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Determination of peptide antibiotic in milk by high performance liquid chromatography tandem mass spectrometry

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Abstract: **High performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) was developed for the determination of bacitracin in milk. Acidified acetonitrile was used as the extraction solution and to remove the protein in sample. The sample was purified with PSA and C18. The chromatographic and mass spectrometric conditions were optimized, Electrospray ionization (ESI^+), multiple reaction detection (MRM) mode and external standard method were used for quantification. The results show: The target compound have a good linear in the range of 50~1000 μg/kg. The correlation coefficient is greater than 0.999. The limit of detection (LOD) was 15μg/kg and the limit of quantification (LOQ) was 50μg/kg. The average recoveries were 89.4~101.2% at different spiked levels, and the relative standard deviations were 5.4~7.1%. The pretreatment method is simple and rapidly, the developed method has the advantages of high sensitivity, good accuracy and precision, and can meet the confirmation and quantitative analysis of polypeptide antibiotic residues in milk.**

Keywords: HPLC-MS/MS; Peptide antibiotics; Milk

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1. Introduction

Peptide antibiotics are small peptide peptides that are used primarily to promote animal growth and treat animal diseases. At present, peptide antibiotics are widely used in animal feed additives and veterinary drugs [1]. Peptide antibiotics compared with the traditional antibiotics, have different targets and the role of time, so do not make pathogens resistant. However, due to peptide antibiotics with certain neurotoxicity and nephrotoxicity [2], it is possible through animal residues, and then through milk and other animal-derived food to humans, thus causing human health risks. Therefore, many countries have developed a limited standard. Among them, the EU on the milk bacitracin and polymyxin E limit of 100 and 50μg/kg, Japan on milk bacitracin and polymyxin E limit of 400 and 10 μg/kg, and China on milk The limits of bacitracin and polymyxin E were 500 and 50μg/kg, respectively. Bacitracin was against Gram-positive bacteria and polymyxin E was against Gram-negative bacteria. At present, the use of QuEChERS pretreatment method for the detection of peptide antibiotics in milk samples has not been reported.

At present, the detection methods of peptide antibiotics are mainly microbial method, enzyme-linked immunosorbent assay [3], high performance liquid chromatography [4], capillary electrophoresis [5] and high liquid chromatography-tandem mass spectrometry [6-10]. Among them, the microbial method is cumbersome, enzyme-linked immunoassay ELISA antibody vegetation is difficult, prone to false positive and false negative phenomenon, the reproducibility of capillary electrophoresis is poor, the method of high performance liquid chromatography need to be derived, and these methods has relatively low sensitivity, it is difficult to meet the current detection needs. The HPLC-MS/MS has the characteristics of high selectivity and high sensitivity, but most of them have been reported by solid-phase extraction, and the steps are cumbersome and time-consuming, and the matrix effect is seriously.

In this study, the modified pretreatment method was used to treat the milk samples. The method was rapid and simple. The LC-MS/MS method for the simultaneous detection of two kinds of peptide antibiotics was highly sensitive and accurate. It could be used for the accurate determination of two antibiotics in milk.

2. Materials and method

2.1. Instruments and reagents

1260 high performance liquid chromatography (Agilent, USA); API5500 triple quadrupole mass spectrometer (AB, USA) with electrospray ionization source (ESI); Milli-Q ultra pure water system (Millipore, USA); vortex KY512 nitrogen blown dryers (Jintan Shenglan Instrument Manufacturing Co., Ltd.);

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KQ-500DE CNC ultrasonic cleaning device (Kunshan ultrasound Instrument Co., Ltd.).

The standard of Bacitracin (78%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Methanol, acetonitrilen and all HPLC grade reagents were obtained from Sigma-Aldrich (St. Quentin Fallavier, France), Formic acid (HPLC grade) was purchased from Baillingwei Company (Beijing, China), Ultra pure water produced by Milli-Q water-purification system (Millipore, Brussels, Belgium) was used for the mobile phase and the preparation of standard solutions. PSA and C18 purifying agent was purchased from Agela Technologies. The milk samples were purchased in the local supermarket.

2.2. Preparation of standard solution

Individual stock standard solutions of bacitracin were prepared respectively by precisely weighing appropriate amount standard (weigh the quality of the standard is the modified quality by purity) into a 10 ml volumetric flask and dissolved with 0.1% formic acid then diluted to volume with 0.1% formic acid in ultra purified water (1000 mg $\cdot L^{-1}$).

The dissolved solution of standards was consisted of 0.1% formic acid and MeOH (4:1, v/v). The extraction solution was prepared by 20% acetic acid and MeOH (2:3, v/v). All standard solutions were stored in dark amber bottle and kept in -20 ℃ where they can stable for half a year. If necessary, dilute the standard working solution with the mixed calibration solution to the required concentration.

2.3. Sample pretreatment

2.0 g (accurate to 0.01 g) milk sample was precisely weighed in 50 mL stoppered centrifuge tube, 20 mL 1% acetic acid-acetonitrile (40:60, v/v) was added, mix for 1 minute, then add 1 g NaCl and 2 g anhydrous magnesium sulfate, the mixture was vortexed for 1 min, horizontal oscillation extraction 15 min, 5000 r/min centrifugation 5 min, the supernatant (10 mL) was transferred to another centrifuge tube include 1 g anhydrous magnesium sulfate, 100 mg PSA and 150 mg C18, vortex mixing and horizontal oscillation, 5000 r/min centrifugation for 10 minutes, then 2 mL supernatant was transferred to 10 mL glass tube, nitrogen concentrated to near dry, add 1 mL 0.1% formic acid water - acetonitrile (4:1, v/v) dissolved, the sample extract was vortexed 30s to mixed, and filtered through 0.22 μm nylon membrane for LC-MS/MS detection.

2.4. LC-MS/MS condition

The analysis was performed by using HPLC series 1260 (Agilent Technologies, USA) integrated system consisting of a binary pump, a degasser, and auto-sampler. Chromatographic separation was performed on a 4.6×150 mm， 5 μm ZORBAX SB-C18 column (Agilent Technologies, USA).

The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile. The flow rate was 1.0 ml/min and the injection volume was 5 μL. The column temperature was 30 °C and the following gradient elution program: 0-4 min, 90% A and 10% B; 4-6 min, 50% A and 50% B; 6-13 min, 10% A and 90% B; 13-14 min, 80% A and 20% B; 14-15 min, 90% A and 10% B. The detector was API 5500 (Applied Biosystems) triple quadrupole mass spectrometer with ESI source. The analyst 1.6.1 software was used to monitor the experiment and data management. The follow mass spectrometer conditions were set: the collision gas was nitrogen, ion source gas 1 (GS1) and ion source gas 2 (GS2) was 55psi and 50 psi, respectively; Ion spray voltage (IS) 5500 V; ion source temperature 550 ℃ and curtain gas 0.2 Mpa. All qualitative and quantitative data in this work were acquired in multiple reaction monitoring (MRM) mode. The MRM transitions of all analytes and other parameters are showed in Table 1.

Table 1 Mass spectrometer parameters of the analytes

Analytes	Precursor ion (m/z)	Product ions (m/z)	CE/eV	DP/V	CXP/V
Bacitracin	712.2	199.2*	45	110	20
		227.3	40	110	20

*: Quantitative ion.

3. Results and discussion

3.1. Optimization of chromatographic conditions

In this study, the effects of different mobile phase systems on the antibiotic analysis of three peptides were investigated. The results showed that the responses of the three kinds of peptide antibiotics in the 0.1% formic acid-acetonitrile system were the highest, and the peak type was the best in the 0.1% formic acid-methanol, 0.1% formic acid-methanol, water-acetonitrile and 0.1% formic acid-acetonitrile. Since the addition of formic acid to the

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mobile phase can provide a source of protons, which facilitates the ionization of the target and the formation of positive ions, thereby increasing the ionization efficiency. In addition, because the peptide antibiotics itself is not stable, the temperature of its impact is relatively large. So the choice of column temperature is also very important, the column temperature is too high may be degraded, the column temperature is too low will lead to increased mobility of the mobile phase, reducing the theoretical column number of the column, thus affecting the separation effect of the target on the column. So the experimental column temperature is selected as 30 ℃.

3.2. Optimization of mass spectrometric conditions

Bacitracin belongs to the peptide antibiotics. Polypeptide Antibiotics usually carry multiple charges, which may have two charges, three charges, or more charges after being ionized. According to the structural characteristics of peptide antibiotic and the previous literature, this experiment uses ESI positive ion mode as ionization mode. The parent ion and the characteristic ion were determined by direct mass spectrometry with 1 mg/L standard solution and the mass spectral parameters were optimized.

The molecular weight of the bacitracin was greater than 1000, after the first-order mass spectrometry, $(M+2H)^{2+}$) m/z 712.2 as the precursor ion of bacitracin. And the parent ions enter the secondary mass spectrum after the break or rearrangement and then produce different debris ions. The precursor ion of bacitracin (m/z 712.2) produce greater abundance of m/z 199.2 and 227.3 ion fragments, where m/z 199.2 abundance is greatest and used as a quantitative ion. The optimized collision energy and other mass parameters of bacitracin were shown in Table 1. Figure 1 shows the MRM chromatograms of bacitracin in spiked milk samples.

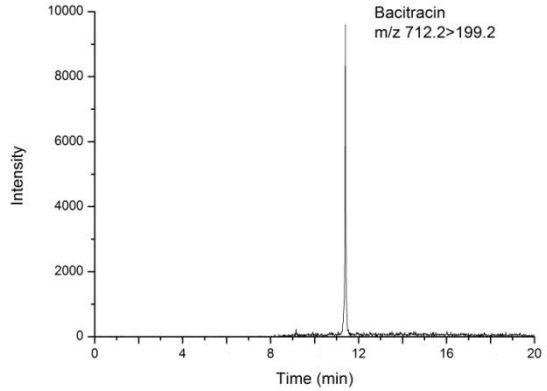

Figure 1. MRM chromatogram of target compound of spiked milk sample (100 μg/kg).

3.3. Optimization of sample preparation

Traditional pretreatment methods commonly used acetonitrile or acidified acetonitrile as the extraction solvent, mainly because of the acetonitrile precipitation protein and extraction ability is relatively strong. In this experiment, 1% acetic acid-acetonitrile was used as the extraction solvent, and the extraction efficiency of 1% acetic acid-acetonitrile (5: 5, 4: 6, 3: 7) in different volume ratio was selected because of the stability of the peptide antibiotics under acidic conditions. The results showed that the best recoveries of V (1% acetic acid): V (acetonitrile) = 40: 60 were the best, and the highest recovery rate (92%) could be obtained by three kinds of peptide antibiotics. 1% acetic acid acetonitrile (40: 60, v/v) was chosen as extraction solvent.

Anhydrous magnesium sulfate as a water-removing agent has a strong water absorption capacity, PSA is mainly used for adsorption of sugars, fatty acids and other organic acids, pigments and polar impurities, C18 is mainly used for adsorption of non-polar substances, and grease removal effect is better, taking the use of anhydrous magnesium sulfate into account, PSA (PSA preferred to absorb more polar lactose and free fatty acids in milk) and C18 as a mixed-type dispersed solid-phase purification sample, in order to prevent PSA excess may reduce the recovery of the target. And the maximum amount of lipid was removed to optimize the amount of them, and the amount of them was determined as follows: 1 g anhydrous magnesium sulfate, 100 mg PSA and 150 mg C18.

3.4. Method validation

3.4.1.Standard curve, detection limit and quantitation limit

In order to reduce the effect of matrix effect, we used blank matrix method to establish matrix matching standard curve for external standard method to quantitation. 5~1000 μg/L concentration range of standard working solution was prepared, the optimized chromatography and mass spectrometry conditions for determination. The peak area of the target is the ordinate (Y), and the corresponding concentration is the abscissa (X), which is used to plot the standard curve. The signal-to-noise ratio (S/N) is 3 as the detection limit and the signal-to-noise ratio (S/N) is 10, and the results are shown in Table 2.

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Table 2 Regression equation, linear range, correlation coefficient, LOD and LOQ of target compound

Target	Standard curve	Linear range (μg/kg)	R^2	LOD (μg/kg)	LOQ (μg/kg)
Bacitracin	Y=3485X+6439	50~1000	0.9996	15	50

3.4.2. Method recovery and precision

The experiment was carried out on the blank sample by sample pretreatment method. The parallel experiments were carried out for each of the five levels. The analysis was carried out according to 2.4. The recoveries and precision results are shown in Table 3. It can be seen from the table that the average recoveries of the method are 81.2~93.6% and the relative standard deviation is 3.6~8.9% at three addition levels.

Table 3 Average recoveries and RSD of the target compound (n= 5)

Target	Add level (μg/kg)	Average recoveries/%	RSD/%
Bacitracin	10	89.4	5.4
	50	95.3	6.2
	200	101.2	7.1

3.5. Real sample detection

The developed determination method has been employed to a series of milk which was purchased from local large supermarkets. The sample matrix was extracted and analyzed though above mentioned processing procedure. Besides, the procedural blank was analyzed in the same process. The results of the detection demonstrated four target analytes not detected in any of samples matrix. Figure 1 shows the chromatograms of a spiked blank of formula infant milk powder sample.

4. Conclusion

A simple and accurate method of simultaneous determination of bacitracin in milk with simple sample preparation process was established. The sample was purified with PSA and C18. The chromatographic and mass spectrometric conditions were optimized, Electrospray ionization, multiple reaction detection (MRM) mode and external standard method were used for quantification. The limit of detection (LOD) and the limit of quantification (LOQ) of target compound were 15μg/kg and 50μg/kg. The average recoveries were 89.4~101.2%. The method was successfully applied to the real samples. The method validation indicating that the developed method is robust and reliable for simultaneous determination bacitracin in milk.

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