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Residual determination of Ceftiofur in Raw Bovine Milk by Liquid Chromatography-Electrospray Mass Spectrometry

Jong-hwan Lim, Byung-kwon Park, Myoung-seok Kim, Beom-Su Jang, Doo Kim[1] and Hyo-in Yun*

Division of Veterinary Pharmacology and Toxicology, Chungnam National University, Daejeon 305-764, Korea
[1]Department of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea
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Abstract: This report describes the determination of ceftiofur residues in milk from treatment of lactating dairy cattle by intramuscular injection of three consecutive daily doses of about 1 mg/kg BW, the recommended label dosing. The separation of ceftiofur was achieved on C_{18} reverse phase column. The mobile phase consisted of 0.1% trifluoracetic acid in water (A) and 0.05% acetic acid in acetonitrile (B) and grediently flowed at the flow rate of 0.4 mL/min. As a result of analysis of blank raw bovine milk samples, matrix interference was not shown. Limit of detection and limit of quantitaion was 0.5 ng/mL and 1 ng/mL, respectively. The values of precision and recovery satisfied the guideline of National Veterinary Research and Quarantine Service (NVRQS, Korea). The mean residual concentration of ceftiofur in milk did not exceed 3.71 ng/mL when ceftiofur was administered intramuscularly to lactating dairy cattle for 3 consecutive days at 1 mg/kg of BW per day. It is much lower than the proposed MRL (100 ng/mL) of ceftiofur in milk.

Key words : ceftiofur, raw bovine milk, LC/MS

Introduction

Ceftiofur has been approved by the Food and Drug Administration (FDA) for intramuscular injection only in cattle to treat bovine respiratory disease associated with *Pasteurella hemolytica*, *Pasteurella multocida* and *Haemophilus somnus* [1, 3].

Maximum residue limits (MRLs) have been established for ceftiofur in tissues and milk. In the European Union and United States, MRLs have been established for ceftiofur (100 ng/mL) in raw bovine milk [6]. Ceftiofur can readily be detected by microbiological methods [5, 11, 12, 15]. However, these methods generally lack selectivity and only produce qualitative or semiquantitative results [11, 15]. Chromatographic procedures have been described for determination of a single cephalosporin or simultaneous determination of two cephalosporins in biological materials [2, 10, 13]. Methods have been reported for determinations of ceftiofur and cephalosporin in milk [8, 10, 13, 14]. Based on the presence of 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 [8]. Many investigators have analyzed ceftiofur by positive-ion detection electrospray mass spectrometry [4, 8, 14]. In consideration of the safety tolerance limit of ceftiofur (50 ng/mL), the detection limits of these methods achieved only relatively high in the range of several hundreds ng/mL or close to the safety tolerance limit. Therefore, more sensitive method is required.

This report describes the magnitude and nature of ceftiofur residues in milk from treatment of lactating dairy cattle by intramuscular injection of three consecutive daily doses of about 1 mg/kg BW, the recommended label dosing and aims to develop the more rapid, simple and sensitive method for the purpose of determining ceftiofur in bovine raw milk by liquid chromatograph/ mass spectrometer (LC/MS) with electrospray (ES) interface.

*Corresponding author: Hyo-in Yun
Division of Veterinary Pharmacology and Toxicology, Chungnam National University, Daejeon 305-764, Korea
[Tel: +82-42-821-6759, Fax: +82-42-822-5780, E-mail: hiyun@cnu.ac.kr]

Materials and Methods

Chemicals

Ceftiofur and cepahlexin for standard were given by Deahan New Pharm (Seoul, Korea). HPLC grade water, methanol, acetonitrile, acetic acid and hexane were purchased from TEDIA (Ohio, USA). The test drug, Ex-cell Suspension® (ceftiofur sodium 5,000 mg, isopropyl myristate q.s. 1 L), was provided by Dae Han New Pharm (Seoul, Korea).

Animals

The study was a randomized, two-way treatment crossover design and was conducted in 8 healthy lactating dairy cows. Four animals were high milk producers in the initial milk producing stage, while the remaining 4 animals were poor milk producers in the late milk producing stage. Dairy cows were raised in Chulwon (Gangwon, Korea) and randomly allocated into two groups of four animals each. One group was treated with the ceftiofur sodium-sterile suspension (Ex-cel1 Suspension®, 50 mg/mL, Daehan New Pharm) and was intramuscularly administered at a dose rate of 1 mg as ceftiofur/kg BW with the dose interval of 24 hours for 3 consecutive days by the recommended label dosing. The other group was treated with the same method but different dose rate of 2 mg as cefiofur/kg BW by intramuscular injection. With the wash-out period of 2 weeks, the administration method was crossed-over. Milk was collected at 0.5, 12, 24, 36 and 48 hours after treatment. The collected samples were immediately kept at -70°C until analysis.

Instrumentation and chromatographic conditions

Samples were analyzed on a Hewlett-Packard 1100 series LC/MSD system. Separation was achieved on Watchers® 120-ODS-BP C_{18} reverse phase column (5 μm, 4.6 mm×150 mm I.D., Daiso, Japan). The mobile phase consisted of 0.1% trifluoracetic acid in water (A) and 0.05% acetic acid in acetonitrile (B). Gradient runs were programmed as follows: B increase from 0% to 100% B for 30 min, re-equilibration with 100% A for 5 min, until the next sample injection at 0.7 m*l*/min of flow rate.

The ES-MS analysis was performed on a Hewlett-Packard 5989 electrospray mass spectrometer with a Hewlett-Packard Atmospheric Pressure Ionization (API) interface fitted with a hexapole ion guide. The instrument was tuned and optimized for the transmission of the [M+H] ion of ceftiofur at m/z 524. The optimal condition for the analysis of ceftiofur employed pneumatic nebulization with nitrogen (45 p.s.i.) and a counterflow of nitrogen (9 L/min) heated to 350°C for the nebulization and desolvation of the introduced liquid. Mass spectrometer was performed using positive ion mode and selected ion monitoring (SIM), detecting m/z 524 with a dwell time of 300 ms.

Sample preparation

A simple and rapid liquid-liquid extraction procedure was employed for the determination of ceftiofur in bovine raw milk. This could be completed in less than 60 min. The procedures were as follows: (1) for the internal standard (I.S.), spike 1 m*l* of milk with 100 μ*l* of a 100 μg/mL of cephalexin (ca. 10 μg/mL milk); (2) to each add 4 mL of acetonitrile for deprotenization; (3) shake for 5 min and then centrifuge at 100 g for 5 min; (4) transfer the supernatants into other tubes and evaporate at 30°C under a stream of nitrogen; (5) reconstitute the residue with 1 mL of methanol and add to 4 mL of *n*-hexane; (6) shake for 5 min and then centrifuge at 100 g for 5 min; (7) transfer the lower phase into other tubes and evaporate at 30°C under a stream of nitrogen; (8) reconstitute the residue with methanol : water (50 : 50, v/v) and inject the samples of 10 μ*l* after filtration.

Treatment of data

Concentrations of ceftiofur in bovine serum were calculated from the standard curves constructed by plotting the area ratio of ceftiofur and cephalexin (internal standard, I.S.) against the working standard concentrations of ceftiofur (0.001, 0.01, 0.1, 0.5, and 1.0 μg/mL). Results are presented as mean ± standard deviation (S.D.). The recovery of ceftiofur was assessed in triplicate determinations in spiked milk. The responses from the spiked sample were compared with those from the blank milk sample and the precision was expressed as coefficient of variation (C.V.). Recovery and precision met certain criteria for the guideline of residual analysis of veterinary drugs by National Veterinary Research and Quarantine Service (NVRQS, Korea). Limit of detection (LOD) and limit of quantitation (LOQ) were based on the signal-to-noise ratio based on their areas. The signal-to-noise ratio of 3 was accepted for the LOD and that of 10 for the LOQ.

Results and Discussions

The highly sensitive and specific method for the determination of ceftiofur in the bovine raw milk by LC-MS has been established. The peak of ceftiofur was shown at about 15.2 min and increased in proportion to its concentrations (Fig. 1). The liner regression line for ceftiofur in the range of 1 ng/mL~1000 ng/mL showed high correlation coefficients (r) of 0.999. A calibration curve described by the equation $y = mx + b$, where y represents the response value of peak area ratio against internal standard, and x the concentration of ceftiofur, was $y = 0.6922x + 0.053$, revealing high linearity. The recovery and precision were shown in Table 1. The recovery rate ranged from 96~105% for 0.01. 0.1 and 0.5 μg/mL, which satisfies the allowance recovery ratio of 70~110% mentioned in the Guidance of Residue Study for Veterinary Drugs (NVRQS, Korea). The C.V. at 0.01 μg/mL, 0.1 μg/mL and 0.5 μg/mL ranged from 6.11~10.66% and the recovery of ceftiofur in bovine raw milk showed 105.10±6.42% for 0.01 μg/mL, 96.41±10.28% for 0.1 μg/mL and 97.93±7.51% for 0.5 μg/mL.

Several manufacturers have developed commercially available tests with the aim of detecting drug residues in milk such as; *Bacillus stearothermophilus* disk sssay, colorimetic bacterial inhibitor test, receptor bindig test and competitive fluorescent immunoassays [5, 11, 12, 15]. These methods are routinely used by the dairy industry to screen milk for antibiotic residues and presented the minimum sensitivity of ceftiofur higher than the 50 ng/mL [7]. Various analytical methods have been developed such as liquid chromatography, capillary electroporesis and mass spectrometry for the qunatitation of ceftiofur or other cephalosporins. The LOQ of this method is more sensitive than previously reported [4, 8, 9, 10, 13, 14].

The concentration of ceftiofur in bovine raw milk after the final administration of ceftiofur of 1 mg/kg and 2 mg/kg BW per day for 3 consecutive days was detected at 3.71 ng/mL and 9.94 ng/mL, respectively, which are much lower than the proposed MRL of ceftiofur in milk being 100 ng/mL (Table 2). Jaglan *et al.* showed that the total radioactivity in milk was

Table 1. Recovery, precision and accuracy

Added conc. (μg/mL)	Detected conc. (μg/mL)	Recovery (Mean±SD, %)	C.V. (Mean±SD, %)
0.5	0.49±0.04	97.93±7.51	7.67
0.1	0.01±0.01	96.41±10.28	10.66
0.01	0.01±0.00	105.10±6.42	6.11

*The recovery and precision of ceftiofur were assessed in triplicate determinations in spiked raw bovine milks.

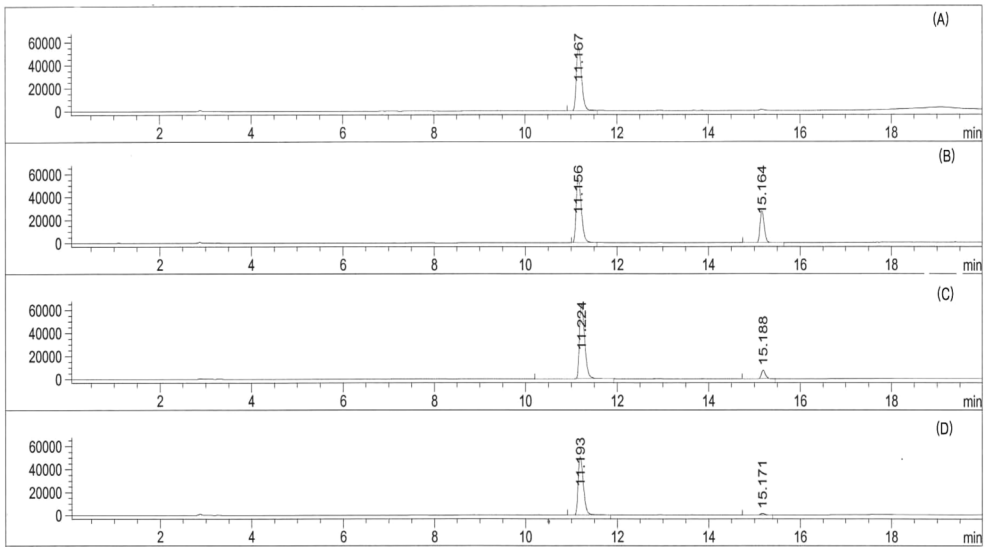

Fig. 1. Total ion chromatograph (TIC) for blank (A) and the 500, 100 and 10 ng/mL of ceftiofur spiked in bovine raw milk (B, C and D). The [M+H]+ ion of ceftiofur (m/z 523.8) as a selected ion monitoring mode. The peak of ceftiofur and cephalexine (internal standard) were shown at about 11.2 and 15.2 min, respectively.

Table 2. Concentration of ceftiofur in milk obtained at different time points after intra muscular administration of ceftiofur sodium (1 mg/kg BW or 2 mg/kg BW) for 3 consecutive days

Dose rate	Ceftioufur conc. (ng/mL) in milk					
	Pre	0.5h	12h	24h	36h	48h
1 mg/kg BW	–	3.71±1.94	4.06±2.19	–	–	–
2 mg/kg BW	–	9.94±6.26	6.43±5.69	1.55±0.63	–	–

– is under limt of quantitation.

highest at 12 h post-treatment (82.51±14.54 to 11.54 ±23.15 ng/mL) and declined to 59.81±13.12 ng/mL at 24 h after the last dose in a lactating cow following intramuscular injection of 1.1 mg of [^{14}C]ceftiofur/kg BW [7]. Ceftiofur undergoes rapid metabolism and degradation to form desfuroylceftioufur and furoic acid [2]. Confirmatory analysis of milk samples collected 12 h after the last dose by thermospray LC/MS showed that the total desfuroyl ceftiofur residues were about 90% the amount of the total residues determined by radioactive counting of the samples. In other words, the parent drug of ceftiofur in milk following intramuscular injection of 1.1 mg of ceftiofur/kg BW showed about 80 ng/mL at 12 h post-treatment [7]. This value is in agreement with the present study.

In conclusion, LC/MS is a simple, rapid and effective technique for the determination of ceftiofur in bovine raw milk. The ceftiofur residues in milk will not exceed 3.71 ng/mL total residues when ceftiofur is administered intramuscularly to lactating dairy cattle for 3 consecutive days at 1 mg/kg of BW per day. It is much lower than the proposed MRL of ceftiofur in milk.

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