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Short communication

LC-MS/MS and centrifugal ultrafiltration method for the determination of novobiocin in chicken, fish tissues, milk and human serum

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ABSTRACT

We present a rapid and simple method for detecting novobiocin in biologic samples using a methanol-based extraction of the tissue matrix and liquid chromatography with electrospray tandem mass spectrometry (LC-ESI-MS/MS) on positive mode. The sample, prepared using centrifugal ultrafiltration with 5.0% SDS, was directly injected into the LC-MS/MS. Chromatographic separation was performed on a TSK-GEL ODS 100 V column using 0.5% formic acid in water/methanol. The method was validated according to the Japanese Maximum Residue Limits recommendations. Detection was linear over a range of 5–100 ppb matrix solution ($r > 0.998$). Novobiocin recovery values from chicken (0.05 ppm) and fish tissues (0.05 ppm), milk (0.08 ppm), and human serum (0.05 and 0.01 ppm) samples ranged from 71 ± 1 to 95 ± 2%.

1. Introduction

Novobiocin (NOV) is a coumarin-containing antibiotic. NOV is an acidic substance comprising a central coumarin nucleus, a benzene residue, and a monosaccharide residue. NOV is well absorbed but poorly metabolized and excreted, and has a longer half-life in various animals than other veterinary medicines [1]. Methods for monitoring NOV in various biologic samples are not well developed, and even less is known about NOV residue levels in animal tissues. Chromatographic methods using thin-layer chromatography (TLC) and liquid chromatography (LC) have been described [2–5]. Recently, Chonan et al. reported a multi-residue method for determining various veterinary drug levels in animal tissues using LC and alumina column extraction; however, recovery for NOV was only approximately 50% [6]. Heller and Nochetto published a procedure for screening ionophores, macrolides, and NOV in egg samples using silica solid phase extraction (SPE) and LC ion trap mass spectrometry (MS) [7]. Miao and Metcalfe investigated the LC-electrospray triple–quadrupole MS/MS (LC-ESI-MS/MS) for analysis of NOV in water samples [8]. However, none of these methods were aimed to quantify NOV in biologic samples. In addition, only two analytical methods of NOV for human blood samples using LC/UV detection have been reported [4,9]. LC/UV methods, however, are not adequate for monitoring trace levels of NOV in human blood. The aim of the present study was to develop a simple analytical method for determining NOV in various biologic samples using LC-MS/MS.

2. Experimental

2.1. Reagents, samples, and standard solutions

Novobiocin was obtained from Hayashi Pure Chemical Ind., Ltd (Osaka, Japan). A NOV 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, formic acid (FA, LC/MS-grade), acetic acid, phenyltrimethylammonium, hexadcyltrimethylammonium, (1-dodecyl) trimethylammonium, sodium dodecyl sulfate (SDS), polyoxyethylene (23) lauryl ether (Briji 35), polyoxyethylene (20) sorbitan monolaurate (Tween 20), and Tween 80 were obtained from Wako Chemical Co., Inc. (Osaka, Japan). Purified water was obtained from a Milli-Q purifying system (Millipore, Bedford, MA). Chicken, fish tissues, and milk were obtained from a local store in Nagoya, Japan. Standard human serum (Consera) was obtained from Nissui Pharmaceutical Co., Tokyo, Japan.

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2.2. LC-MS/MS analysis

LC-MS/MS analyses were performed using a Waters Alliance 2695/Micromass Quattro Premier system (Waters, Milford, MA). LC column was a TSK-GEL ODS 100 V (2.0 mm × 150 mm, 3 μm: Tosoh Co., Tokyo, Japan) maintained at 40 °C. The mobile phase consisted of 0.5% FA in water (Solvent A) and 0.5% FA in methanol (Solvent B). The LC stepwise gradient was as follows: 72% Solvent B at 0 min, 80% Solvent B at 15 min, 98% Solvent B at 15.1 min, 98% Solvent B at 20 min, and 72% Solvent B at 20.1 min with a flow rate of 0.2 mL/min. The injection volume was 10 μL. The ESI source conditions in the positive ionization mode were as follows: cone voltage 20 V, capillary voltage 2.8 kV, extractor voltage 4 V, RF lens voltage 0 V, source temperature of 100 °C, and desolvation temperature of 400 °C. The cone and desolvation gas flows were 50 L/h and 1000 L/h, respectively, and were obtained using a nitrogen source. We used argon as the collision gas and regulated it at 0.35 mL/h, setting the multipliers to 650 V.

2.3. Extraction procedure

The solid sample with a blender was homogenized using YELLOW LINE, D125 basic (IKA Japan K.K. Inc., Nara, Japan). For recovery test, the necessary standards were spiked in samples at this stage. Two grams of sample were added to 10 mL of methanol for optimal extraction. The mixture was homogenized for 1 min, and then centrifuged at 6000 rpm (6842 g) for 15 min by using Kubota 5420 type (Tokyo, Japan). For the milk sample, the mixture was vortex-mixed for 1 min, stored at 4 °C for 10 min, and then centrifuged at 6000 rpm (6842 g) for 15 min. For the human serum sample, the mixture (0.5 mL of sample and 1.5 mL of methanol) was vortex-mixed for 1 min, stored at 4 °C for 10 min, and then centrifuged at 12,000 rpm (14,825 g) for 5 min by using Sorvall fresco (Kendoro Laboratory, NC, USA). The pellet was then blended and centrifuged three times with the extracted solution. This solution was evaporated to dryness at 30 °C. The samples were then adjusted by water/methanol (50/50, v/v, 4.0 mL for chicken, fish tissues, and milk, and 0.5 mL for human serum).

2.4. Centrifugal ultrafiltration

These supernatant solutions were applied to centrifugal ultrafiltration using Amicon Ultra-15 (Ultracel-10 K, regenerated cellulose 10,000 M.W., Millipore Co. Ltd., Billerica, MA). The first step, 1.5 mL 5.0% SDS in water was added, and then the solution was centrifuged at 6000 rpm (6842 g) for 15 min. The second step, 1.5 mL water was added and the mixture was centrifuged at 6000 rpm (6842 g) for 15 min. Then, a 1.5-mL aliquot of extraction solution was applied to the centrifugal ultrafiltration. The sample was centrifuged at 6000 rpm (6842 g) for 15 min and measured by LC-MS/MS.

2.5. Analytical validation

2.5.1. Matrix effects

Based on the approach of Matuszewski et al. [10] and Villagrasa et al. [11], the matrix effects were evaluated by comparing the MS/MS responses of the standard and the test solution. Chicken muscle samples (0.05 μg/g) 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 and dilution by comparing the absolute peak areas for target compounds.

2.5.2. Recovery

NOV was quantified by a 6-point matrix matching calibration curve (5–100 ppb). The calibration curves were prepared daily using stock solutions. The area of each NOV was plotted against analyte concentration. Spiked levels for the recovery test in chicken, fish tissues, and milk were based on the Japanese Maximum Residue Limit (MRL) in Positive List Decision for Agricultural Chemical Residues.

3. Results and discussion

3.1. LC-MS/MS analysis of novobiocin

We recorded the mass spectrum of NOV in the positive mode because under our conditions NOV was barely detectable in the negative mode. The precursor peak corresponded to protonated molecular ion $[M+H]^+$ (*m/z* 613), which comprises the major collision-induced fragments. Fig. 1 shows the MS/MS spectra based on collision energies of 14, 16, and 26 eV. The *m/z* 189 from $C_{12}H_{12}O_2$ had a higher intensity than other ions with collision energy of 26 eV. The major NOV fragment ion at *m/z* 613 → 189 in the multiple reaction monitoring mode can determine lower quantities of 0.05 ng/mL standard solution with S/N = 3 (LOD: 0.5 pg) than other ions. The relative standard deviations ($n = 5$, intra-day) of the NOV standard were 0.07% (10 ng/mL) and 0.09% (100 ng/mL) for retention time, and 0.63% (10 ng/mL) and 1.03% (100 ng/mL) for peak area.

3.2. Extraction of novobiocin in biologic samples

We investigated the recovery by several extraction solvents such as acetonitrile, ethanol, methanol, and 1.0% FA and acetic acid in methanol from chicken tissue samples. Methanol produced higher relative recoveries of NOV than the other solvents. Therefore, methanol extraction of NOV from biologic samples was useful.

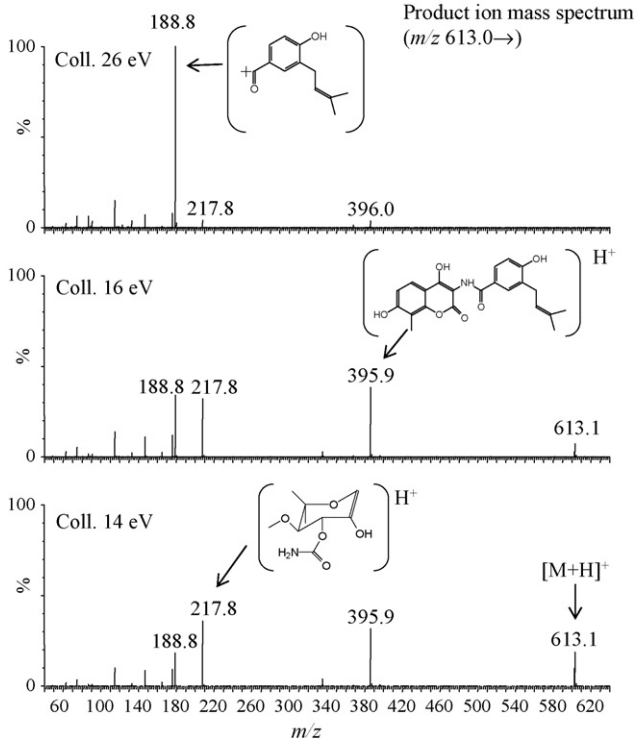

Fig. 1. MS/MS spectra of NOV standards based on various collision energies (14, 16, and 26 eV). MS/MS ionization: electrospray positive mode. Precursor ion: *m/z* 613.0.

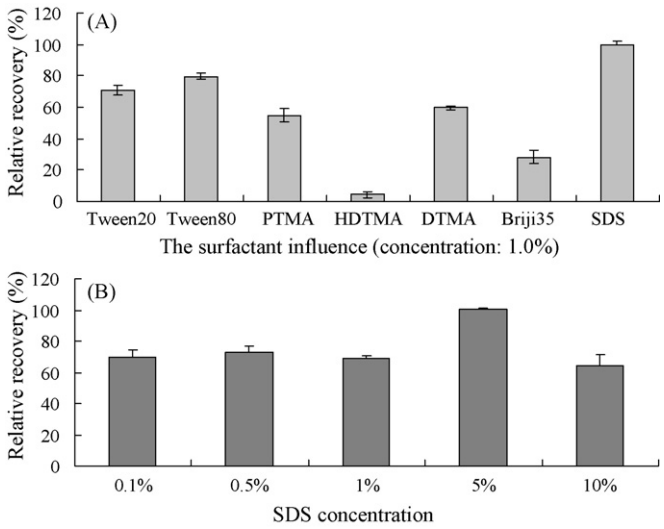

Fig. 2. Surfactant effects on centrifugal ultrafiltration for NOV sample preparation. (A) Effect of various surfactants: concentration 1.0% (polyoxyethylene sorbitan monolaurate: Tween 20 and 80, phenyltrimethylammonium: PTMA, hexadecyltrimethylammonium: HDTMA, (1-dodecyl)trimethylammonium: DTMA, polyoxyethylene (23) lauryl ether: Briji 35, and sodium dodecyl sulfate (SDS)) and (B) Effect of various SDS concentrations (0.1, 0.5, 1.0, 5.0, and 10%)., $n = 3$.

3.3. Centrifugal ultrafiltration of novobiocin in biologic samples

The centrifugal ultrafiltration procedure is easy to use and requires little time and solvent, but usually results in high recovery and reproducibility [12,13]. Samples containing NOV were prepared by methanol extraction of chicken muscle followed by a standard centrifugal ultrafiltration procedure. The recovery of NOV from these samples, as detected by LC-MS/MS, however, was only 57%. In addition, the recovery of NOV in methanol/water (50/50, v/v) was almost same values (58%). There preliminary data indicated that NOV has the behavior of sticking together to this filter. Therefore, we investigated a detergent-treated centrifugal ultrafiltration because the Tween 20 has the good effect to improve the membrane characteristics [14]. Thus, the influence of various surfactants (concentration: 1.0%) on the relative recovery of the centrifugal ultrafiltration was investigated in extracted chicken muscle samples spiked with NOV (Fig. 2-(A)). In this result, this is fortunate to find the good effects using SDS. Then, we investigated that the available concentration of SDS (5%) provided the greatest increase in NOV recovery compared to others (Fig. 2-(B)). The preparation with 5% SDS indicated that satisfactory values of recovery were detected using various biologic samples.

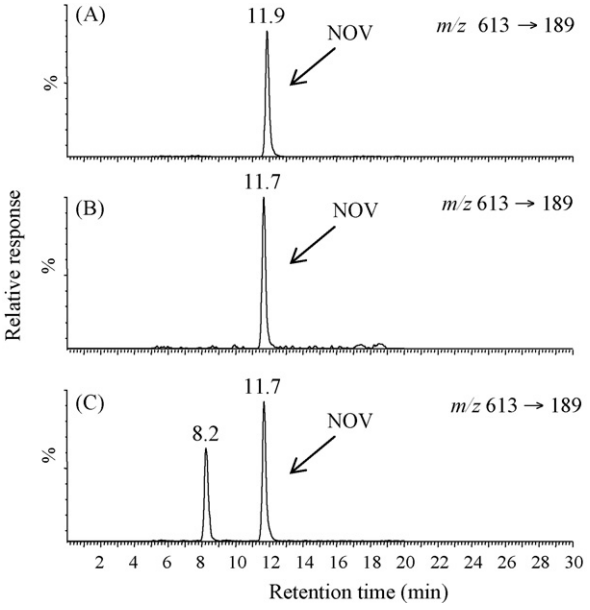

Fig. 3. LC-MS/MS chromatograms of small amounts of NOV in biologic samples. (A) Chicken liver sample (5 ng/g), (B) fish tissue sample (5 ng/g), and (C) human serum sample (5 ng/mL).

3.4. Validation of the analytical method

We evaluated ion suppression of NOV subjected to the centrifugal ultrafiltration procedure. The NOV value obtained from spiked extract of chicken muscle samples (50 ppb) was compared with that of the same analyte prepared in a water/methanol solution (50/50, v/v). Spiked chicken muscle extract that was concentrated 2-fold had an MEV of 53%, indicating that concentrating the biologic

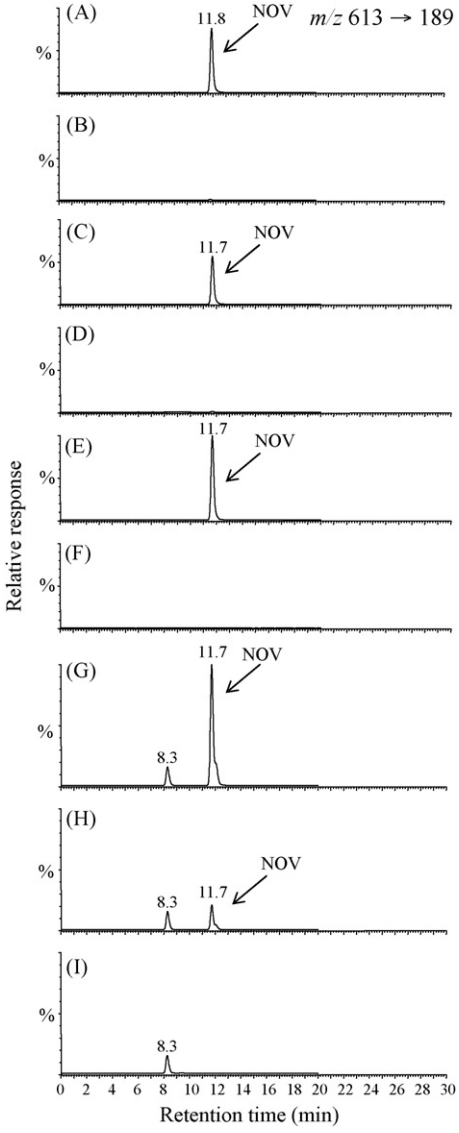

Fig. 4. LC-MS/MS chromatograms of NOV for recovery test and blank chicken, fish tissue, milk, and human serum samples. (A) Chicken liver sample (0.05 μg/g, 0.05 ppm), (B) chicken liver sample (non-spiked), (C) fish tissue sample (0.05 μg/g, 0.05 ppm), (D) fish tissue sample (non-spiked), (E) milk sample (0.08 μg/g, 0.08 ppm), (F) milk sample (non-spiked), (G) human serum sample (0.05 μg/mL, 0.05 ppm), (H) human serum sample (0.01 μg/mL, 0.01 ppm), and (I) human serum sample (non-spiked).

Table 1
The results of recovery test for determination of NOV in biologic samples.

Sample	Spiked levels (ppm)	Recovery (%)±SD (n = 3)	Linearity of matrix matching calibration (r)
Chicken muscle	0.05	75 ± 3	0.998
Chicken liver	0.05	95 ± 2	0.999
Fish tissue	0.05	72 ± 4	0.999
Milk	0.08	71 ± 1	0.999
Human serum	0.05	94 ± 3	0.998
	0.01	91 ± 7	

samples induced ion suppression of NOV in ESI-MS/MS. Villagrasa et al. suggested that the use of internal standard, the application of standard dilution method, and the dilution of the extracts before instrumental determination were useful for avoiding matrix signal suppression [11]. The commercially-supplied internal standard such as substituted d- and/or ^{13}C-NOV can not be obtained from reagent's company. Thus, we tried to study other two methods for avoiding ion suppression of NOV on LC-MS/MS. The dilution of extracts (diluted 2-fold) indicated that the MEV was almost 100%. In this study, this dilution of extracts was very useful for avoiding ion suppression. Moreover, poorly reproducible ME values (SD > 10%) were indicated by using only dilution of extracts. Thus, we tried to study the application of standard dilution (matrix matching standard) for getting the good-reproducible quantitative values (Table 1). Based on these results, the dilution of the sample solution completely inhibited ion suppression in ESI-MS/MS detection. Special attention must be paid to the possible determination levels of NOV from chicken tissue and milk samples according to the MRL. Based on the linear matrix matching calibrations, it appears that even with 2-fold dilution it is possible to detect NOV based on a low MRL (0.05 or 0.08 ppm). The chromatograms of small amounts (5 ppb) of NOV in typical chicken liver, fish tissue, and human serum are shown in Fig. 3. Based on these LC-MS/MS responses of NOV in biologic samples, the coupling method of the application of standard dilution and the dilution of the extracts was useful to decrease ion suppression and sensitive detection of NOV in biologic samples.

The NOV recovery test results, based on an MRL of 0.05 ppm in chicken and fish tissues, and 0.08 ppm in milk, are summarized in Table 1. The chromatograms of the recovery tests and blanks from typical chicken liver, fish tissues, and milk samples are shown in Fig. 4. Overall recovery from chicken and fish tissue and milk samples ranged from 71 ± 1 to 95 ± 2%. The matrix matching calibrations had good linearity over a range of 5–100 ng/mL (Table 1). The results of the NOV recovery test in human serum samples are shown in Table 1. There was good recovery (84–98%) and linearity (r = 0.998). Based on the chromatograms of human serum, peak (8.2–8.3 min) for endogenous substances derived from the human serum sample was observed. However, the good separation of these peaks is useful for monitoring NOV in human serum.

This method has higher sensitivity and selectivity for NOV detection in human serum than other previously reported methods, and is clinically applicable.

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