kg body weight. The drug was injected intravenously rather than intramuscularly to maximize serum concentrations which would facilitate any tendency of the drug to diffuse into milk. Intramammary infusion of 50 mg ceftiofur into a single mammary gland was performed on two lactating dairy cows. Milk samples were collected prior to dosing and at 30 min, 1, 2, 4, 8, 22, 24 and 48 h after dosing from the right and left front glands. Serum samples were collected at the same time as milk samples. Pretreatment control serum and milk samples were used for blank analysis, *in vitro* studies or were spiked with ceftiofur for the assay validation.

Sample preparation procedure

Aliquots (500 μl) of serum or milk were diluted with an equal volume of acetonitrile–water (50:50, v/v) in the microseparation system equipped with a 10 000 dalton molecular mass cut-off filter. Samples were vortex-mixed for 10–15 s and centrifuged for approximately 30 min at 4000 *g* with a 45° fixed-angle rotor. A 10–100 μl aliquot of colorless ultrafiltrate was injected into an LC system equipped with a UV–VIS 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). The LC separations were performed using a mobile phase consisting of 20% acetonitrile in water (v/v), 0.25% of 80% phosporic acid (v/v) and 0.25% triethylamine solution containing 0.0025 *M* octanesulfonate and 0.0025 *M* dodecanesulfonate. The mobile phase flow-rate was 0.8–1.0 ml/min giving a 6–7 min retention time for desfuroylceftiofur and 11–16 min for ceftiofur on a Ultremex 3-μm phenyl column, 250 mm × 4.6 mm I.D. (Phenomenex, Torrance, CA, USA). The column effluent was analyzed in the wavelength range 200–350 nm using the PDA detector. The recoveries of ceftiofur from bovine milk and serum samples were determined by analyzing five to six spiked samples at the 1- and 10-ppm levels at 289.6 nm (Table I). After comparing the areas of ceftiofur standard and bovine serum/milk samples, the quantity of ceftiofur determined by LC–UV detection was calculated as follows: μg/ml = [ceftiofur (ng) × 2]/[injection volume (μl)]. Usually the injection volume was between 10 and 100 μl. The multiplication by 2 in the equation accounts for the dilution of serum (1:1) with the solution for releasing protein-bound drug. Desfuroylceftiofur standard was not available, therefore the quantities of this compound were calculated as ceftiofur equivalent at 265.8 nm.

Liquid chromatography–mass spectrometry

A thermospray mass spectrum of ceftiofur was acquired using a mobile phase of 25% acetonitrile in 0.1 *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 110°C, respectively. A Finnigan MAT 4800 quadrupole mass spectrometer was operated in positive-ion detection mode under full-scan conditions of 150–1000 daltons for 0.2 s. Confirmation of desfuroylceftiofur was performed using samples collected from bovine serum (15 min post-dose) under the LC–UV–VIS conditions described in the Experimental section (loop injection). Confirmation of the dimer of desfuroylceftiofur was performed after collecting this com-

pound from bovine serum incubated with ceftiofur for 20 h (loop injection).

In vitro defuroylation of ceftiofur in bovine serum and milk

Bovine milk and serum were fortified to achieve concentrations of 200 μg/ml ceftiofur, then incubated at 37°C for 20 h. At the time of sampling (2 min, 15 min, 30 min, 1 h, 8 h and 20 h), 500-μl aliquots were vortex-mixed with an equal volume of 50% acetonitrile and placed in a microseparation system with 10 000 daltons for centrifuging. Ultrafiltrates were injected into the LC–UV PDA system.

RESULTS AND DISCUSSION

Previous work in our laboratory on developing analytical procedures for β-lactam antibiotics shows that dilution of milk with water-miscible solvents, ultrafiltration through 10 000 dalton molecular mass cut-off filters and direct injection into a HPLC system was successful for the determination of penicillin G, cloxacillin, amoxicillin, ampicillin [9,10] and cephapirin [11] in bovine milk. Separation of these β-lactam antibiotics from the other milk components was performed by ion-paired chromatography using a reversed-phase phenyl column.

Several solutions were tested for their ability to free ceftiofur from bovine serum and milk proteins. The optimal solution was 50% acetonitrile; it allowed over 90% recovery of ceftiofur from bovine serum and milk in spiked samples of 10 and 1 ppm (Table I). Average recoveries of ceftiofur from milk were 95.9% (1 ppm) and 97.0% (10 ppm) with coefficients of variation (C.V.) of 3.69 and 2.51%, respectively. Average recovery of ceftiofur from bovine serum spiked with 10 ppm of ceftiofur was 90.4% with a C.V. of 5.29%. The study on linearity of UV–VIS detector response was performed by injection of ceftiofur standard containing increasing amounts from 10 to 1000 ng. Each level was analyzed in duplicate. The relationship between peak area and concentration of aqueous ceftiofur standard solution was linear within this range with a correlation coefficient of 0.9982 ($n = 5$). The limit of detection for the determination of ceftiofur in bovine milk and serum, defined as the analyte concentration yielding a peak approximately three times the noise level, was about 50 ppb, using an injection volume of 100 μl. Optimal separation of ceftiofur and its metabolite was obtained with a mobile phase consisting of 20% acetonitrile, 0.25% phosphoric acid and 0.25% triethylamine in 0.0025 *M* octanesulfonate and 0.0025 *M* dodecanesulfonate solution.

Fig. 2A shows a chromatogram of bovine serum ultrafiltrate collected after 30 min incubation with ceftiofur at 37°C. The chromatography was generated at 265.8 nm, at maximum wavelengths for desfuroylceftiofur. Fig. 2B exhibits the UV–VIS absorption spectral curve for desfuroylceftiofur acquired at the 200–350 nm range.

Ceftiofur was rapidly degraded *in vitro* in bovine serum, but not in bovine milk (Table II). Only 53.3 ppm ceftiofur remained from the 200 ppm spiked in serum following 15 min incuba-

TABLE I

STATISTICAL SUMMARY OF LC–UV ANALYSIS OF CEFTIOFUR IN BOVINE MILK AND SERUM (λ = 289.6 nm)

Matrix	n	Concentration spiked (ppm)	Recovery (%)		Coefficient of variation (%)
			Range	Mean ± S.D.	
Milk	6	1	91–99.5	95.9 ± 3.54	3.69
Milk	5	10	94.0–100.1	97.0 ± 2.44	2.51
Serum	5	10	87.5–97.6	90.4 ± 4.78	5.29

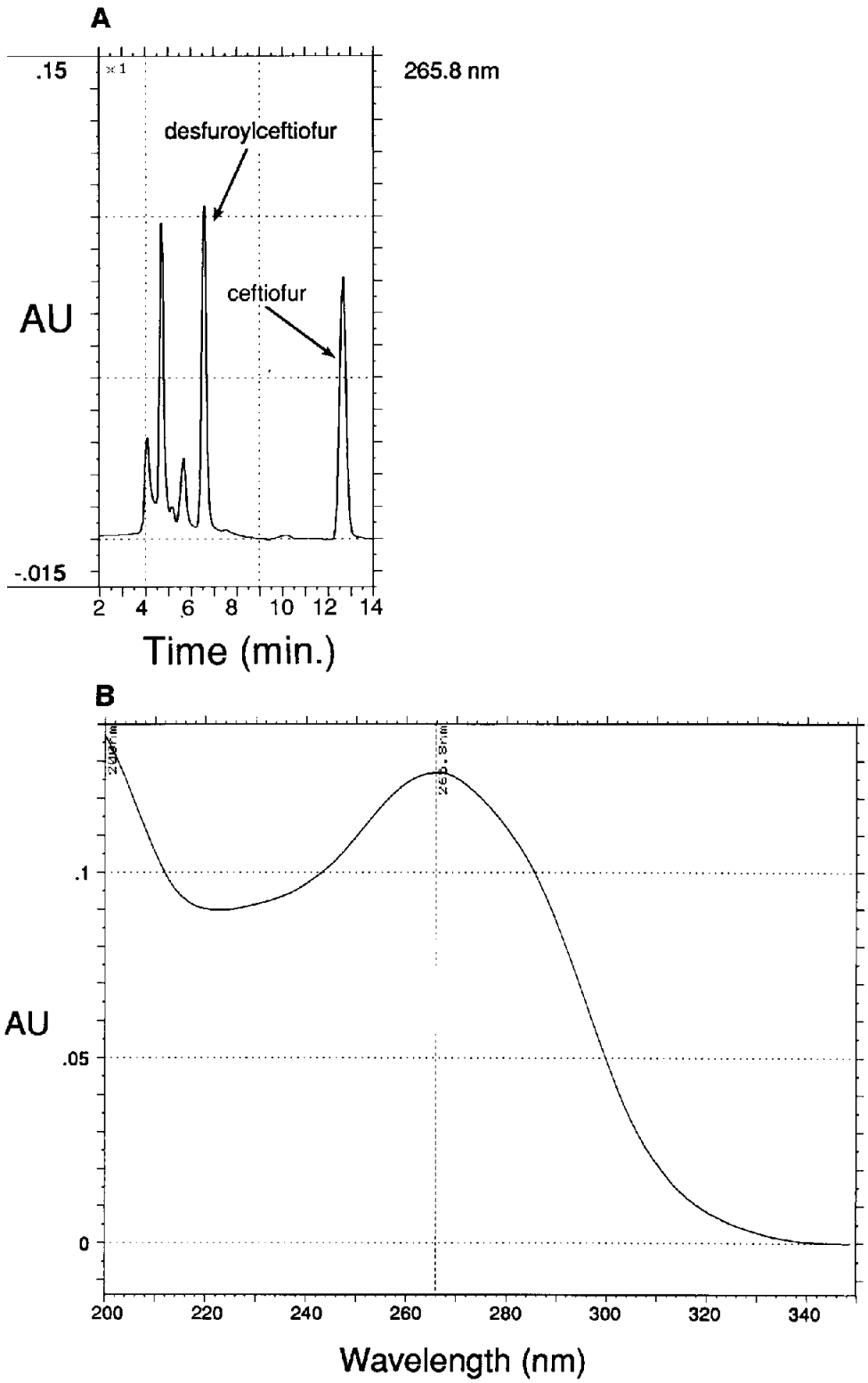

Fig. 2. (A) LC–UV PDA chromatogram of a bovine serum sample collected after 30 min incubation with ceftiofur at 37°C, injected at 0 min and acquired at 265.8 nm (λ_{max} for desfuroylceftiofur). Injection volume was 20 μl. (B) UV–VIS spectral curve for desfuroylceftiofur.

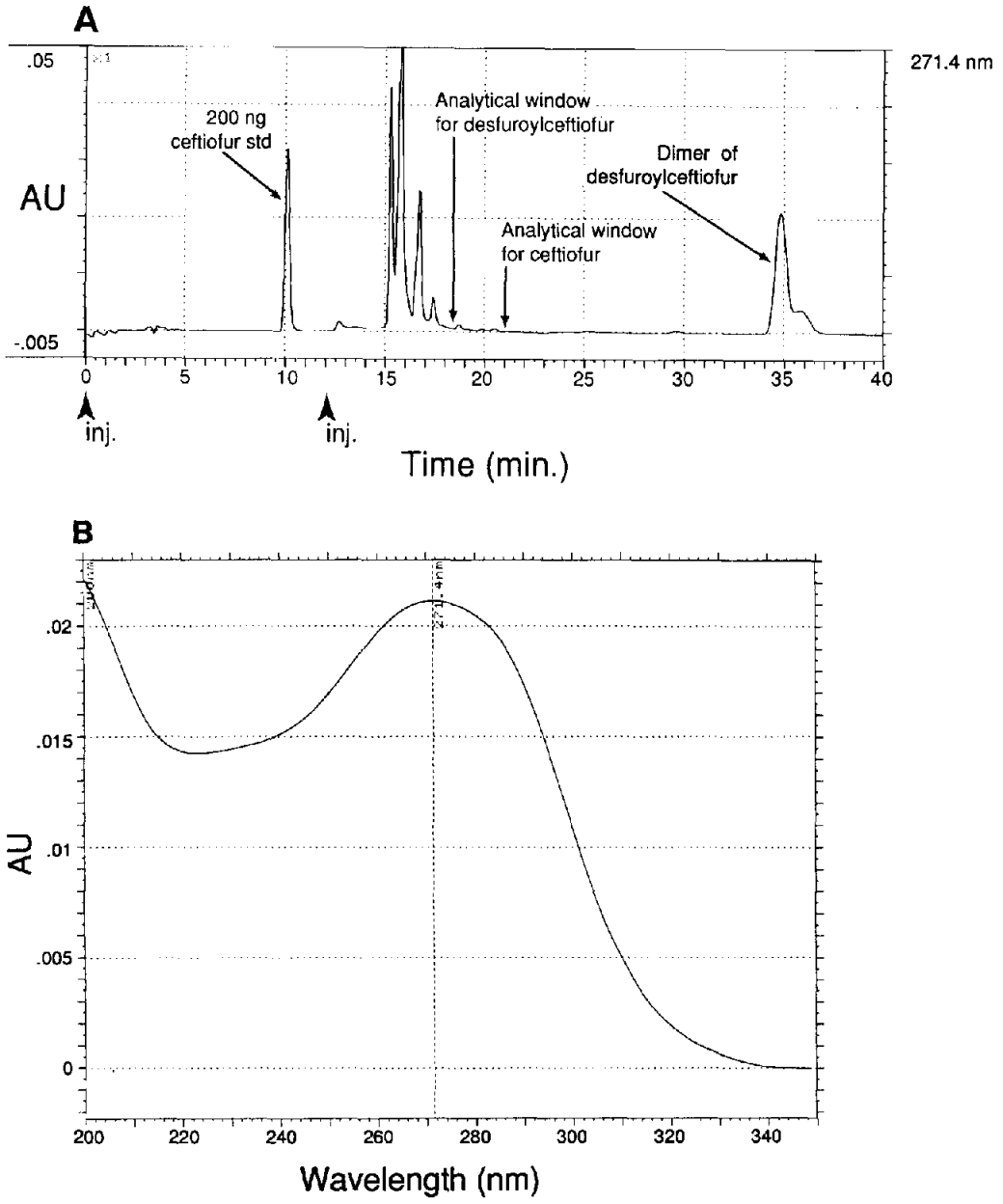

Fig. 3. (A) LC–UV PDA chromatograms of a 200-ng ceftiofur standard (0–12 min) and a bovine serum sample collected after 20 h incubation with ceftiofur at 37°C (12–40 min), acquired at 271.4 nm (λ_{max} for dimer of desfuroylceftiofur). Injection volume was 20 μl. (B) UV spectral curve for the dimer of desfuroylceftiofur.

tion. In comparison, 190 ppm of ceftiofur remained in milk. Ceftiofur was converted to desfuroylceftiofur in serum, whereas no such a conversion occurred in milk. After 8 h incubation of ceftiofur with bovine serum, a new compound containing an S–S bond appeared which was related to ceftiofur and identified by MS as the dimer of desfuroylceftiofur (Table II). Fig. 3A shows the chromatograms of a 200-ng ceftiofur standard (0–12 min) and a bovine serum sample

TABLE II

IN VITRO CEFTIOFUR BIOTRANSFORMATION TO DESFUROYL CEFTIOFUR AND ITS DIMER IN BOVINE SERUM AND MILK AT 37°C (200 ppm CEFTIOFUR)

Time	Bovine serum			Bovine milk		
	Ceftiofur (ppm)	Desfuroylceftiofur (ppm)	Dimer of desfuroylceftiofur (ppm)	Ceftiofur (ppm)	Desfuroylceftiofur	Dimer of desfuroylceftiofur
2 min	89.1	2.1	Neg[a]	–	–	–
15 min	53.3	3.4	Neg	190.0	Neg	Neg
30 min	7.6	3.4	Neg	192.9	Neg	Neg
1 h	0.3	3.4	Neg	191.7	Neg	Neg
8 h	Neg	1.2	1.0	–	–	–
20 h	Neg	Neg	3.4	175.9	Neg	Neg

[a] Neg = below 0.05 ppm.

TABLE III

IN VIVO CEFTIOFUR BIOTRANSFORMATION TO DESFUROYLCEFTIOFUR IN BOVINE SERUM

Bovine milk samples taken at the same times as serum were all negative for both cows.

Time	Cow 1 dosed 2.2 mg/kg		Cow 2 dosed 11 mg/kg	
	Desfuroylceftiofur (ppm)	Ceftiofur	Desfuroylceftiofur (ppm)	Ceftiofur
Pretreatment	Neg[a]	Neg	Neg	Neg
15 min	2.1	Neg	13.5	Neg
30 min	1.8	Neg	9.6	Neg
45 min	Neg	Neg	7.3	Neg
60 min	Neg	Neg	7.3	Neg
24 h	Neg	Neg	Neg	Neg

[a] Neg = below 0.05 ppm.

TABLE IV

CONCENTRATIONS OF CEFTIOFUR (ppm) IN MILK AT VARIOUS TIMES AFTER INTRAMAMMARY INFUSION OF 50 mg CEFTIOFUR INTO THE RIGHT FRONT GLAND OF COW 1 AND THE LEFT FRONT GLAND OF COW 2

Bovine serum samples taken at the same times as milk were all negative for ceftiofur and its metabolite for both cows.

Time	Cow 1		Cow 2	
	Right front gland	Left front gland	Right front gland	Left front gland
Pretreatment	Neg[a]	Neg	Neg	Neg
30 min	1605.7	Neg	Neg	545.9
1 h	1252.4	Neg	Neg	458.0
2 h	549.6	Neg	–	–
4 h	230.1	Neg	Neg	237.1
8 h	57.0	Neg	Neg	57.2
22 h	0.8	Neg	Neg	Neg
24 h	0.3	Neg	Neg	Neg
48 h	Neg	-	Neg	Neg

[a] Neg = below 0.05 ppm.

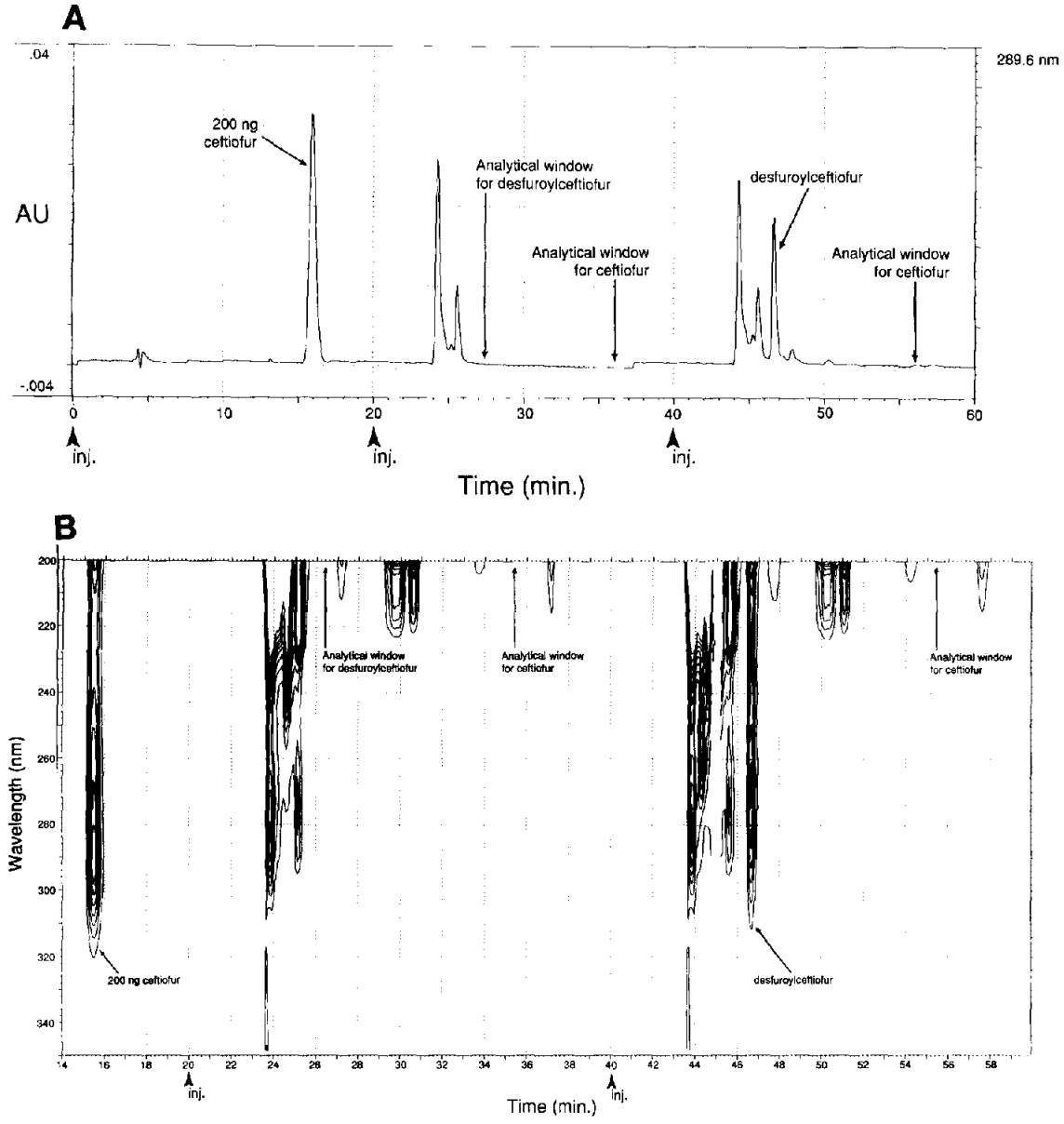

Fig. 4. (A) LC–UV PDA chromatograms of a 200-ng ceftiofur standard (0–20 min), a blank bovine serum sample (20–40 min) and bovine serum sample collected 15 min after intravenous injection of ceftiofur, acquired at 289.6 nm (λ_{max} for ceftiofur). Injection volume was 20 μl. (B) UV–VIS absorbance contour plots for ceftiofur standard (0–20 min), blank bovine serum (20–40 min) and bovine serum collected 15 min after intravenous injection of ceftiofur.

collected after a 20-h incubation period with ceftiofur at 37°C (12–40 min). The dimer of desfuroylceftiofur had a retention time of 23 min (sample was injected at 12 min). The UV absorbance spectral curve for the dimer of desfuroylceftiofur (Fig. 3B) shows similarities to desfuroylceftiofur (Fig. 2B).

Fig. 4A shows chromatograms of a ceftiofur standard (0–20 min), a control bovine serum (20–40 min) and serum from a cow taken 15 min after the intravenous injection of ceftiofur. There were no interfering peaks near the retention times of either ceftiofur or desfuroylceftiofur compounds.

The UV absorbance contour plots for peaks introduced in Fig. 4A are shown in Fig. 4B. The analytical windows for ceftiofur and its metabolite in blank bovine serum are clear.

To assure repeatability of the chromatographic separation of ceftiofur and its metabolites, two phenyl columns (250 mm × 4.6 mm I.D.) were used. Both of them separated these analytes from serum and milk components. The only variability observed was a change in retention times.

In vivo studies were performed on two cows injected intravenously with ceftiofur at 2.2 and 11 mg/kg body weight, respectively (Table III). Ceftiofur was not detected even in serum samples taken 15 min after the injection of 11 mg/kg, which represented five times the regular dose. Ceftiofur and desfuroylceftiofur were not detected in any of the milk samples which were taken at the same time as the serum samples.

Fig. 5A shows a chromatogram of a 150-ng ceftiofur standard (0–10 min), a bovine milk sample collected 8 h after intramammary infusion of

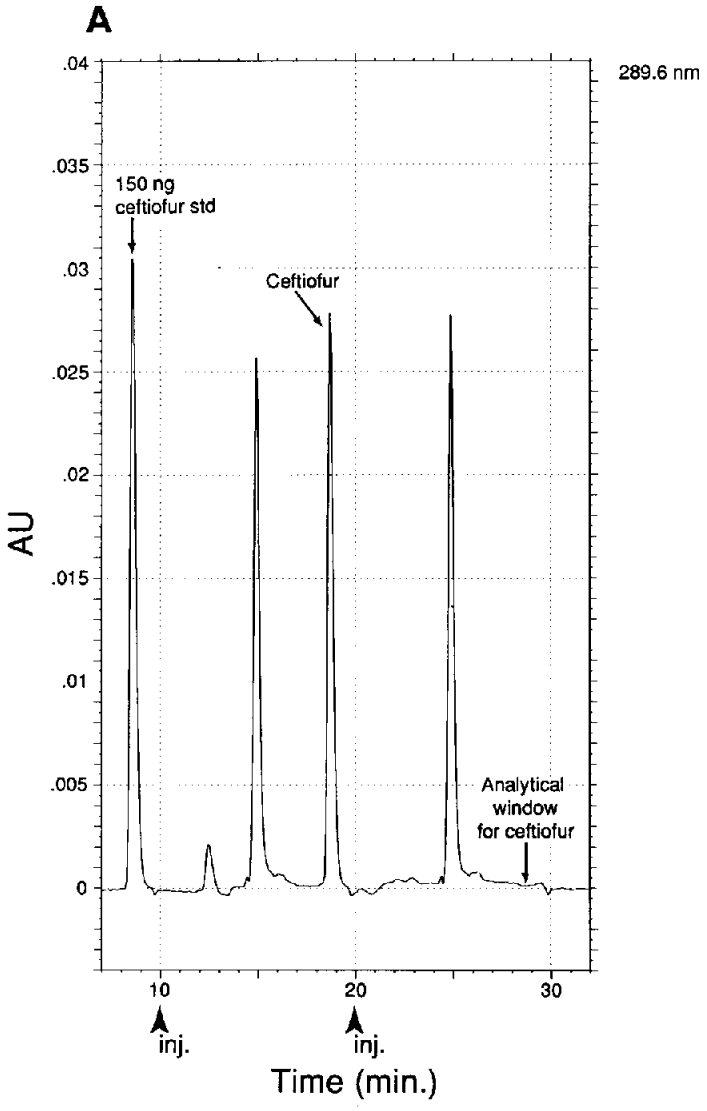

Fig. 5.

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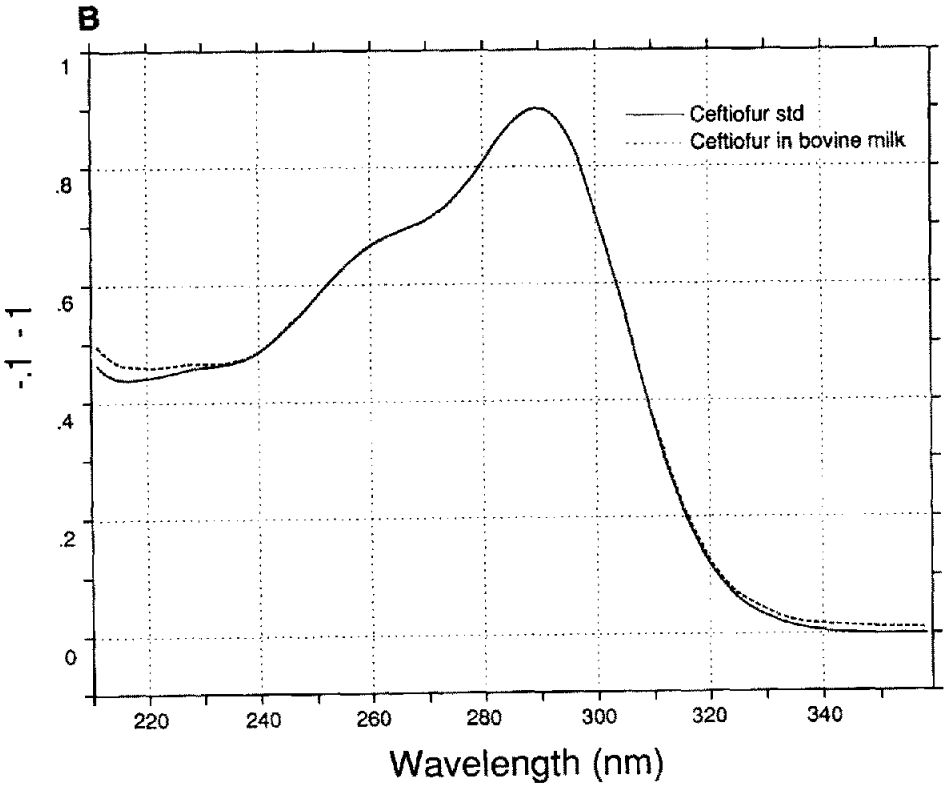

Fig. 5. (A) LC–UV PDA chromatograms of a 150-ng ceftiofur standard injected at 0 min (not shown), a bovine milk sample collected 8 h after intramammary infusion of ceftiofur (10–20 min) and a blank bovine milk sample (20–30 min). Injection volume was 5 μl. (B) UV–VIS spectral curves for ceftiofur aqueous standard (solid line) and ceftiofur found in bovine milk collected 8 h after intramammary infusion of this drug (dashed line).

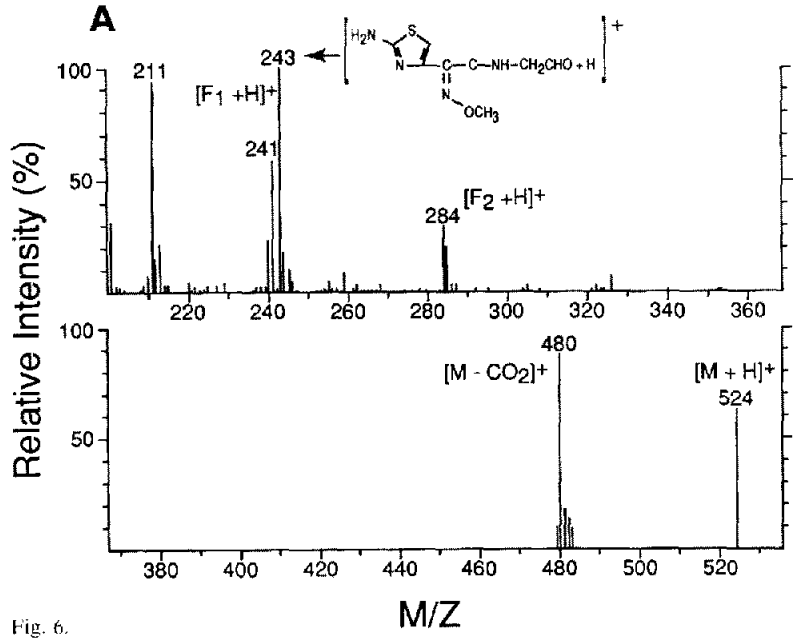

Fig. 6.

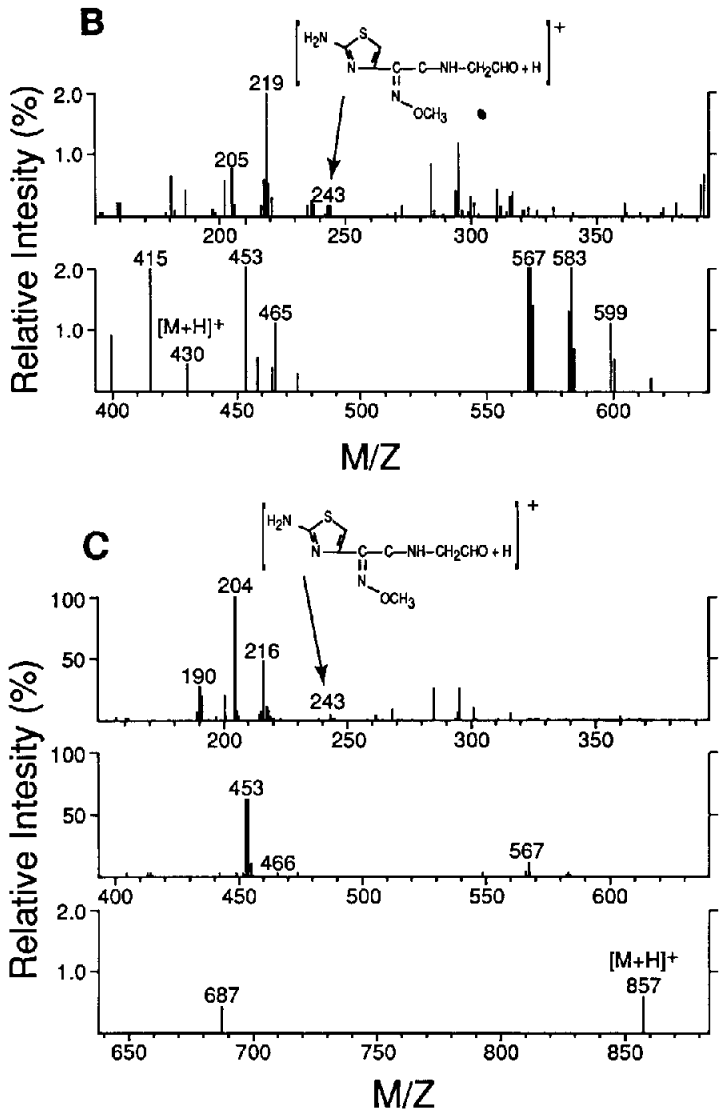

Fig. 6. (A) Thermospray LC–MS spectrum of ceftiofur standard. (B) Thermospray LC–MS spectrum of desfuroylceftiofur collected from bovine serum sample under the LC–UV conditions. (C) Thermospray LC–MS spectrum of the dimer of desfuroylceftiofur collected from bovine serum under LC–UV conditions.

50 mg ceftiofur (10–20 min) and a blank bovine milk sample (20–32 min). Fig. 5B exhibits UV–VIS spectral curves (210–360 nm) for the ceftiofur standard (solid line) and milk sample (dashed line). There was a good agreement beween these two curves except at the lower wavelength (210–230 nm).

Table IV depicts results of an *in vivo* study performed on two cows infused intramammary with 50 mg of ceftiofur. Desfuroylceftiofur was not detected in any milk samples. Bovine serum samples taken at the same times were negative for ceftiofur and its metabolite.

Thermospray LC–MS provided the specificity to identify and confirm the presence of desfuroylceftiofur and its dimer in bovine serum collected under the conditions described for the LC–UV method. Fig. 6A exhibits the thermospray spectrum for ceftiofur standard (10 μg) which showed an $[M + H]^+$ ion (m/z 524) and several fragment

ions at *m/z* 241, 243, 284 and 480. The fragment ion at *m/z* 243 is indicative for the cephalosporin ring connected to the thiazole ring of the molecule (MW 242). To confirm the presence and identity of desfuroylceftiofur and its dimer in bovine serum, a concentrated sample was analyzed in order to obtain a full-scan (150–1000 daltons) mass spectrum (Fig. 6B and 6C). The thermospray LC–MS spectrum for desfuroylceftiofur exhibited a weak $[M + H]^+$ ion at *m/z* 430 (Fig. 6B) and one fragment ion related to its structure at *m/z* 243 identified as aldehyde (MW 242). The same characteristic ion at *m/z* 243 was present on the thermospray LC–MS spectrum for the dimer of desfuroylceftiofur (Fig. 6C), together with the $[M + H]^+$ ion at *m/z* 857. These identifications are consistent with proposed structures for ceftiofur metabolites reported in the literature [5].

CONCLUSIONS

A simple and sensitive method is presented for the simultaneous determination of ceftiofur and its metabolite desfuroylceftiofur in bovine serum and milk without derivatization. The limit of detection was estimated to be 50 ppb.

Ceftiofur is handled by the body differently following intravenous and intramammary routes of administration. (a) Ceftiofur is rapidly metabolized to desfuroylceftiofur following intravenous administration, but neither ceftiofur nor desfuroylceftiofur could be detected in milk at concentrations of 50 ppb or above. (b) Ceftiofur is rapidly cleared from milk with no evidence of metabolism to desfuroylceftiofur or diffusion into serum following intramammary infusion. Should ceftiofur be detected in milk, it would indicate that the intramammary route of administration was used.

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