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<Original Article>

Analysis of antibiotic residues in milk from healthy dairy cows treated with bovine mastitis ointment using ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry

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Abstract: This study was conducted to analyze penicillin G (PEG), streptomycin (STR) and neomycin (NEO) residues in milk of healthy lactating cows. Milk samples were collected from all four quarters of 12 dairy cows 2−7 days after intramammary infusions of an ointment containing PEG, STR and NEO once (n = 4; group I) or twice (n = 4, group II) daily. Ultra-performance liquid chromatography coupled with electrospray tandem mass spectrometry was used to determine the antibiotic residues in the samples. The correlation coefficient (r^2) of the calibration curves for all antibiotics was > 0.999 and the limits of detection and quantification were 0.002–0.005 μg/mL and 0.007–0.02 μg/mL, respectively. Recovery rates were ranged from 75.5 to 92.3%. In group I, PEG, STR and NEO residues were detected in milk at 2, 3 and 2 days post-treatment, respectively, which were below the maximum residue limit (MRL). In group II, PEG, STR and NEO residues were detected in milk at 2, 3 and 3 days post-treatment, respectively, which were bellow the MRL. These results suggest that a 3-day for milk withdrawal period after the ointment treatment might be sufficient for reduction of the antibiotic residues below the MRL.

Keywords: UPLC-MS/MS, bovine milk, neomycin, penicillin G, streptomycin

Introduction

Bovine mastitis is the most common bacterial disease in dairy cows and one of the most economically to the dairy farm. It is an inflammatory condition of the mammary glands, most often caused by a bacterial intramammary (IMM) infection [3]. Economic losses from bovine mastitis are mainly caused by the increase of treatment cost and culling risk, loss in milk production and reduction in milk quality due to increased somatic cell count [4].

Nowadays, antibiotic therapy is the most common method to treat bovine mastitis. However, the emergence of antibiotic-resistant bacteria has become a critical problem in dairy industry [12]. The incriminate use of antibiotics against bovine mastitis may cause a grave problem because of the advent of antimicrobial resistance, the creation of resistant bacteria within the food chain [26], and drug residues in milk, which is consumed by humans and expresses concern about adverse health effects on consumers [15].

Residual antibiotics in milk can cause allergic reactions in some hypersensitive persons [14], and lead to resistant bacterial strains that do not respond to drugs commonly used for human diseases [2]. In addition, drug residues change the raw milk processing qualities by hindering the propagation of starter cultures used for the production of dairy products such as cheese and yogurt [1]. Pasteurization and ultra-high temperature treatment get rid of pathogenic bacteria in raw milk but do not affect to reduce or eliminate residual drugs [21].

As penicillin G (PEG) suppresses bacterial proliferation by interfering with cell wall assembly, the drug has been used for the treatment of bovine mastitis caused by susceptible bacteria such as *Streptococcus agalactiae* and *Streptococcus dysgalactiae* [14]. IMM therapy of PEG alone can treat only sub-acute mastitis revealed by mild inflammatory changes in the bovine udder [30]. Streptomycin (STR) is a water-soluble aminoglycoside antibiotic derived from *Streptomyces gri-*

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seus and has broad-spectrum bactericidal activity against various pathogenic bacteria [25]. With the mechanism of action of aminoglycoside antibiotics, STR inhibits bacterial growth by interfering with peptide synthesis systems of bacteria [24]. Neomycin (NEO) is one of aminoglycoside antibiotics with antibacterial activity against Gram-positive and many Gram-negative pathogens by binding the region for translation of mRNA and message readout, and disrupting its functions [29]. The sulfate salt of NEO has been used worldwide to combat bacterial gastro-intestinal infections in poultry and livestock. NEO sulfate is also used in IMM infusions to treat bovine mastitis [11]. In the treatment of bovine mastitis, aminoglycosides such as STR and NEO have been used mostly in combination with PEG [27]. For the reasons mentioned above, a bovine mastitis ointment containing PEG, STR and NEO was used in this study.

In order to protect humans from the harmful effects of antibiotic residues in milk, the Food and Agriculture Organization (FAO) and European Union (EU) have set maximum residue levels (MRLs) of 1.5, 0.2 and 4.0 μg/mL for the milk residues of NEO, STR and PEG, respectively [5, 8, 22]. In addition, Korean Ministry of Food and Drug Safety (KMFDS) has established MRLs for the residues of NEO, STR and PEG in milk exactly like FAO and EU [18].

The aim of this study was to evaluate PEG, STR and NEO residues in bovine milk collected from individual dairy cows after IMM administration of a bovine mastitis ointment containing PEG, STR and NEO.

Materials and Methods

Chemicals and reagents

PEG, STR and NEO were purchased from Sigma-Aldrich (USA). Acetonitrile, methanol, formic acid and acetic acid were HPLC gradient grade and purchased from Merck (Germany). Sodium dihydrogen phosphate (> 99.0%) and disodium hydrogen phosphate (> 99.0%) were also obtained from Merck. Double-deionized water was obtained using a Milli-Q Gradient water system (Millipore, USA).

Stock standard solutions of PEG, STR and NEO were prepared to 10 μg/mL by dissolving the compounds in a suitable solvent, such as acetonitrile (PEG) or methanol (STR and NEO). These standard solutions were stored in dark-brown glass bottles at −20°C.

Drug

The bovine mastitis ointment (Synerject) was obtained from Daehan New Pharm (Korea). The IMM syringes (5 g) contain 100,000 IU of PEG procaine, 100 mg of STR sulfate and 100 mg of NEO sulfate.

Animals

The study was carried out using twelve lactating Holstein cows with no history of antimicrobial use in the previous 21 days at the experimental animal farm at the Gyeongsang National University (Jinju, Korea) (GNU-LA-0116). The animals were 5 to 7 years old with a body weight of 400 to 450 kg, and were milked by machine twice (6 AM and 6 PM) a day.

Drug administration and milk sampling

The animals were randomly divided into a control (CON) and two treatment groups (group I and II), containing four animals per group. In the treatment groups, the four quarters of each dairy cow were administered with one (group I) or two (group II) syringes per quarter once after morning milking, respectively, while CON was not treated. Before drug treatment, the quarter was thoroughly milked out by hand and the teat was cleaned using a cloth towel soaked with 70% alcohol. Milk samples were collected from each quarter of the dairy cows during routine morning milking in sterilized vials 2, 3, 4, 5, 6 and 7 days after administration of the drug. The four milk samples collected from a dairy cow per day were combined in the same ratio to give one sample per dairy cow per day. Milk samples were instantly moved to the laboratory and frozen at −20°C until they were analyzed for antibiotic residues.

Instrumentation

Milk samples were analyzed by ultra-performance liquid chromatography (UPLC) system, and separations were carried out with an Acquity UPLC BEH C_{18} column (1.7 μm particle size, 50 mm × 2.1 mm; Waters, USA). Analytes were separated using a mobile phase consisting of acetonitrile (eluent A) and 0.1% formic acid in water (eluent B) at a flow rate of 0.3 mL/min. Separation was carried out at 40°C, according to the following gradient program: 0–0.5 min, 5% eluent A; 0.5–1 min, linear increase to 10% eluent A; 1–3 min, linear increase to 40% eluent A; 3–4 min, linear increase to 90% eluent A; 4–4.1 min, decrease to 40% eluent A; and finally, 4.1–6.5 min, 5% eluent A. Samples were kept in an auto-sampler at 15°C.

Analyses using mass spectrometry were performed on a Waters Acquity TQS Micromass Quattro Ultima triple-quadrupole MS quadrupole equipped with an electrospray ion source (Micromass, UK). The instrument was run using an electrospray (ESI) source in positive mode with the following parameters: 0.5 kV capillary voltage, 30 V cone voltage, 500°C desolvation temperature, and 1000 L/h desolvation gas (nitrogen > 99.999%) flow. Data acquisition was carried out using MassLynx V 4.1 software with the Quanlynx program (Waters). Electronic balance (0.0001 g precision; Shimazu, Japan), vortex mixer (Scinco, Korea), CR22G centrifuge (Hitachi, Japan), and pressure N_2 gas blowing concentrator (Organomation, USA) were used for sample preparation.

Extraction procedure

One milliliter of blank fresh milk was put into a 10-mL centrifuge tube, and then 0.5 and 3 mL of water and acetonitrile were added to extract the residual antibiotics, respec-

tively. The mixture was mixed by vortex mixer for 5 min and centrifuged at 6,500 × g for 10 min. The upper layer was transferred into a centrifuge tube and evaporated at 48°C to 1 mL under nitrogen stream. The residue was reconstituted using 3 mL of 0.1 mol/L phosphate buffer solution (PBS) and the pH was adjusted to 8.5 with 1.0 M NaOH. The mixture was vortexed for 5 min and then extracted with an Oasis HLB cartridge at a flow rate of 0.5 mL/min. Before extraction of the mixture, the cartridge had been activated with 2 mL of methanol, 2 mL of water and 2 mL of PBS (pH 8.5). After the cartridge was washed with 2 mL of PBS (pH 8.5) and 1 mL of water, the analytes were eluted from the cartridge added with 3 mL of an acetonitrile/water solution (1 : 1, v/v). After the eluates were concentrated at 48°C to approximate 1 mL under nitrogen stream, 0.44 mL of acetonitrile was added. Then, the mixture was reconstituted to 4 mL with water. After the solution was filtered through a 0.22-μm sterile syringe filter (Acrodisc PVDF membrane; Sigma-Aldrich, USA), 5 μL of the final filtrate were injected into the UPLC–MS/MS system, and the analytes were monitored in multiple reaction monitoring (MRM).

Validation

Based on a previous study [16], the linearity and analytical limits were validated. The linearity was evaluated by matrix-matched calibration curves at different spiked levels. PEG was spiked with the following levels: 0.02, 0.04, 0.08, 0.16 and 0.32 μg/mL. STR was spiked with the following levels: 0.3, 0.6, 1.2, 2.4 and 4.8 μg/mL. NEO was spiked with the following levels: 0.5, 1.0, 2.0, 4.0 and 8.0 μg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the lowest concentrations with a signal-to-noise (S/N) ratio of 3 for LOD and 10 for LOQ.

Recovery rates

The recovery study was carried out using two different spiked concentration levels for each compound, as follows: PEG, 0.05 and 0.1 μg/mL; STR and NEO, 0.5 and 1.0 μg/mL. Three spiked solutions per concentration were prepared. According to the extraction procedures described above, the spiked samples were extracted in triplicate and analyzed for PEG, STR and NEO using UPLC-MS/MS. After analysis, the recovery rate of the antibiotics was calculated and presented as the mean ± SD.

Results

Chromatograms and calibration curves

Optimized extraction was validated for quantification of PEG, STR and NEO in PEG, STR and NEO-spiked blank bovine milk.

Figure 1 shows the chromatograms of the blank milk spiked with PEG (at 0.3 μg/mL; Fig. 1A), STR (at 0.3 μg/mL; Fig. 1B) and NEO (at 0.75 μg/mL; Fig. 1C). The retention time

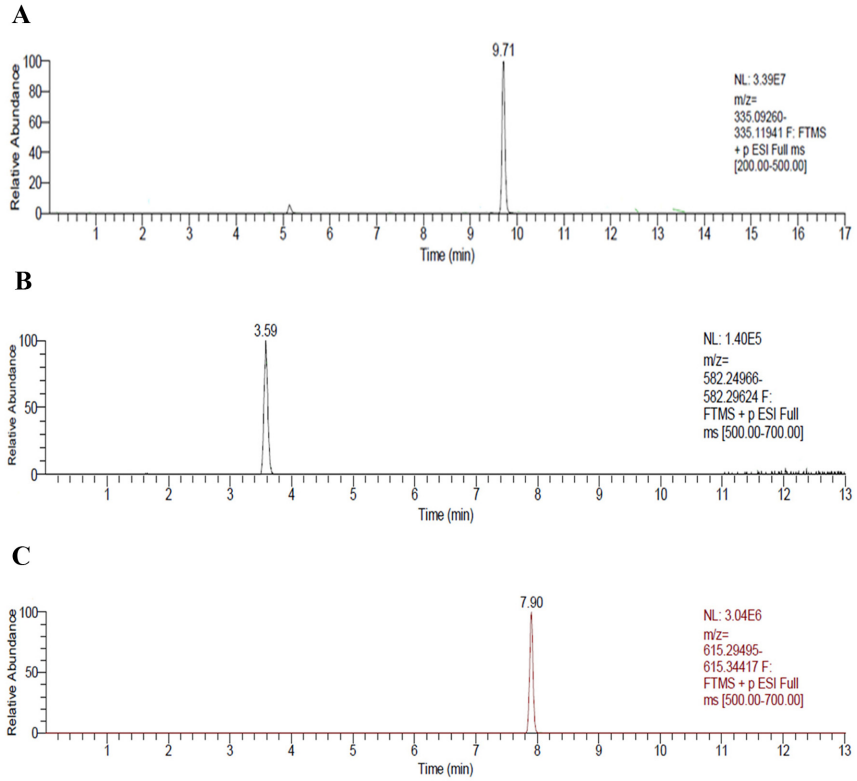

Fig. 1. UPLC-MS/MS MRM chromatograms for penicillin G (A), streptomycin (B) and neomycin (C) in a blank bovine milk spiked with penicillin G (0.3 μg/mL), streptomycin (0.3 μg/mL) and neomycin (0.75 μg/mL).

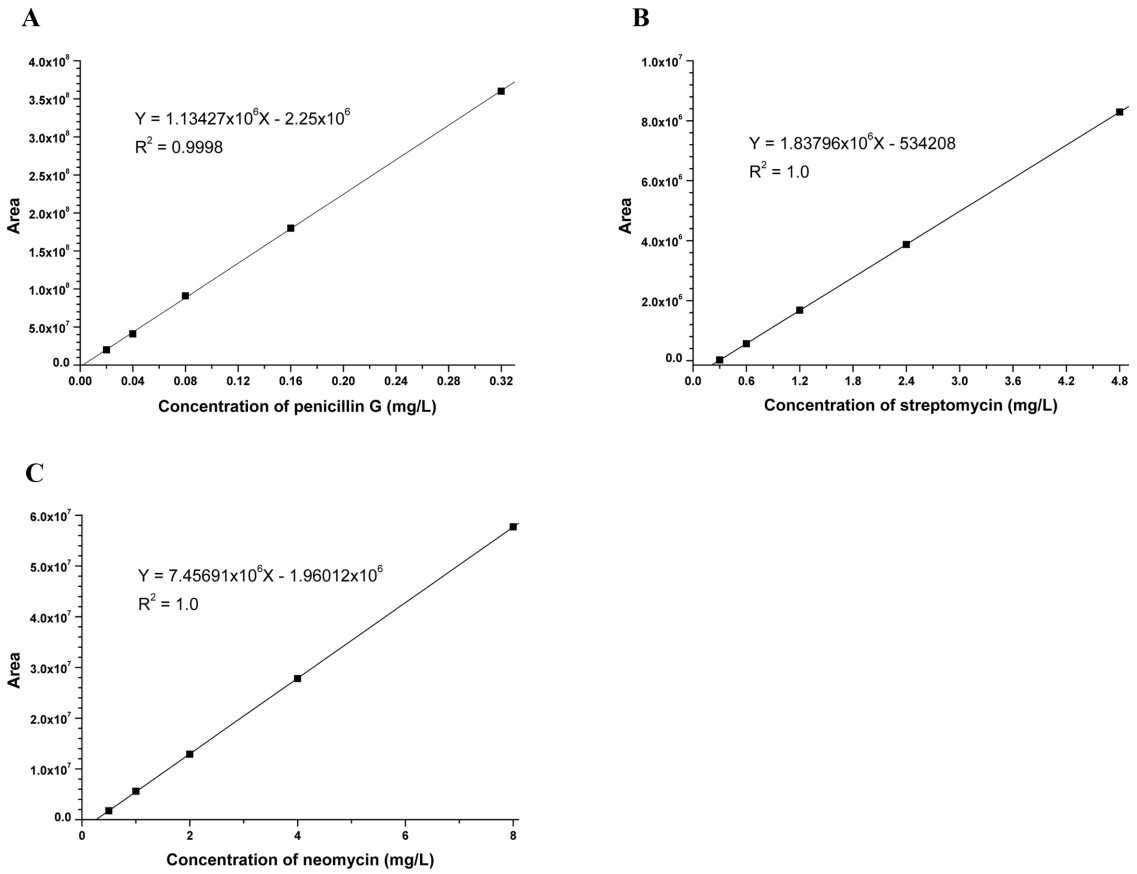

Fig. 2. Linearity of calibration curves using blank milk samples with the addition of 0.02–0.32 mg/L penicillin G (A), 0.3–4.8 mg/L streptomycin (B), and 0.5–8.0 mg/L neomycin (C) at all five concentration levels.

for PEG, STR and NEO was 9.71, 3.59 and 7.90 min, respectively.

Calibration curves for PEG, STR and NEO are shown in Figure 2. Linearity was defined using calibration curves prepared with blank raw milk spiked with PEG, STR and NEO. These curves were prepared using five concentrations of each drug. The correlation coefficient (r^2) was calculated through linear regression. As shown in Figure 2, all analytes had a satisfactory linearity with r^2 values of greater than 0.999.

LOD and LOQ

LOD and LOQ values for PEG, STR and NEO were calculated to determine the lowest concentration of the compounds detectable as required by the guidelines for implementation of Commission Decision 2002/657/EC (LOD and LOQ) [8]. The LOD values for PEG, STR and NEO was 0.002, 0.005 and 0.003 μg/mL, respectively, and the LOQ value for PEG, STR and NEO was 0.007, 0.02 and 0.01 μg/mL, respectively, based on a S/N ratio of 3 for LOD and 10 for LOQ.

Recovery rates

The accuracy of the method was evaluated through spike recovery tests using blank milk. Table 1 shows the recovery rates of PEG, STR and NEO in PEG-, STR- and NEO-spiked blank bovine milk. Recoveries of PEG, STR and NEO were 89.6–91.8%, 88.2–92.3% and 75.5–82.8%, respectively.

Table 1. Recoveries for penicillin G, streptomycin and neomycin spiked into bovine milk

Antibiotics	Spiked concentration (μg/mL)	Recovery (%)
Penicillin G (PEG)	0.05	91.8 ± 6.8
	0.1	89.6 ± 7.4
Streptomycin (STR)	0.5	92.3 ± 7.1
	1.0	88.2 ± 6.5
Neomycin (NEO)	0.5	82.8 ± 5.6
	1.0	75.5 ± 7.2

Antibiotic residues in milk

Table 2 shows the concentration of antibiotic residues in milk from the dairy cows after IMM administration of the bovine mastitis ointment at different doses. PEG detected in all treatment groups was below the LOQ of 0.007 μg/mL on day 2 after IMM administration of the ointment. STR in group I and II was detected until 4 and 5 days after drug treatment, respectively. In addition, NEO in group I and II was detected

Table 2. Residue concentrations of antibiotics in milk of dairy cows after intramammary administration of bovine mastitis ointment at different concentrations

Drugs	Groups*	Days after administration (μg/mL)					
		2	3	4	5	6	7
PEG	Control	ND†	ND	ND	ND	ND	ND
	Group I	ND	ND	ND	ND	ND	ND
	Group II	ND	ND	ND	ND	ND	ND
STR‡	Control	ND	ND	ND	ND	ND	ND
	Group I	0.225 ± 0.036	0.092 ± 0.019	0.011 ± 0.005	ND	ND	ND
	Group II	0.398 ± 0.040	0.142 ± 0.025	0.084 ± 0.020	0.009 ± 0.003	ND	ND
NEO	Control	ND	ND	ND	ND	ND	ND
	Group I	0.350 ± 0.051	0.152 ± 0.021	ND	ND	ND	ND
	Group II	0.593 ± 0.071	0.272 ± 0.037	0.123 ± 0.042	ND	ND	ND

*Group I, intramammary administration of one bovine mastitis ointment dose (5 g) per udder; Group II, intramammary administration of two bovine mastitis ointment doses (10 g) per udder. †No detection due to the concentration being below the limit of quantification, which is that was 0.007, 0.02 and 0.01 μg/mL for PEG, STR and NEO, respectively. ‡Detection of STR residue in two of four dairy cattle.

until 3 and 4 days after drug treatment, respectively. As the MRL of STR and NEO in milk established by the KMFDS is 0.2 and 0.5 μg/mL, respectively, the concentrations of STR and NEO in group II was below each MRL 3 days post-treatment.

Discussion

Several previous studies have described the detection methods for antibiotics in bovine milk, including high-performance liquid chromatography (HPLC) [19], HPLC-MS/MS [13], UPLC-MS/MS [28], screening methods and immunoassays [7].

In the present study, PEG, STR and NEO residues in milk from dairy cows after IMM administration of bovine mastitis ointment at different concentrations, were determined using UPLC-MS/MS.

The calibration curves for PEG, STR and NEO residues showed good linearity with the correlation coefficient (r^2), in the range of 0.9998–1.0. In previous studies using UPLC-MS/MS, the correlation coefficient of the PEG calibration curve was > 0.998 [20], and that of the STR and NEO calibration curve was greater than 0.996 [28]. In this study, the correlation coefficients are similar with those of the previous studies above.

The LOD value for PEG, STR and NEO in milk ranged from 0.002 to 0.005 μg/mL, and the LOQ value for PEG, STR and NEO in milk ranged from 0.007 to 0.01 μg/mL, which is below the MRLs in milk established by the FAO, EU and KMFDS [5, 8, 18]. A previous study reported that the LOD for PEG was 0.25 ng/mL [20], and the LOQs for STR and NEO in a previous study were both 2.0 ng/mL [28]. Compared with the results of previous studies, the LOD for PEG and LOQ for PEG, STR and NEO in this study were very low.

In this study, the recovery rates for PEG, STR and NEO ranged from 75.5 to 92.3%, which are in agreement with the European Commission Decision 2002/657/EC guidelines for validation methods, where the acceptable measurement trueness is determined through recovery of known amounts of the analytes added to a blank sample. Data adjusted with the mean recovery are only acceptable when they fall within 50 to 120% for less than 1.0 μg/kg, 70 to 110% for > 1.0 to ≥ 10.0 μg/kg and 80 to 110% for 10.0 μg/kg [9].

In a previous study for PEG residue in milk by liquid chromatography-electrospray tandem mass spectrometry, recovery rates of PEG in PEG-spiked blank milk with 2 and 6 μg/L were 105 and 100%, respectively [17]. In another previous study for determination of aminoglycosides by liquid chromatography-tandem mass spectrometry, recovery rates in spiked raw milk with 0.05 and 0.1 μg/mL were 95.5 and 97.5% for STR and 75.1 and 70.1% for NEO, respectively [21]. In this study, recover rates of NEO were higher than those in the above study, but those of PEG and STR were lower than those in above studies. With the results, the variation of recoveries may be caused by the extraction method and the amount of an analyte spiked in milk.

In the present study, PEG residue in milk was not detected 2 days after administration of the drug, whereas STR and NEO residues in milk were detected until 4 and 3 days after treatment of the drug following the manufacturer's recommended dose of 1 syringe (5 g) per quarter per day. However, the concentrations of STR and NEO residues 3 and 2 days, respectively, after drug treatment, respectively, were each below the MRL established by the FAO, EU and KMFDS [5, 8, 18]. In milk from dairy cows following three successive IMM infusions of 375,000 IU per 12 h for PEG, the drug residue detected was under the MRL (4.0 μg/mL) 3 days post-treatment [10]. In a previous study to evaluate STR residue in milk [6], the concentration of STR 24 h post-treatment was below the MRL of 0.2 μg/mL, after daily intramus-

cular administrations of STR (10 mg/kg body weight) for 3 days. In another study [23], both NEO and dihydrostreptomycin residue detected 5 days post-treatment was under the MRL in milk from dairy cows administered with a drug (6 g) containing 200 mg of NEO once daily for 2 days and a drug (4 g) containing 100 mg of dihydrostreptomy once. Considering the drug dosage, treatment route and treatment times, the concentrations of antibiotic residues in this study were similar to the above studies.

In conclusion, this study shows that all antibiotics in the bovine mastitis ointment detected were below the MRL 3 days after IMM administration using one- and two-fold the manufacturer's recommended dose. Therefore, a 3-day milk withdrawal period for the drug might be sufficient to decrease all antibiotic residues to a level below each MRL.

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