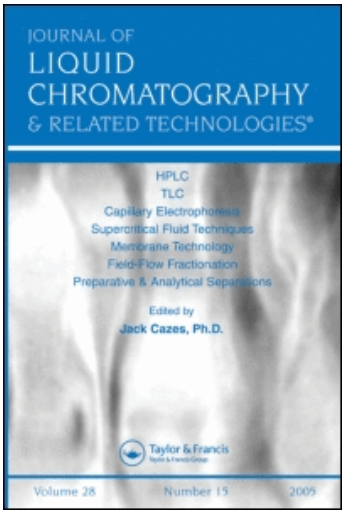

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Abstract: A fast and simple method for the quantitative determination of bicozamycin (BCM) in milk samples by hydrophilic interaction liquid chromatography with electrospray tandem mass spectrometry (HILIC-ESI-MS/MS) and centrifugal ultrafiltration (CUF) was presented. The milk sample was extracted with an acetonitrile/water (4/1, v/v) and CUF procedure. After preparation, the sample solution was directly injected onto the HILIC-MS/MS. Chromatographic separation of the components was performed on a TSK-GEL NH_2 column using 50 mM ammonium acetate in water (pH 4.0 adjusted with acetic acid) and acetonitrile. The mass spectrometer was operated in the negative ESI-MS/MS mode (m/z $301 \rightarrow 209$). The LOD and LOQ were 2.5 ng/mL (25 pg) and 5 ng/mL, respectively. This method was validated according to the Japanese maximum residue limit (0.1 μg/g) of BCM. The matrix matched calibration of BCM was linear over the calibration range from 50 to 500 ng/g ($r^2 = 0.999$). Recovery values were from 82.6 to 109.9% (RSD 7.5%, $n = 27$). The present method could be applied to measure BCM in milk samples.

Keywords: Bicozamycin, Centrifugal ultrafiltration, Hydrophilic interaction liquid chromatography tandem mass spectrometry, Milk

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INTRODUCTION

Bicozamycin (BCM: Figure 1) is an antibacterial agent for animals and fishes that is produced from the fermentation harvest of *Streptomyces* sapporonensis.[1] BCM has been used for the treatment of nonspecific diarrhea in animals.[2,3] Biological studies have suggested that BCM exerts its activity by binding at the interface of the adjacent C-terminal domains of Rho.[4–7] BCM is the only natural product inhibitor of the transcription termination factor Rho. Therefore, the evidence has been worth noting the BCM structure activity studies, site directed mutagenesis investigations, affinity labels of BCM, and biochemical and biophysical measurements of the BCM-Rho complex.[8,9] On the other hand, the potential monitoring of BCM residues in foods is not well developed, and even less known are the residue levels in foods such as milk samples.

The chromatographic methods for analysis of BCM in yellowtail tissues have been described using high performance liquid chromatography (HPLC).[10] This reported method could not be enough for the required validation in accordance with the Japanese maximum residue limit (MRL) in Positive List Decision for Agricultural Chemical Residues.[11] The Japanese Ministry of Health, Labour, and Welfare has set the MRLs for BCM at 0.2–0.05 ppm in animal tissues and 0.1 ppm in milk samples.[11] To the best of our knowledge there are no reports of selective and sensitive analytical methods for determination of BCM in food samples. There is a growing interest to apply liquid chromatography tandem mass spectrometry (LC-MS/MS) in control of various veterinary drug residues from milk to ensure food safety.[12] Currently, milk in Japan is monitored for the presence of veterinary drug residues by a cooperative agreement between the governments and manufacturers for the new required validation of MRLs.[11] Many researchers have tried to develop the multi-residue screening and

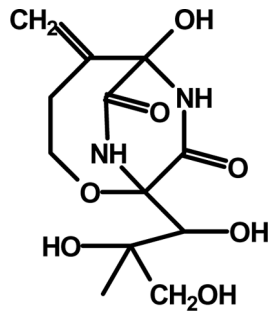

Figure 1. Structural formula of bicozamycin (BCM).

confirmation methods for the analysis of veterinary drugs in milk samples.[13] However, it is hard to develop the simultaneous analysis of BCM and other veterinary drugs because of the very different polarity of BCM. Therefore, we suggest that an individual and specific analytical method for the determination of BCM in milk samples will be needed. So, the separation with a C_{18} based column was limited because of the inherently poor interaction of the highly polar BCM. Recently, hydrophilic interaction chromatography (HILIC) is widely used for the separation of highly polar substances including biologically active compounds such as pharmaceutical drugs, neurotransmitters, nucleosides, nucleotides, and amino acids.[14] HILIC on silica columns with low aqueous/high organic mobile phase was emerging as a valuable supplement to the reversed phase LC-MS/MS.[15] Moreover, it is hard to make the cleanup procedures of the highly polar BCM from milk samples using the liquid–liquid and solid phase extractions. Thus, the novel and easy centrifugal ultrafiltration (CUF) was applied to the cleanup procedure of BCM from milk samples because we reported that the CUF procedure was easy to use and had a shorter operation time, resulting in good recovery and reproducibility of highly polar nucleotides in infant formula.[16] The objective of this study was to develop a quantitative LC/MS/MS and CUF method that would be highly sensitive and selective for the regulatory analysis of residues of BCM in milk samples.

EXPERIMENTAL

Reagents, Samples and Standard Solution

The structural formulas of the compounds studied are shown in Figure 1. Bicozamycin (BCM: purity 91.7%) was obtained from Fujisawa Pharmaceutical Ind., Ltd (Tokyo, Japan). BCM stock solution (1.0 mg/mL) was prepared by dissolving the appropriate amount of standard in methanol. Pure standard solutions were prepared by diluting an aliquot of the stock solution in water/methanol (50/50, v/v). HPLC grade water, methanol, acetonitrile, acetic acid, and ammonium acetate were obtained from Wako Chemical Co., Inc. (Osaka, Japan). Purified water was obtained from a Milli-Q purifying system (Millipore, Bedford, MA. USA). Milk samples were obtained from a local store in Nagoya, Japan.

HILIC-MS/MS Analysis

LC analyses were performed using a Waters Ailiance 2695 system (Waters, Milford, MA, USA). LC separation was performed using a

TSK-GEL Amide-80 (2.0 × 150 mm, 3 μm: Tosoh Co., Tokyo, Japan) maintained at 40°C. The mobile phase consisted of 50 mM ammonium acetate in water (Solvent A: pH 4.0 adjusted with acetic acid) and acetonitrile (Solvent B). The LC gradient was as follows: 98% Solvent B at 0 min, 50% Solvent B at 15 min, 2% Solvent B at 15.1 min, 2% Solvent B at 20 min, and 98% Solvent B at 20.1 min with a flow rate of 0.2 mL/min. The injection volume was 10 μL. The mass spectrometer (a Waters Micromass Quattro Premier triple quadrupole mass spectrometer) was operated with an ESI source in the negative ionization mode. The ionization source conditions were as follows: cone voltage of 20 V, collision energy of 15 eV, capillary voltage of 2.8 kV, extractor voltage of 4 V, RF lens voltage of 0 V, source temperature of 120°C, and desolvation temperature of 400°C. The cone and desolvation gas flows were 50 L/hr and 900 L/hr, respectively, and were obtained using a nitrogen source (N_2 Supplier Model 24S, Anest Iwata Co., Yokohama, Japan). We used argon as the collision gas and regulated it at 0.35 mL/hr, setting the multipliers to 650 V.

CUF Preparation of Milk Samples

Two grams of a milk sample was added to 10 mL of acetonitrile/water (4/1, v/v). The mixture was vortex mixed for 1 min (IKA MS3 basic, Wilmington, NC, USA), stored at 4°C for 10 min, and then centrifuged at 6000 rpm for 15 min. The pellet was then blended and centrifuged two times with 10 mL of extracted solution, respectively. This solution was evaporated to dryness at 30°C. Then, the samples were adjusted by 2 mL of water/methanol (50/50, v/v), and then centrifuged at 10,000 rpm for 5 min. The supernatants obtained were applied in a centrifugal ultrafiltration (CUF) step using Amicon Ultra-15 (Ultracel-10K, regenerated cellulose 10,000 M.W., Milliore Co. Ltd., Billerica, MA, USA). The CUF was centrifuged at 6000 rpm for 15 min and measured by LC-MS/MS.

Analytical Validations

Matrix Effects and Matrix Matched Calibration

Based on the approaches of Matuszewski, the matrix effects were evaluated by comparing the MS/MS responses of the standard and the test solution.[17,18] The 0.1 μg/g of samples were used for validating the standard dilution and the absence of the matrix effect of the sample preparation method. The validation data obtained in the above manner

enabled the determination of the matrix effect value (MEV) for the extraction procedure by comparing the absolute peak areas for BCM obtained in standard solution and the spiked solution after CUF preparation. By calculating for the peak areas obtained using standard solution, and the corresponding peak areas for the standards spiked after CUF preparation, MEV was calculated as follows: MEV (%) = (peak area of standard/peak area of spiked solution after CUF) × 100. In addition, matrix matching calibration of BCM was conducted using the sample originating from six different concentrations (50–500 ng/g) and spiked after CUF preparation.

Precision, Quantitation, Limits of Detection (LOD), and Limit of Quantity (LOQ)

BCM in milk samples were quantified by a six-point matrix matching calibration curve (50–500 ng/g). These calibration curves were prepared daily by prepared matrix matching solutions. The area of each BCM was plotted against analyte concentration. Precision (intra- and inter-day) was calculated from the analysis of BCM standard and recovery tests by the same operator. Precision within the laboratory was tested using the standard solution (100 ng/mL, n = 6) of retention time and peak area. Inter-day reproducibility was performed for nine days using the recovery tests of different milk samples (n = 3). The limits of detection (LOD) and quantity (LOQ) were calculated from the signal to noise ratio (S/N) using MassLynx V. 4.0 software. The S/N values for LOD and LOQ were three and ten times, respectively, using the standard solution of the averages within the laboratory.

RESULTS AND DISCUSSION

HILIC-MS/MS Analysis of BCM

We investigated the ionization modes of BCM by infusion system. The mass spectra of BCM in both the positive of $[M+Na]^+$ (m/z 324.8) and negative of $[M-H]^-$ (m/z 301.1) ions were detected. From early experiments, it was clear that a positive ionization might give greater sensitivity than a negative mode. However, it seemed possible that the $[M+Na]^+$ adduct ion might be unstable, and the $[M-H]^-$ ion is amenable. Therefore, the precursor ion was selected to the corresponding deprotonated ion $[M-H]^-$ (m/z 301.1). Figure 2a shows the influence of con voltages on the response of m/z 301.1. In this result, the 20 V was selected for optimal cone voltage of m/z 301.1. Then, two product ions were observed by MS/MS spectrum (Figure 3). In addition, these

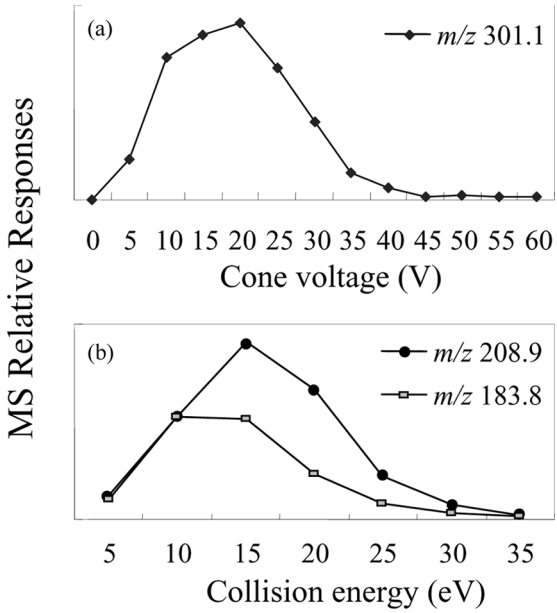

Figure 2. Investigation of optimal MS/MS conditions such as cone voltage and collision energy. (a) Investigation of cone voltages for BCM. (b) Investigation of collision energy for BCM of precursor ion (m/z 301).

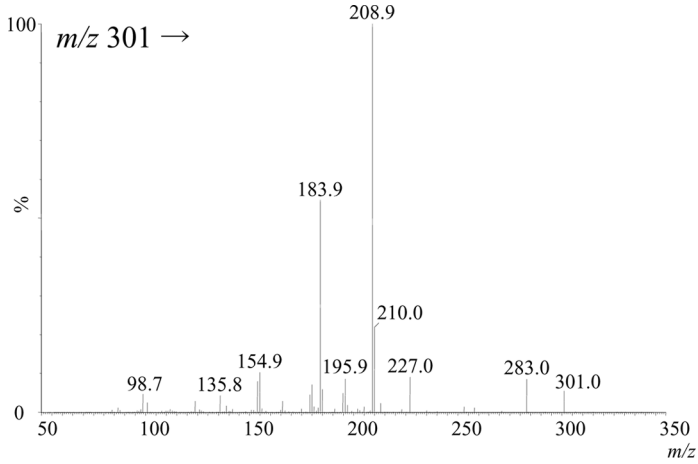

Figure 3. MS/MS spectrum of BCM. Ionization conditions as follows: ESI-positive mode, cone voltage of 20 V, collision energy of 15 eV, capillary voltage of 2.8 kV, extractor voltage of 4 V, RF lens voltage of 0 V, source temperature of 120°C and desolvation temperature of 400°C.

m/z 301 → 138.8 and 208.9 responses were investigated by the changing collision energies (Figure 2b). In these results, the m/z 208.9 was the highest intensity than other ions in collision energy of 15 eV. The major product ion of BCM at m/z 301 → 209 in multiple reaction monitoring (MRM) mode would be useful in monitoring BCM in samples.

Recently, HILIC separation for determination of highly polar substances has been reported. HILIC separation of BCM was investigated using a mobile phase comprising 50 mM ammonium acetate at pH range from pH 3.0 to 6.0 with acetic acid. When we tried to measure the BCM standard, only the mobile phase condition at pH 4.0 showed good peak shape. Therefore, we decided to measure BCM at MRM in HILIC with the mobile phase adjusted to pH 4.0, and to measure milk samples. To achieve successful HILIC-MS/MS analysis of the BCM, we investigated the precision of intra-day (retention time and peak area), LOD and LOQ of BCM. The repeatability test of retention time was that the RSDs (n = 6, intra-day) of the BCM standard (100 ng/mL) was 0.37%. The RSD of peak area was 2.95% (n = 6). The chromatogram of BCM standard (100 ng/mL) is shown in Figure 4a. The calibration graphs obtained for the BCM standards were linear over the calibration range (5–500 ng/mL) and showed good correlation values ($r^2 = 0.999$). The calculated LOD and LOQ were 2.5 ng/mL (25 pg) and 5 ng/mL, and the S/N was three and ten times, respectively. This method could be used to evaluate BCM in milk samples.

Centrifugal Ultrafiltration of BCM in Milk Samples

The sample preparation is necessary to eliminate the various matrixes from Biological samples. The liquid–liquid and solid phase extractions were reported for determination of various veterinary drugs from bovine milk. However, there is no useful sample technique for routine quantitation of highly polar BCM in milk samples. Thus, novel and easy centrifugal ultrafiltration (CUF) was applied to the cleanup procedure of BCM from milk samples. Using acetonitrile/water (4/1, v/v),[10] the extraction of BCM in milk was achieved following the CUF procedure. As a result using matrix matched calibration of BCM, the values of recovery (0.1 μg/g) in various milk samples was shown in Table 1. Average of total recovery from all samples was 97.0% and the relative standard deviation (RSD) was 7.5% (n = 27). The MRM chromatogram of BCM in the recovery test was shown in Figure 4b. This CUF preparation was found to be highly selective and recovery of BCM in milk samples.

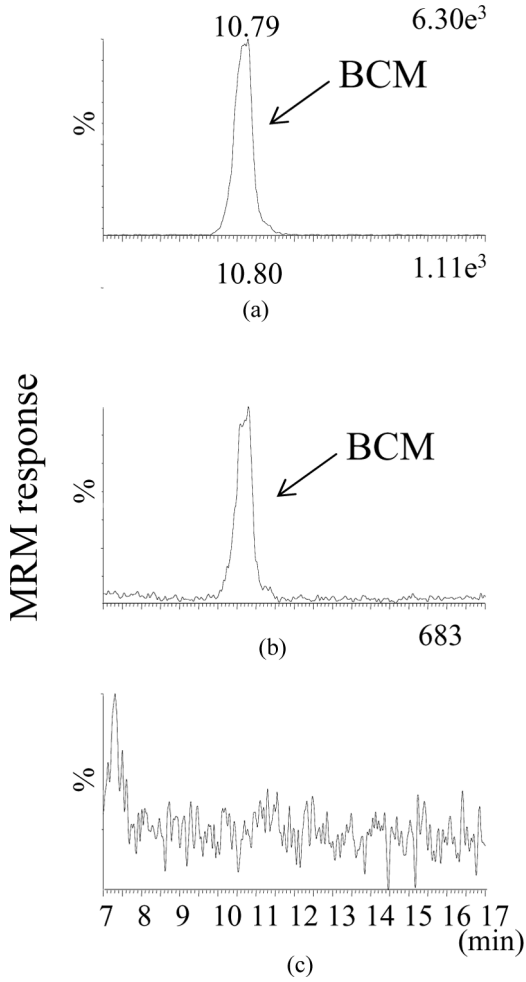

Figure 4. MRM chromatograms for monitoring BCM recovery test and application of commonly milk samples. (a) Standard solution of 100 ng/mL. (b) Recovery test of BCM (0.1 μg/g) in milk sample. (c) Monitoring BCM in the commercially available milk.

Validation and Matrix Effects of Analytical Method

The matrix effect of BCM in extracted milk was evaluated. This purpose was to examine whether the matrix matched calibration is useful for the analysis of BCM in milk sample by LC-MS/MS.[19] We evaluated the matrix effect of BCM subjected to CUF for milk samples. The response obtained of BCM on the spiked extract of samples (0.1 μg/g) was

Table 1. Sample. Recovery tests of BCM in milk samples for inter-day reproducibility (9 day from different milk samples, $n = 3 \times 9$ day)

	1	2	3	4	5	6	7	8	9
1	88.7	92.1	97.9	101.3	90.9	91.4	100.5	101.6	101.0
2	109.9	84.1	98.7	92.4	97.5	92.3	100.9	108.8	100.8
3	95.4	88.8	100.7	90.7	82.6	91.5	105.3	106.9	105.2
Average	98.0	88.3	99.1	94.8	90.3	91.7	102.2	105.8	102.3
SD	10.8	4.1	1.4	5.7	7.4	0.5	2.7	3.7	2.5

compared with the response of the same analyte prepared in water/methanol solution (50/50, v/v). When ionization suppression occurs, the MEV (%) of analyte in spiked matrix was lower than 100%. The MEV of BCM in milk was $18.4 \pm 1.4\%$ ($n = 3$). Kang et al. indicated that matrix effects were assessed using the post-extraction addition method such as matrix matched calibration.[19] Using matrix matching calibration, the recovery values of BCM from milk samples ranged from 82.6 to 109.9%. This postextraction addition method is considered to decrease abundance of target compounds in MS/MS detection. Thus, special attention must be paid to the possible determination levels of BCM from milk samples according to MRL (0.1 μg/g). Based on calibration range and linearity curves, we decided that the matrix matched calibration of BCM should be used for milk samples because LC-MS/MS response can detect BCM based on lower MRL (50 ng/g), based on very simple CUF preparation. The matrix matched calibration graphs obtained for the BCM in milk were linear over the calibration range (50–500 ng/g) and showed good correlation values (Figure 5). Moreover, inter-day

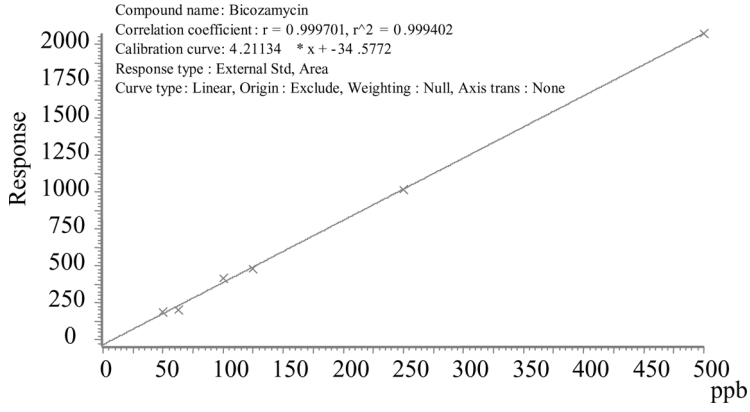

Figure 5. Matrix matching calibration of BCM in milk sample (50–500 ng/g).

reproducibility (9 day from different milk samples) was shown Table 1. It is proven that this postextraction addition and high sensitive LC-MS/MS could assess ion suppression and detect MRL of NVB in milk samples.

Application of Measurement of BCM in Commercially Available Milk Samples

This developed method was applied to monitoring BCM in commercially available milk ($n = 10$) in Japan. The BCM in all samples was not detected by this analytical method with the LC-MS/MS and CUF method. This chromatogram is shown in Figure 4c.

CONCLUSIONS

A simple, sensitive, and specific HILIC-MS/MS method was developed for the quantitative determination of BCM in milk at levels of MRL (0.1 μg/g). This reliability of the LC-MS/MS and CUF procedure was more useful than other chromatographic methods, and fulfilled the Japanese validation criteria for the performance of analytical methods and interpretation of results.

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