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Development of certified reference materials for accurate determination of fluoroquinolone antibiotics in chicken meat

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ABSTRACT

Certified reference materials (CRMs; KRISS CRM 108-03-003, 108-03-004) were developed for the accurate determination of fluoroquinolones (enrofloxacin and ciprofloxacin, respectively) in chicken meat. Two groups of chickens were cured with feeds containing enrofloxacin and ciprofloxacin, respectively. After slaughter, the thigh and breast meats were combined for the respective groups and the meat was freeze-dried, pulverized, sieved, and V-mixed. The final bulk material was bottled in 10 g portions. For certification of the CRMs, isotope dilution-liquid chromatography/tandem mass spectrometry was used. The certified values of the CRMs were (19.06 ± 0.86) mg/kg for enrofloxacin and (1.095 ± 0.038) mg/kg for ciprofloxacin. The stabilities of the CRMs were monitored at −70 °C for 12 months, at −20 °C for 2 months, and at room temperature for 1 month. Both CRM candidates were stable during the monitoring period for each temperature.

1. Introduction

A variety of antibiotics have been developed to protect people and livestock from bacterial infections. Among them, fluoroquinolones (derived from quinolones) are a widely used class of synthetic antibiotics that have good oral absorption and tissue penetration rates, and show broad-spectrum antibacterial activity (Van Bambeke, Michot, Van Eldere, & Tulkens, 2005). Side effects of fluoroquinolone antibiotics include hepatotoxicity, tendon disorders, disturbances in the central nervous system (CNS) and gastrointestinal tract, and allergic reactions (Blanca-López et al., 2013; Kim & Del Rosso, 2010; Orman et al., 2011; Owens & Ambrose, 2005). Another more noteworthy problem associated with antibiotic use is the emergence of drug-resistant bacteria, and much effort is now dedicated to elucidating the resistance mechanism (Aldred, Kerns, & Osheroff, 2014; Redgrave, Sutton, Webber, & Piddock, 2014). Unfortunately, the problems of antibiotic resistance can be aggravated by human consumption of animal products derived from animals that were supplemented with antibiotics. Residual antibiotics in meat and side products like milk or eggs provide the potential for microbial resistance to develop against the antibiotics present (Cho et al., 2008; Jiang et al., 2013; Zeng, Dong, Yang, Chen, & Huang, 2005). As a result, the use of fluoroquinolones and other antibiotics for food-producing animals is now regulated and many countries have established maximum residue limits (MRLs) for antibiotics in animal products (European Commission, 1999; Health Canada, 2015; MFDS, 2015; The Japan Food Chemical Research Foundation, 2006; USDA, 2001; Xiao et al., 2013).

Accordingly, well-established analytical methods for accurate determination of antibiotics are required to enforce the regulation with confidence. This necessity has led to the development of many analytical methods based on liquid chromatography/mass spectrometry (LC/MS), LC/fluorescence spectroscopy, and LC/ultraviolet spectroscopy (Andreu, Blasco, & Picó, 2007; Hermo, Barrón, & Barbosa, 2006; Schneider, Braden, Reyes-Herrera, & Donoghue, 2007). However, laboratories using such methods should regularly check the reliability of their analytical results. In this respect, certified reference materials (CRMs) are one of the most preferred tools for this purpose. Nevertheless, to the best of our knowledge, we are unaware of the existence of animal meat CRMs for the analysis of fluoroquinolone antibiotics.

With this deficiency in mind, our laboratory at the National Metrology Institute (NMI) of Korea launched a project for the development of CRMs for accurate determination of fluoroquinolones in chicken meats. Among several fluoroquinolones, enrofloxacin and ciprofloxacin were selected; these compounds are widely used because of their effectiveness against *Haemophilus* sp., *Pasteurella* sp., and *Actinomyces* sp. (Prescott & Yielding, 1990)

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and they are strongly regulated in many countries. Initially, our laboratory developed an isotope dilution liquid chromatography/-tandem mass spectrometry (ID-LC/MS/MS) method as a higher-order reference method for certification of CRMs (Lee, Kim, & Kim, 2013). In this study, we developed CRMs for the analysis of enrofloxacin and ciprofloxacin. The two CRMs were prepared from two groups of chickens that were separately cured with enrofloxacin and ciprofloxacin, because meats fortified or spiked with target analytes will behave differently under analysis from those with naturally accumulated analytes. The CRMs were certified with the ID-LC/MS/MS method, and their stabilities at various temperatures were monitored.

2. Experimental

2.1. Reagents

Primary reference materials for enrofloxacin and ciprofloxacin (hydrochloride salt form) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Purities of the primary reference materials was determined by using in-house protocols (LC/UV analysis of structurally related impurities, thermo-gravimetric analysis for non-volatile impurities, Karl-Fischer Coulometry for water contents, headspace GC/MS for residual solvents). Enrofloxacin-d_5 (hydrochloride salt form) was obtained from Sigma-Aldrich (Kyounggi-do, Korea). Ciprofloxacin-$^{13}C_3{}^{15}N$ was a product of Cambridge Isotope Laboratories (Andover, MA, USA). HPLC-grade organic solvents (methanol, acetonitrile, *n*-hexane, and dichloromethane) were purchased from Burdick and Jackson (Muskegon, MI, USA). Ammonium acetate, sodium acetate, acetic acid, sodium phosphate monobasic, sodium hydroxide, formic acid, ammonia (2.0 mol/L in methanol), and ethylenediaminetetraacetic acid (EDTA; 0.1 mol/L in water) were purchased from Sigma-Aldrich. SupelMIPTM SPE-Fluoroquinolones (Molecularly Imprinted Polymers, 25 mg, 3 cc) was obtained from Sigma-Aldrich and Oasis MAX (Mixed-mode anion exchange sorbent, 60 mg, 3 cc) was obtained from Waters (Seoul, Korea). Ultra-pure water was prepared using a Milli-Q integral water purification system (Merck Millipore, Germany).

2.2. Preparation of CRM candidates

CRM for the analysis of enrofloxacin in chicken meat (KRISS CRM 108-03-003) was prepared as follows. Chickens were raised at the Live Stock Experimental Station of KonKuk University with feed containing 150 μg/kg of enrofloxacin for 3 weeks and then the level of enrofloxacin was raised to 300 μg/kg for the next 3 weeks. The chickens were slaughtered when their body weight exceeded 2 kg. The thigh and breast parts were combined after removing the skin and fatty tissue. The chicken meat was converted to powder using processes established in our laboratory (Ahn, Lee, Lee, & Kim, 2016), including grinding, freeze drying, pulverizing, sieving, and mixing in a V-mixer for 12 h. The chicken powder was bottled in 10-g portions under Ar gas and the samples were numbered in order of bottle filling. The bottles were capped and sealed with heat-shrink film and stored at −70 °C (designated as storage condition) until use. A total of 240 bottles were prepared for the CRM.

Preparation of CRM for the analysis of ciprofloxacin in chicken meat (KRISS CRM 108-03-004) was prepared in the same way as described above from chickens cured with ciprofloxacin. A total of 173 bottles were prepared and these were also stored at −70 °C.

All the chickens used in this study were raised and slaughtered in accordance with the ethics guidelines of the Konkuk University Institutional Animal Care and Use Committee.

2.3. Analytical methods

The ID-LC/MS/MS method reported in our previous article (Lee et al., 2013) was used for certification of the two CRMs, after slight modification of the extraction conditions.

2.3.1. Preparation of standard solutions

For each target analyte (enrofloxacin and ciprofloxacin), four standard solutions were gravimetrically prepared with mobile phase A (0.1% formic acid + 10 μmol/L EDTA in water) at concentrations of 20 mg/kg and 5 mg/kg, respectively. Isotope standard solutions of enrofloxacin-d_5 (20 mg/kg) and ciprofloxacin-$^{13}C_3{}^{15}N$ (1 mg/kg) were also prepared. For each target analyte, two isotope ratio standard (1:1) solutions were prepared for each of the four standard solutions. The eight isotope ratio standard solutions for each target analyte were cross-checked using the LC/MS/MS conditions described below. Based on the cross-check results, one isotope ratio standard solution for each target analyte was used as a calibrant for the ID-LC/MS/MS analysis of its respective CRM.

2.3.2. Extraction and clean-up

Extraction and clean-up of samples taken from the two CRMs followed the method described in our previous article (Lee et al., 2013) with minor changes. Briefly, approximately 1 g of chicken meat powder was placed in a 50-mL conical tube. One milliliter of enrofloxacin-d_5 solution or ciprofloxacin-$^{13}C_3{}^{15}N$ solution (depending on the CRM target analyte) was spiked into the sample to make a 1:1 isotope ratio. Three milliliters of deionized water was added to reconstitute the sample. Acetonitrile was added (10 mL) to the sample and loaded on a mechanical shaker for 1 h for mixing and proper equilibrium of the endogenous analytes and its isotope standard. In this study, equilibrium time was extended to 1 h from 25 minutes, used in the previous study, after checking proper equilibrium time for cured materials used for the CRMs. The sample was centrifuged at 1520*g* for 10 min and then the acetonitrile layer was transferred into a 50-mL conical tube and mixed with 10 mL of *n*-hexane. The mixture was again blended via mechanical shaker for 10 min and centrifuged at 1520*g* for 5 min. The supernatant (*n*-hexane layer) was discarded and the remaining acetonitrile layer was dried under nitrogen using a TurboVap evaporator (Biotage, Sweden). The residue was reconstituted with 2 mL of 50 mmol/L monosodium phosphate buffer (pH 7.4) for solid-phase extraction (SPE) clean-up. The sample was cleaned by tandem SPE treatment on two different cartridges using a vacuum manifold. SupelMIPTM SPE-fluoroquinolone cartridge was preconditioned with 1 mL of methanol and 2 mL of ultra-pure water before the reconstituted sample was loaded onto the cartridge. The SPE cartridge was washed consecutively with 3 mL of ultra-pure water, 1 mL of acetonitrile, 1 mL of 0.5% acetic acid in acetonitrile (v/v), and 1 mL of 0.1% ammonia in ultra-pure water. Elution of the target analyte was performed using 2 mL of 2% ammonia in methanol and the collected solution was dried under nitrogen. The residue was reconstituted with 2 mL of 50 mmol/L monosodium phosphate buffer (pH 7.4) for the SPE treatment on an Oasis MAX cartridge. The cartridge was conditioned in series with 1 mL of methanol, 1 mL of 5 mol/L NaOH, and 1 mL of ultra-pure water. The reconstituted sample was loaded onto the cartridge, washed with 1 mL of 5% ammonia in water and 1 mL of methanol, and the target analyte was eluted with 2 mL of 0.2 mol/L HCl in methanol. The collected solution was dried under nitrogen, and the sample was reconstituted with 10 mL of mobile phase A (0.1% formic acid and 10 μmol/L EDTA in H_2O) for enrofloxacin or 1 mL of mobile phase A for ciprofloxacin.