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# Quantitative determination of ceftiofur in milk by liquid chromatography–electrospray mass spectrometry

Jeffrey Keever[a], Robert D. Voyksner[a,*], Krystyna L. Tyczkowska[b]

[a]Analytical and Chemical Sciences, Research Triangle Institute, P.O. Box 12194, Research Triangle Park, NC 27709, USA

[b]Department of Anatomy, Physiology Sciences and Radiology, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, NC 27606, USA

**Abstract**

A liquid chromatography–electrospray mass spectrometry (LC–ES-MS) was developed for the quantitation of ceftiofur in milk at the 50 ppb tolerance level set by the US Food and Drug Administration (FDA) for the drug. The method used ultrafiltration as a simple and rapid means to prepare the sample for analysis. A 100 μl volume of ultrafiltrate containing ceftiofur was concentrated on-column for LC–MS analysis. The LC separation was accomplished using an acetonitrile gradient with the ion-pair reagent heptafluorobutyric acid (HFBA). Propionic acid was added after the LC column to minimize electrospray signal suppression, enhancing the response for ceftiofur by a factor of 10. The transmission ions from the electrospray interface to the MS was enhanced by a factor of 7 by using a Rf ion guide. The development method could detect ceftiofur to 10 ppb and quantitate the antibiotic from 25–200 ppb (linear correlation coefficient of 0.993). The analysis indicated that bovine milk collected 32 h after dosing with ceftiofur was above the FDA tolerance of 50 ppb, while milk collected 48 h after dosing was found to contain 24–31 ppb of ceftiofur. © 1998 Elsevier Science B.V.

*Keywords:* Mass spectrometry; Ceftiofur

## 1. Introduction

A number of veterinary drugs which are frequently used to prevent infections, can contaminate meat and milk products destined for human consumption. Ceftiofur (Fig. 1) is a cephalosporin β-lactam antibiotic which is finding widely increased use in the treatment of bacterial infections. While residues of ceftiofur and its metabolites have been reported in bovine milk and serum [1–3], mass spectrometry (MS) methodology has not been developed for the confirmation of this antibiotic at the 50 ppb tolerance level set by US Food and Drug Administration (FDA) [4]. Based on the presence of a basic nitrogen on ceftiofur and its polar and thermally unstable properties, electrospray positive-ion detection (analyzing ceftiofur at low pH to protonate the molecule in solution) should be feasible [5,6]. Also, ceftiofur and other antibiotics from the cephalosporin class have been analyzed by positive-ion detection electrospray mass spectrometry (ES-MS) [7–9].

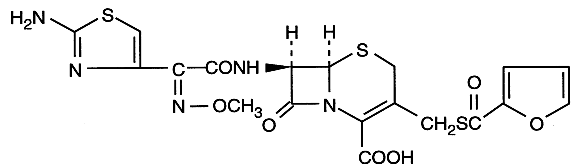


Fig. 1. Structure of ceftiofur.

To achieve the ES-MS sensitivity necessary to detect residues of ceftiofur in milk, ES-MS required the use of an Rf ion guide to improve ion transport.

*Corresponding author.

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The Rf ion guides offer advantages over electrostatic lens systems for transmissions of ions into the mass analyzer [10–15]. Rf ion guides involving quadrupole, hexapole and octapole configurations are now commonly used in the ion transport region of the atmospheric pressure ionization (API) interface for introduction of ions into a mass analyzer.

This paper presents the research into developing a quantitative confirmation methodology based on liquid chromatography–electrospray-mass spectrometry (LC–ES-MS) for ceftiofur in milk at the 25 ppb level.

## 2. Experimental

### 2.1. Materials and reagents for LC–MS analysis

Water was purified with a Milli-Q water system (Millipore, Bedford, MA, USA) prior to use. Acetonitrile (ACN) was of LC–GC grade quality (Baxter Healthcare, Muskegon, MI, USA). The ion-pair reagent, heptafluorobutyric acid (HFBA) was obtained from Aldrich (Milwaukee, WI, USA). Ceftiofur hydrochloride standard was supplied by the Upjohn (Kalamazoo, MI, USA). The FDA milk samples collected 32 and 48 h after dosing with ceftiofur were supplied by the FDA Center of Veterinary Medicine, Beltsville, MD, USA.

### 2.2. Sample preparation procedure

Aliquots (500 μl) of milk were diluted with an equal volume of acetonitrile–water (50:50, v/v) in the microseparation system (Amicon Division of W.R. Grace, Danver, MA, USA) equipped with a Centrion-10, 10 000 molecular-mass cutoff filter. Samples were vortex mixed for 10–15 s and centrifuged for approximately 30 min at 4000 *g* with a 45° fixed-angle rotor. All samples were protected from light in amber vials covered in aluminum foil and stored at −20°C until analyzed.

### 2.3. LC–MS analysis

The LC separation was performed on a Nova Pak $C_{18}$ 75×3.9 mm (Waters, Milford, MA, USA) using a gradient of 5% ACN to 95% ACN in 9 min at a flow-rate of 1 ml/min. The mobile phase was delivered by two Waters 6000 A pumps. The mobile phase contained 1% acetic acid and 25 m*M* HFBA. A post-column split reduced the flow to about 100 μl/min. An ISCO 100D syringe pump (Isco, Lincoln, NE, USA) was used to post-column deliver a 50 μl/min flow-rate of priopionic acid–isopropanol (75:25) solution to the split flow (100 μl/min) from the LC column. This results in a total flow-rate 150 μl/min to the ES-MS system. A 100-μl aliquot of the colorless ultrafiltrate was injected for the analysis.

The ES-MS analysis was performed on a Hewlett-Packard 5989 electrospray mass spectrometer (Palo Alto, CA, USA) with a Hewlett-Packard API interface fitted with a hexapole ion guide (Analytica of Branford, Branford, CT, USA). The instrument was tuned and optimized for the transmission of the $[M+H]^+$ ion of ceftiofur at *m/z* 524 by infusing a 100 pg/ml solution of the antibiotic into the system. The optimal condition for the analysis of ceftiofur employed pneumatic nebulization with nitrogen (80 p.s.i.; 1 p.s.i.=6894.76 Pa) and a counterflow of nitrogen (6–8 l/min) heated to 290°C for the nebulization and desolvation of the introduced liquid. The API (electrospray) interface was typically operated using the following voltage settings: needle 0 V (grounded), $V_{cylinder}$ at −4.5 kV, $V_{endplate}$ at −4 kV, $V_{capillary}$ at −4 kV, capillary exit at 125 V, and skimmer 1 at 14 V. An octapole Rf ion guide was used to transmit ions from skimmer 1, through a stage of pumping and into the entrace lens cap of the ion trap. The Rf ion guide had a radius of 1.75 mm, was 6.2 cm long, and was operated at 5.3 MHZ, 500 Vpp with 4.4 Vbias. The entrance lens was at 75 V, the HED at 9500 V and the quadrupole temperature was set to 100°C. MS analysis was performed using selected ion monitoring (SIM), detecting *m/z* 524 with a dwell time of 300 ms.

## 3. Results and discussion

### 3.1. Sample preparation

The development of the method took into account the need to handle large numbers of samples. For this reason the simple and rapid procedure of ultrafil-

tration using a 10 000 molecular-mass cutoff filter was chosen for sample clean-up. A 100-μl aliquot of the filtrate was concentrated on column. Ultrafiltration has been previously optimized in our laboratory for the extraction of β-lactams in milk [16]. This optimization demonstrated the need of the addition of an organic solvent to the milk to minimize the milk protein binding to the drug. The acetonitrile–water (50:50) solution proved optimal for the extraction of penicillin G [14], resulting in excellent recoveries for ceftiofur. A 95±6.8% ($n$=6) recovery was obtained for ceftiofur in milk at the 50 ppb level.

### *3.2. Post-column modification of solvent*

In order to achieve sufficient retention for ceftiofur and to insure a clean analytical window for its detection, the separation used an electrospray compatible ion-pair reagent HFBA. This ion-pair reagent still results in some electrospray signal depression due to the formation of neutral ion-pairs. The addition of propionic acid post-column has been reported to minimize the signal suppression from ion-pair formation with trifluoroacetic acid (TFA) [17]. The weaker ion-pair reagent, propionic acid, displaces the stronger ion-pair reagent TFA based upon volatility (boiling point of propionic acid>> boiling point of TFA). Likewise this displacement should work for the use of HFBA, which has a boiling point less than that of propionic acid. To determine the improvement in the electrospray signal intensity when propionic acid is added to solvent systems containing HFBA, various ratios of HFBA to propionic acid were evaluated for the detection of ceftiofur. Fig. 2 shows that greater than a ten-fold increase in signal intensity is obtained when propionic acid is added post-column. A mobile phase containing HFBA–75% propionic acid in isopropanol (IPA) (1:0.5, v/v) resulted in the largest signal enhancement. Higher post-column mixing volume of propionic acid (e.g., HFBA–propionic acid in IPA, 1:1) showed less enhancement due to the simple dilution of the sample. On the other hand, lower post-column mixing volumes did not provide sufficient propionic acid to reduce the ion-pair suppression from HFBA.

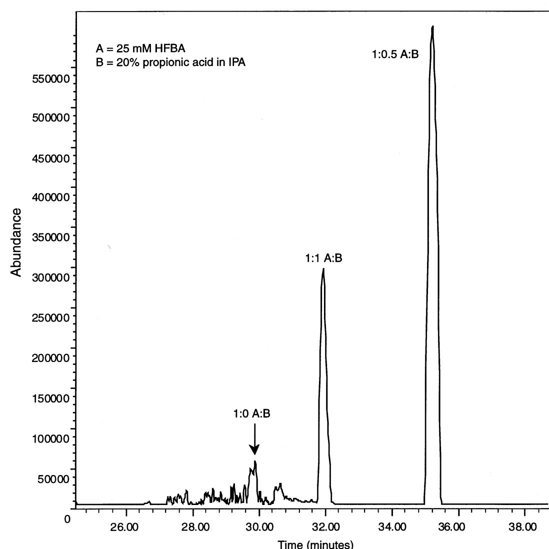


Fig. 2. The $[M+H]^+$ ion current for ceftiofur ($m/z$ 524) for the same quantity flow injected into the electrospray system in solutions of 25 m$M$ HFBA and mixtures of 25 m$M$ HFBA and propionic acid.

### *3.3. Rf ion guide*

In order to achieve the sensitivity required to detect and quantitate ceftiofur down to one half of the FDA tolerance level (25 ppb), it was necessary to improve the instrument's ion transmission by incorporating a Rf ion guide. Ion guides have been used to improve transmission of ions into various mass analyzers [12,14]. The comparison for the detection of a 100 ppb standard of ceftiofur with the conventional lens electrostatic stack versus Rf ion guide is shown in Fig. 3. The Rf ion guide shows a signal-to-noise ratio for the detection of ceftiofur approximately a factor of 7 better than the conventional electrostatic lens stack. This signal enhancement also improved the precision for the determination of ceftiofur at 100 ppb to 7% R.S.D. ($n$=5) compared to 12% R.S.D. ($n$=5) for the conventional lens stack.

### *3.4. Electrospray mass spectra*

The selection of the ion or ions to monitor for the detection of ceftiofur must take into account both sensitivity and specificity. While the best sensitivity would be obtained if all the ion current is in a single

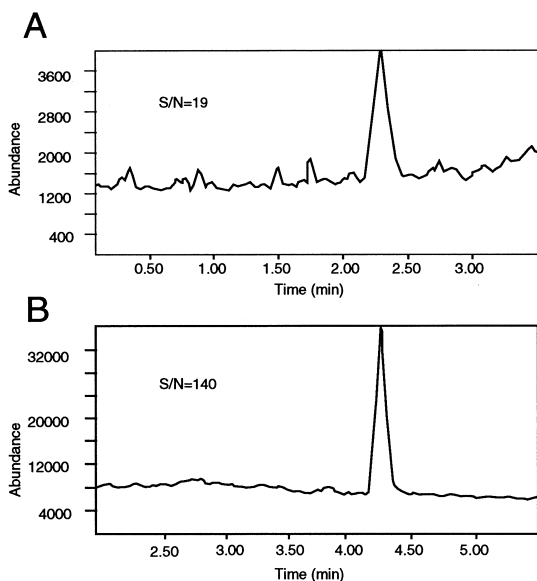


Fig. 3. LC–MS selected ion current chromatogram for the $[M+H]^+$ ion of ceftiofur (*m/z* 524) for the injection of a 100 ppb standard. (A) Analysis using the original electrostatic lens stack. (B) Analysis with a hexapole Rf ion guide.

*m/z* ion, the detection of 2–3 confirming ions would be beneficial for reasons of specificity. The electrospray mass spectra of ceftiofur only exhibited a $[M+H]^+$ ion at *m/z* 524 (capillary exit voltage of 125 V). Increasing the capillary exit voltage did not yield significant intensity fragment ion from the collision induced decomposition in the transport region. At capillary voltages of >200 V only a few fragment ions below *m/z* 200 were detected, but the overall signal intensity for these ions and the $[M+H]^+$ ion had decreased by more than a factor of 5. For this reason it was decided that quantitation of ceftiofur would be based upon only the detection of the $[M+H]^+$ ion at *m/z* 524 with the capillary exit voltage optimized for the transmission of that ion (125 V).

### 3.5. On-column concentration

To achieve 25 ppb detection of ceftiofur (half of the FDA tolerance for the drug) by LC–MS could prove difficult when working only with ultrafiltration sample preparation. To ultrafiltrate dilutes the original milk sample by a factor of two (addition of solvent to reduce protein-drug binding) and provides no enrichment of the drug. To minimize sample handling and loss due to thermal degradation or handling, it was decided to try on-column concentration of the ultrafiltrate versus concentration of the ultrafiltrate prior to injection for LC–MS analysis. The relatively hydrophobic nature of ceftiofur and the broad change in solvent strength during the gradient from water to acetonitrile should enable the trapping of the drug upon injection during the initial gradient (95% water) conditions. Injections of 20, 50, 100 and 200 μl of milk ultrafiltrate were evaluated to determine both gain in signal intensity for the drug and the possibility of interference or injection volumes increased. The LC peak width was nearly constant from 20–100 μl injected, with the peak areas for ceftiofur increasing by a factor of 2.4 and 5.1 for the larger volume injections relative to a 20 μl injection. The 200 μl injection showed some peak broadening and only a 6.2 factor increase (versions a theoretical increase of factor 10) in signal intensity. The milk background steadily increased from 20 to 200 μl injected, with interference appearing in the analytical window of ceftiofur for the largest volume injection. Based upon these observations it was decided that a 100 μl injection of milk ultrafiltrate was optimal for the detection of ceftiofur.

### 3.6. Quantitation of ceftiofur in milk

The sensitivity gains from on-column concentration, addition of propionic acid post-column and the Rf ion guide permitted the detection of ultrafiltrate samples down to about 10 ppb for ceftiofur. A five point calibration curve from 0–200 ppb for the $[M+H]^+$ of ceftiofur (*m/z* 524) was linear with a correlative coefficient of 0.993 ($n=19$) (Fig. 4). The ion chromatograms for the $[M+H]^+$ ion of ceftiofur at the 50 ppb level (ceftiofur tolerance in milk) and blank are shown in Fig. 5A and 5B. The relative standard deviation (R.S.D.) for replicate determinations of ceftiofur at the tolerance level (50 ppb) and limit of quantitation (LOQ) (25 ppb) were 5.4% and 9.1%, respectively ($n=4$). The milk background generated at *m/z* 524 was less than 15% of the response for the ceftiofur at the LOQ level of 25 ppb. The electrospray conditions developed were optimized to generate only $[M+H]^+$ ions therefore,

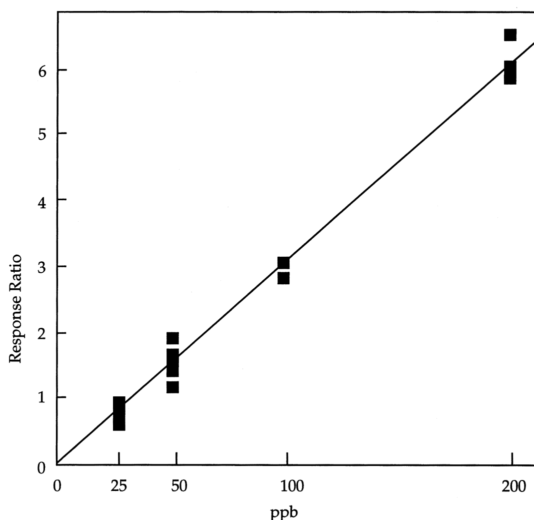


Fig. 4. Calibration curve for ceftiofur in milk ultrafiltrate monitoring the $[M+H]^+$ ion of ceftiofur ($m/z$ 524) for 0, 25, 50, 100 and 200 ppb spiked samples. The linear correlation coefficient was 0.993 ($n$=19).

other molecular-mass cephalosporins found in milk would not interfere with the analysis for ceftiofur. The method was tested on samples of bovine milk collected after dosing with ceftiofur to determine at what time the milk levels of ceftiofur were below FDA tolerance (50 ppb). The ion chromatogram for the determination of a milk sample collected 32 h after dosing with ceftiofur is shown in Fig. 5C. This sample was determined to contain 71 ppb of ceftiofur. The quantity of ceftiofur measured in other milk samples collected 32 h and 48 h after dosing with ceftiofur is shown in Table 1. The quantitative accuracy at the LOQ showed an error of less than 10% between the spiked value and measured value of ceftiofur in milk ($n$=6). Clearly it appears that milk collected at 32 h after dosing with ceftiofur was above the 50 ppb tolerance. Milk samples collected 48 h after dosing were measured to contain 24–31 ppb of ceftiofur. Therefore, a 48 h withdrawal period may be required to insure the levels of ceftiofur are below the tolerance for the drug in milk.

Table 1
Levels of ceftiofur in milk collected 32 and 48 h after dosing with ceftiofur

| Sample No.[a] | Quantity of ceftiofur in milk (ppb) |
|---|---|
| 1 | 71 |
| 2 | 27 |
| 3 | 54 |
| 4 | 67 |
| 5 | 24 |
| 6 | 31 |

[a] Samples 1, 3 and 4 were collected 32 h after dosing, samples 2, 5 and 6 were collected 48 h after dosing with ceftiofur.

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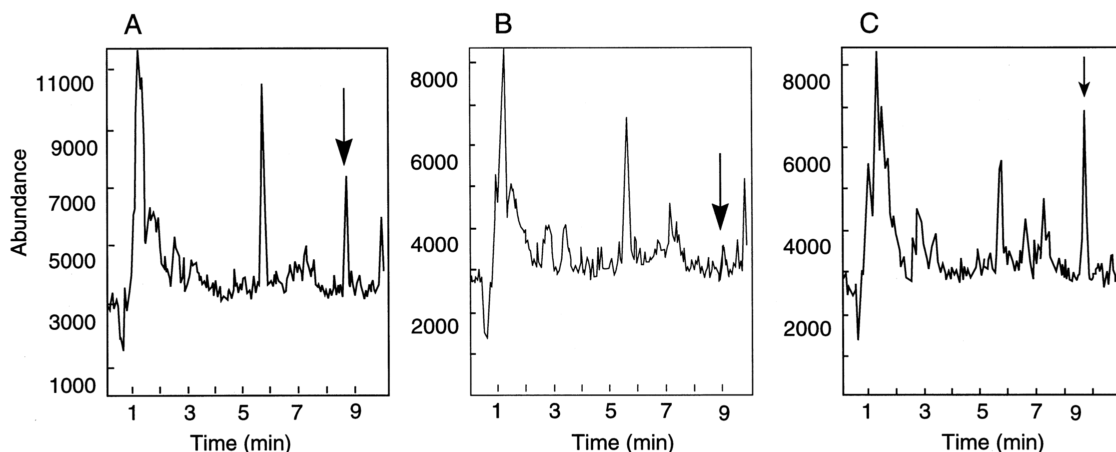


Fig. 5. LC–MS ion chromatograms for the $[M+H]^+$ ion of ceftiofur in milk ultrafiltrate. (A) 50 ppb spiked standard. (B) Blank milk. (C) Milk collected 32 h post-dosing with ceftiofur.

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