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액체크로마토그래프/질량분석기를 이용한 소의 혈청 중 Ceftiofur의 분석

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Determination of Ceftiofur in Bovine Serum by Liquid Chromatography-Electrospray Mass Spectrometry

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Abstract: This study was aimed to develop a more rapid, simple and sensitive method to determine ceftiofur in bovine serum using LC/MS with electrospray interface. Separation was achieved on the Nova-Pak C_{18} reverse phase column. The mobile phase consisted of 0.1% acetic acid in water (A) and acetonitrile (B) and gradiently flowed at the rate of 0.4 mL/min. As a result of analysis of blank muscle samples, matrix interference was not shown. Limit of detection and limit of quantitaion was 5 ng/g and 20 ng/g, respectively. The values of precision and recovery satisfied the guideline of NVRQS. The precision and recovery developed in this method are suitable and sensitive to determine the concentration of ceftiofur in the bovine serum. These results could be applied for the confirmation and quantification in the biofluid.

Key words: ceftiofur, bovine serum, LC/MS

Introduction

Cephalosporins are an important class of antibacterial agents in use today for both humans and animals [1]. Ceftiofur, a third-generation cephalosporin, has been developed exclusively for veterinary use and has worldwide approvals for respiratory disease in swine, cattle, sheep, goats and horses [1, 2].

Ceftiofur is a rather unstable compound for pH, temperature and stability in aqueous solution, in comparison with other soluble antibiotics [19]. Therefore, rapid determination of ceftiofur is in need. Cephalosporins can readily be detected by microbiological methods and receptor tests which are commonly used for screening purposes [6, 16, 20]. However, these methods generally lack selectivity, hence producing only qualitative or semi-quantitative results. HPLC has been by far the most useful tool for the quantitaton of ceftiofur. Chromatographic procedures have been described for determination of single cephalosporin or simultaneous determination of several cephalosporins in biological materials [2, 3, 12, 14, 17].

More recently, liquid chromatograpy-mass spectrometry (LC/MS) has been proposed for the analysis of ceftiofur in

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milk, as well as for the simultaneous analysis β- lactam antibiotics [4, 7, 10, 18]. However, most of studies using LC/MS have been described for determination of ceftiofur or simultaneous determination of cephalosporins in milk. It is seldom reported to determine ceftiofur in biomatrices, such as tissue samples like serum or plasma. We aim to develop the more rapid, simple and sensitive method for the purpose of determining ceftiofur in bovine serum by LC/MS with electrospray interface

Materials and methods

Chemicals

Ceftiofur for standard was given by Deahan Newpharm (Hwasung, Korea). HPLC grade water, methanol, acetonitrile, acetic acid and hexane were purchased from TEDIA (Ohio, USA).

Instrumentation and chromatographic conditions

Samples were analyzed on a Hewlett-Packard 1100 series LC/MSD system. Separation was achieved on the Nova-Pak C_{18} reverse phase column (4 μm, 3.9 mm×150 mm I.D., Waters, USA). Flow rate was operated at 0.4 ml/min. The mobile phase consisted of 0.1% acetic acid in water (A) and acetonitrile (B). Gradient runs were programmed as follows: 30% A for 4 min, increase from 70% to 100% B in 8 min, 100% B for 2 min, re-equilibration with 30% A for 5 min, until the next sample injection. The nebulizing gas was flowed at 45 p.s.i. and 350℃. The quadrupole was heated to 100℃. The mass spectrometer was run in the positive mode and selective ion monitoring mode.

Sample preparation

Each 1 ml bovine serum sample was added to 5 ml of 0.1% acetic acid in methanol for deprotenization and then shaken for 10 min. The samples were centrifuged at 1,300 g for 10 min and the supernatants were transferred into other tubes. They were evaporated to dryness at 30℃ under a stream of nitrogen. The residue was reconstituted with 1 mL of methanol and an aliquot of 10 $\mu\ell$ was injected after filtration with syringe filter.

Treatment of data

Concentrations of ceftiofur in bovine serum were calculated from the standard curves constructed by plotting the area of ceftiofur against the working standard concentrations of ceftiofur (0.01, 0.1, 0.5, 1.0, and 5.0 μg/ml). Results are presented as mean ± standard deviation. The recovery of ceftiofur was assessed in triplicate determinations at spiked muscles. The responses from the spiked sample were compared with those from the blank serum 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 in National Vetrinary Research and Quarantine Service (NVRQS). Limit of detection and limit of quantitation were based on the signal-to-noise ratio based on their areas. The signal-to-noise ratio of 3 was accepted for the limit of detection and that of 10 for the limit of quantiation.

Results

Mass spectra of ceftiofur

The mass spectra of ceftiofur showed that $[M+H]^+$, m/z 524.0, was the predominant ion (Fig. 2). Each relative abundance of adduct ions, $[M+Na]^+$, m/z 546.0, was less than 5% of $[M+H]^+$.

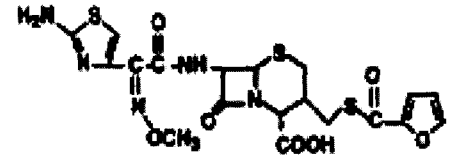

Fig. 1. Structure of ceftiofur.

Limit of quantitation and limit of detection

As a result of analysis of blank muscle samples, matrix interference was not shown (Fig. 3). The peak of ceftiofur was shown at about 14.2 min and increased in proportion to its concentrations. The linearity for ceftiofur showed high correlation coefficients (r) of 0.999. Limit of detection and limit of quantitaion was 5 ng/g and 20 ng/g, respectively.

Recovery and precision

The values of precision and recovery were satisfied with the guideline of NVRQS. The C.V. at 0.1 μg/ml, 1 μg/ml and 10 μg/ml ranged from 4.47% to 1.56% and the recovery of ceftiofur in bovine serum showed 94.2±4.21% for 0.1 μg/ml, 94.9±2.33% for 1 μg/mL and 96.6±1.51% for 10 μg/ml (Table 1).

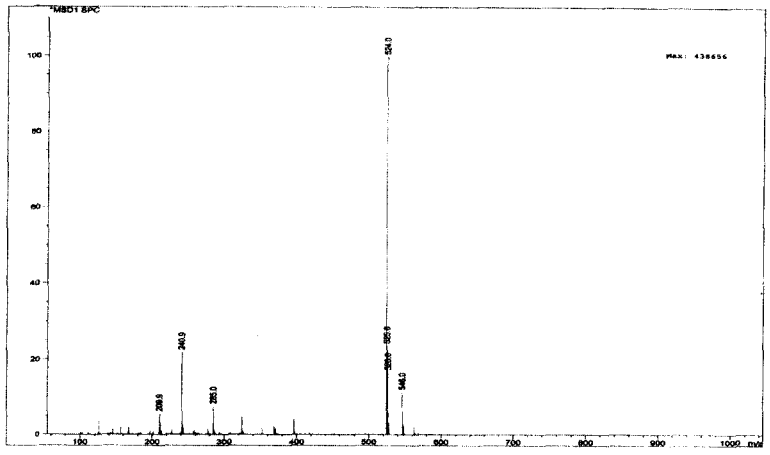

Fig. 2. Representative mass spectrum of ceftiofur.

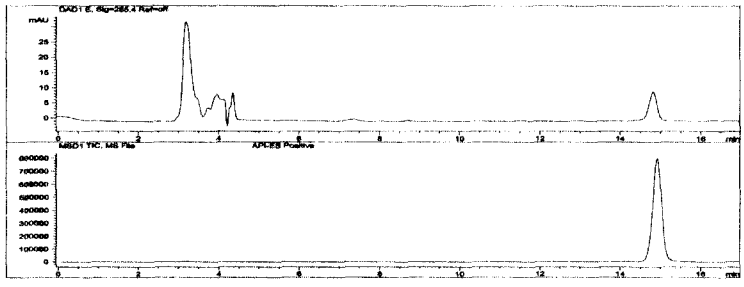

Fig. 3. UV chromatogram and total ion chromatograph (TIC) for the 1 μg/ml of ceftiofur spiked bovine serum. $[M+H]^+$ ion of ceftiofur (m/z, 524.0) as a selected ion monitoring mode.

Table 1. Recovery of ceftiofur from spiked bovine serum

Spiked concentration (μg/ml)	Detected concentration (μg/ml)	Recovery (%)	C.V. (%)
0.1	0.09±0.004	94.2±4.21	4.47
1	0.95±0.023	94.9±2.33	2.46
10	9.66±0.151	96.6±1.51	1.56

Discussion

The highly sensitive and specific method for the determination of ceftiofur in the bovine serum by LC-MS has been established. The extraction of ceftiofur in bovine serum in this study was carried out by the simple liquid-liquid extraction with methanol which was used for the precipitation of protein. The recovery of the ceftiofur from bovine serum was slightly dependent on the sample pH. In the present study, the highest recovery was obtained when extraction with methanol was performed at the acidic pH level of bovine serum. The recovery of ceftiofur may be influenced by its pKa or stability. Sunkara *et al* [19] reported that ceftiofur degradation accelerated greatly at neutral pH and alkaline pH as compared to acidic pH. Alternation of pH has a dramatic effect on the solubility of ionizable agents [19]. Generally, it is known the ionized compounds exhibit much higher solubility in aqueous solution than unionized compounds. Hong *et al* [8] reported that most agricultural penicillins exhibited the highest recovery in serum (70-90%) following extraction with a phosphate buffer (pH 7)-acetonitrile solution and centrifugation. Beconi-Barker *et al* went through a lengthy extraction process (extraction solution, dithioerythritol in borate buffer, followed by clean-up on C_{18}, anion- and cation-exchange SPE cartridges) and obtained recoveries of 70 to 85% in swine

muscle, liver and kidney [2]. Ceftiofur undergoes rapid metabolism and degradation to form desfuroylceftioufur and furoic acid [2, 3, 14, 19]. Therefore, determination of ceftiofur in biomatrices must be based on rapid and simple procedures. In the present study, recovery of ceftiofur in bovine serum (ranged from 96.6% to 94.2%) showed relatively higher values than previous studies, but also extraction procedure was simple rather than sophisticated.

The electric charge depends not only on the number of carboxyl and amino groups of the component, but also on the pH of the mobile phase because the dissociations of these groups are controlled by pH [5]. In a wide range of pH all investigated cephalosporin compounds have a negative net charge [5, 11, 13]. With an increase in the pH the solutes reach the end of column faster but the resolution becomes poor. The resolution would be improved by decreasing the pH, however the time of the retention will be markedly lengthened. In this study, the retention times of ceftiofur were 14.2 min. Taking into consideration the flow rate, these values were very short retention times as compared to other HPLC method [3, 12, 17].

Various analytical methods have been developed for the qunatitation of ceftiofur or other cephalosporins. Microbial methods [15,20], liquid chromatography [2, 3, 12, 14, 17], capillary electroporesis [5, 11, 13] and mass spectrometry have been used [4, 7, 10, 18], but some methods achieved only relatively high detection limits in the range of several hundreads ng/g or ng/ml [2, 3, 15, 17, 20]. In the present study, limit of quantitaion and limit of detection was 20 ng/g and 5 ng/g, respectively. These values satisfied the acceptance criteria of the limit of detection and limit of quantitation. The LOQ of this method is more sensitive than previously reported [2, 3, 5, 11, 12, 14, 15, 17, 20].

In conclusion, LC/MS is a simple, rapid and effective technique for the determination of ceftiofur in bovine serum. The precision and recovery developed in this method are suitable and sensitive to determine the concentration of ceftiofur in the bovine serum. These results could be applied for the purpose of the confirmation and quantification in the biofluid.

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