Analytical Methods

Simultaneous determination of 38 veterinary antibiotic residues in raw milk by UPLC–MS/MS

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A B S T R A C T
A selective and rapid method has been developed to determine, simultaneously, 38 veterinary antibiotic residues in raw milk by ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). One milliliter of raw milk was diluted with 0.5 mL water and 3 mL acetonitrile, then purified using an Oasis HLB cartridge. The eluates were evaporated by nitrogen drying and then reconstituted to 4 mL with water/acetonitrile (8:1) before being injected into the UPLC–MS/MS system. The results indicated recoveries of 68–118% for 14 β-lactams, 79–118% for eight quinolones, 71–106% for eight sulphonamides, 76–116% for four tetracyclines, 78–105% for three macrolides, and 88–103% for one lincomamides, with coefficients of variation less than 15% for intraday and interday precisions. The limit of quantification for all antibiotics was 0.03–10 μg kg⁻¹. This methodology was then applied to field-collected real raw milk samples and trace levels of four antibiotics were detected.

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1. Introduction

Veterinary antibiotics are widely used to treat dairy cattle diseases, such as mastitis, diarrhea, and pulmonary diseases, or to increase milk yield (Erskine, Wagner, & DeGraves, 2003; McEwen & Fedorka-Cray, 2002). However, the overuse of antibiotics results in detectable antibiotic residues in milk, which cannot be completely destroyed with heat treatment and are easily transferred from the raw milk into milk products (Knecht et al., 2004; Wassenaar, 2005; Zorraquino, Althaus, Roca, & Molina, 2011). These residues can cause some adverse human health effects, such as allergic reactions, alterations of the delicate balance of intestinal flora, and antibiotic resistance (Paige, Tollefson, & Miller, 1997; Paige, Tollefson, & Miller, 1999).

In order to ensure the safety of human food, most countries have established official standard documents and have strictly regulated the maximum residue levels (MRLs) of veterinary drugs in animal-origin food. In China, the MRLs of 48 types of different veterinary drugs in cattle milk were set by No. 235 announcements (2002) (Han et al., 2012; MOA, 2002). In the cases of veterinary drugs that were not documented with MRLs, validation levels (VLs) were defined based on the related classes of the drugs and the characteristics of the compounds (Table 1) (Freitas, Barbosa, & Ramos, 2013).

Many papers have been published recently on the analysis methods of veterinary antibiotics in milk, such as microbiological and bioassay techniques, liquid chromatography with UV, photodiode array, and fluorometric detection (Christodoulou, Samanidou, & Papadoyannis, 2007; Knecht et al., 2004; Lamar & Petz, 2007; Pastor-Navarro, Maqueira, & Puchades, 2009). However, these methods lack the sensitivity and specificity required to ensure both the unequivocal identification and reasonable detection limits of veterinary drug residues (Freitas et al., 2013; Gaugain-Juhel et al., 2009; Le Bizec, Pinel, & Antignac, 2009).

Time of flight mass spectrometry (TOF–MS) methods have been used to screen hundreds of compounds in food matrices in one run, but they are less sensitive and repeatable than those based on the triple quadrupole system (Kaufmann, Butcher, Maden, & Widmer, 2008; Peters, Bolck, Rutgers, Stolk, & Nielen, 2008).
2009). Ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) methods have become a significant trend in the confirmation and quantification of as many drug residues in milk as possible. However, the reported use of UPLC–MS/MS has been focused only on single-drug classes of veterinary antibiotic residues in milk, mainly due to the different physico-chemical properties of the various veterinary drugs and the complexity of the extraction and clean-up processes (Koesukwiwat, Jayanta, & Leepipatpiboon, 2007; Lopez, Pettis, Smith, & Chu, 2008; Tang, Lu, Lin, Shihi, & Hwang, 2012).

The extraction and clean-up of drugs are the most important steps in analyzing multiclass antibiotic residues (Aerts, Hogenboom, & Brinkman, 1995). Milk is a complex matrix with high contents of protein and fat, which can bind antibiotics and interfere with their extraction. Acetonitrile is the best solvent for extraction because of its good performance on protein deposition and enzyme denaturation. Cleanup methods include solid phase extraction, QuEChERS, and matrix solid phase dispersion (Garrido-French, Aguileru-Luiz Mdel, Martinez Vidal, & Romero-Gonzalez, 2010; Heller, Nochetto, Rummel, & Thomas, 2006). However, recovery of veterinary antibiotic residues has not been satisfactory (Jimenez, Rubies, Centrich, Companyo, & Guteras, 2011).

The aim of the present study was to determine, simultaneously, 38 selected veterinary antibiotic residues (14 β-lactams, eight quinolones, eight sulfonamides, four tetracyclines, three macrolides, and one lincosamide) in raw milk by UPLC–MS/MS. The extraction and purification conditions of these veterinary antibiotics were optimized, and satisfactory recoveries were obtained. The UPLC–MS/MS analysis of the 38 veterinary antibiotics was applied to raw milk samples collected on dairy farms in Beijing and traces of four veterinary antibiotic residues were detected.

2. Materials and methods

2.1. Chemicals and reagents

Amoxicillin (AMOX), ampicillin (AMP), cloxacillin (CLOX), oxacillin (Oxac), dicloxacillin (DICL), nafcillin (NAFC), cefoperazone (PER), ceftiquinome (QUI), cepahipirin (PIR), ciprofloxacin (CIP), danofloxacin (DAN), enrofloxacin (ENR), flumequine (FLU), marbofloxacin (MAR), difloxacin (DIF), orbifloxacin (ORB), sarafloxacin (SAR), tetracycline (TET), terramycin (TER), chlorotetracycline (CTC), erythromycin (ERY), lincomycin (LIN), tylosin (TYL) and tilmicosin (TIL) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Penicillin G (PENG) and ceftazolin (ZOL) were obtained from USP (Rockville, MD). Cephalaxin (LEX), cefitriof (TIO), cefalomion (LON), sulfaguanidine (SGD), sulfapyridine (SPD), sulfadiazine (SDZ), sulfamonmetoxine (SMM), sulfamethoxazole (SMX), sulfaemetazine (SMZ), sulflachloropryridazine (SP2DZ), trimethoprim (TMP) and doxycycline (DOX) were obtained from Sigma (St. Louis, MO). Acetonitrile, methanol, formic acid and acetic acid were HPLC grade and purchased from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate (>99.0%) and disodium hydrogen phosphate (>99.0%) were obtained from Merck. Double-deionized water was obtained with a Milli-Q Gradient water system (Millipore, Bedford, MA).

Stock standard solutions of all analytes were prepared at 1000 mg L⁻¹ by dissolving the compounds in an appropriate solvent, such as acetonitrile (AMOX, AMPI, CLOX, PENG, DICL, NAFC, PER, QUI, LEX, TIO, ZOL, LON) or methanol (OXAC, PIR, CIP, DAN, ENR, FLU, MAR, DIF, ORB, SAR, SGD, SPD, SDZ, SMM, SMX, SMZ, SPDZ, TMP, DOX, TET, TER, CTC, ERY, LIN, TYL, TIL). These standard solutions were stored at −20°C in dark glass bottles during the three-month validity period and diluted with acetonitrile or methanol to prepare working solutions. The working solutions were kept at −20°C in dark glass bottles for a month, after which they were replaced with fresh solutions. The 0.1 mol L⁻¹ phosphate buffer solution (PBS) was prepared by dissolving 32.3 g disodium hydrogen phosphate and 4.5 g sodium dihydrogen phosphate in 1000 ml ultra-pure water and adjusted to pH 8.5 using 1 mol L⁻¹ hydrochloric acid or 1 mol L⁻¹ sodium hydroxide. Oasis-HLB solid phase extraction cartridges (60 mg, 3 cm³) were obtained from Waters (Milford, MA).

2.2. Instrumentation

The chromatographic analyses were performed on an Acquity UPLC system, and separations were achieved using an Acquity UPLC BEH C18 column (1.7 μm particle size, 50 mm × 2.1 mm; Waters). The analytes were separated with 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⁻¹. The separation was performed at 40°C, applying the following gradient program: 0–0.5 min, 5% A; 0.5–1 min, linear increase to 10% A; 1–3 min, linear increase to 40% A; 3–4 min, linear increase to 90% A; 4–4.1 min, decrease to 40% A; and finally, 4.1–6.5 min, 5% A. The samples were kept in an autosampler at 15°C.

The mass spectrometry analyses were carried out using a Waters Acquity TQSM Micromass Quattro Ultima triple-quadrupole MS quadrupole equipped with an electrospray ion source (Micromass, Manchester, UK). The instrument was operated 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⁻¹ desolvation gas (nitrogen >99.999%) flow. Data acquisition was performed using MassLynx V 4.1 software with the Quanlynx program (Waters).

Other equipment used for sample preparation was as follows: electronic balance (0.0001 g precision; Sartorius, Goettingen, Germany), vortex mixer (IKA Labortechnik, Staufen, Germany), CR22G centrifuge (Hitachi, Tokyo, Japan), pressure N2 gas blowing concentrator (Organonation, Berlin, MA), PB-10 pH meter (Sartorius, Goettingen, Germany).

2.3. Extraction procedures

One milliliter of blank fresh milk was transferred to a polypropylene centrifuge tube (10 mL), and then 0.5 mL water and 3 mL acetonitrile were added to extract the veterinary drug residues. The mixture was vortexed for 5 min and centrifuged at 6500 g for 10 min. The upper layer was removed and evaporated at 48°C to 1 mL under nitrogen. The residue was reconstituted with 3 mL of 0.1 mol L⁻¹ PBS and the pH was adjusted to 8.5. The mixture was vortexed for 5 min and then applied to an Oasis HLB cartridge at a flow rate of 0.5 mL min⁻¹. The cartridge had previously been activated with 2 mL of MeOH, 2 mL of water and 2 mL of PBS (pH 8.5). After washing with 2 mL of PBS (pH 8.5) and 1 mL of water, the analytes were eluted with 3 mL of acetonitrile/water solutions (1:1 by volume). After the eluates were evaporated at 48°C to less than 1.5 mL under nitrogen, 0.44 mL of acetonitrile was added in. Then, the mixture was reconstituted to 4 mL with water. After the solution was filtered through a 0.22 μm polyvinylidene difluoride membrane, 5 μL of the final extract solution was transferred to vials and injected into the UPLC–MS/MS system under multiple reaction monitoring (MRM) optimized conditions for each compound.

2.4. Validation

The parameters of linearity, recovery, precision, and analytical limits were validated mainly based on EU Commission Decision 2002/657/EEC (Commission, 2002). The linearity was evaluated
by matrix-matched calibration curves at different spiked levels. Depending on the response value of each analyte to the mass spectrometry, the drugs were categorized into six groups. Group 1 included FLU, SPD, SMX, and LIN, with the following spiking levels: 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 1 and 2 \( \text{µg kg}^{-1} \). Group 2 included SDZ, SMM, SMZ, SPDZ, ERY, and TYL, with the following spiking levels: 0.03, 0.06, 0.12, 0.24, 0.48, 1, 1.5 and 2 \( \text{µg kg}^{-1} \). Group 3 included AMPI, DAN, ENR, ORB, SAR, DIF, SGD, and TMP, with the following spiking levels: 0.1, 0.2, 0.4, 0.8, 1.0, 1.2, 2 and 2.5 \( \text{µg kg}^{-1} \). Group 4 included CIP, MAR, QUI, PER, TIO, PENG, OXAC, CLOX, DICL, AMOX, LON, DOX, TET, TER, CTC, and TIL, with the following spiking levels: 0.3, 0.6, 0.9, 1.2, 1.8, 2.4, 2.7 and 3 \( \text{µg kg}^{-1} \). Group 5 included PIR, ZOL, and NAFC, with the following spiking levels: 1, 1.2, 1.4, 1.8, 2, 2.5, 3, 4 \( \text{µg kg}^{-1} \). The last group included only LEX, and the spiking levels were 5, 7.5, 10, 12.5, 15, 17.5, 25, and 30 \( \text{µg kg}^{-1} \). Limits of detection (LOD) and quantification (LOQ) were defined as lowest concentrations with a signal-to-noise (S/N) ratio of 3 for LOD or 10 for LOQ. The recovery was evaluated at three spiked levels of each antibiotic. The precision of the method was evaluated in terms of repeatability (intraday precision: single day, five replicates) and intermediate precision (interday precision: three different days) at a spiked concentration.

2.5. Samples

Twenty-five raw milk samples were collected from local dairy farms in Beijing (China). The milk samples were used as blank milk samples in the preparation of calibration standards and during the validation study, and were further analyzed using the procedure described below.

3. Results and discussion

3.1. Optimization of UPLC–MS/MS conditions

In order to achieve better separation and retention of analytes, acetonitrile, methanol, formic acid and ammonium acetate aqueous solutions were investigated. A compromise was required

Table 1

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MRLs (( \text{µg kg}^{-1} ))</th>
<th>VLs (( \text{µg kg}^{-1} ))</th>
<th>Retention time (min)</th>
<th>Parent ion (m/z)</th>
<th>Daughter ion (m/z)</th>
<th>Collision energy (eV)</th>
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<td></td>
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</table>

MRLs, maximum residue levels; VLs, validation levels; /, no MRL.
between mobile phase composition and MS response for the 38 selected veterinary antibiotics. Regarding the organic solvent, acetonitrile showed better sensitivity (S/N) and peak shape than methanol. The addition of formic acid provided better results than ammonium acetate, and the former improved the ionization efficiency. The best results were obtained when acetonitrile was used as an organic modifier and 0.1% formic acid was used in aqueous solution.

The optimization of the MS parameters (collision energy) was performed by infusing a standard solution of 5 µg L⁻¹ of each antibiotic in a mixture of acetonitrile/water (1:8 by volume). Atmospheric pressure chemical ionization and ESI probes were assayed, both in positive ionization mode. Full-scan spectra were acquired in order to select the most abundant \( m/z \) value, optimizing the cone voltage. In all cases, \([\text{M+H}]^+\) ions were found to be the most abundant and were selected as the precursor ions. Collision energies were studied in order to find the most abundant product ions, selecting the most sensitive transition for quantification purposes and another for confirmation. Table 1 shows MS/MS transitions for quantification and confirmation as well as collision energy values optimized for each of the selected compounds. Fig. 1 shows total ion current of a milk sample spiked with 5 µg kg⁻¹ of each of the 38 veterinary antibiotics.

3.2. Optimization of the extraction procedure of antibiotic residues

Three different extraction procedures of veterinary antibiotic residues in raw milk were used to remove protein and lipid components, which can interfere with the analytical process. In extraction procedure A, 1 mL of raw milk sample was diluted with 3 mL water and the mixture was applied directly to the HLB cartridge after centrifugation; in extraction procedure B, 1 mL of raw milk sample was diluted with 0.5 mL water and 3 mL acetonitrile, and the mixture was centrifuged and applied to the HLB cartridge; in extraction procedure C, 4 mL acetonitrile were directly added to a 1-mL raw milk sample, and the mixture was centrifuged and applied to the HLB cartridge. Extraction procedure A was the simplest procedure, but the HLB cartridge was easily blocked and the extraction procedure was stopped. Fig. 2 shows the effects of the extraction procedures on the recovery (%) of the 38 veterinary antibiotics. In most cases, procedures B and C both had good recoveries of the veterinary antibiotics, indicating that acetonitrile was able to prevent the interference of protein and lipids. However, certain antibiotics (i.e., AMOX, AMPI and PENG) had recoveries that were too low (below 60%) with extraction procedure C. When the concentration of acetonitrile in solvent was too high, it was difficult to extract some highly polar components, such as \( \beta \)-lactams. Therefore,
Fig. 2. Effects of extraction procedures on recovery (%) of the 38 veterinary antibiotics. Extraction procedure B: 1 mL of raw milk was diluted with 0.5 mL water and 3 mL acetonitrile. Extraction procedure C: 1 mL of raw milk was diluted with 4 mL acetonitrile.

Fig. 3. Effect of pH on recovery (%) of the 38 veterinary antibiotics.

Fig. 4. Effects of air- and nitrogen-drying on recovery (%) of the 38 veterinary antibiotics.
Table 2
Validation parameters of UPLC-MS/MS at spiked levels for the 38 veterinary antibiotics in raw milk.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Linear equation</th>
<th>Coefficient of determination ($R^2$)</th>
<th>LOD ($\mu$g kg$^{-1}$)</th>
<th>LOQ ($\mu$g kg$^{-1}$)</th>
<th>Recoveries $R$ (%)</th>
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Some tetracyclines were degraded with the heat treatment of air drying (50 °C); Hassani, Lazaro, Perez, Condon, and Pagan (2008) has reported 98% of tetracyclines in milk were destroyed with the traditional pasteurization treatment (65 °C, 30 min) (Hassani et al., 2008). Therefore, nitrogen drying was preferred for evaporation of that sample.

3.3 Method validation

Matrix-matched standard calibration curves were performed to achieve good accuracy and to compensate for the matrix effect and loss in the sample preparation. Good linearity was found in the studied ranges (0.01–2 μg kg⁻¹ for the compounds in group 1; 0.03–2 μg kg⁻¹ for group 2; 0.1–5 μg kg⁻¹ for group 3; 0.3–3 μg kg⁻¹ for group 4; 1–4 μg kg⁻¹ for group 5, and 5–30 μg kg⁻¹ for group 6), with coefficients of determination (R²) higher than 0.990, except for PENG (0.9895), PIR (0.9871), LEX (0.9892), TER (0.9871), TYL (0.9897), and TIL (0.9851). The LODs and LOQs of the 38 compounds calculated for S/N of 3 and 10 were from 0.01–5 μg kg⁻¹ and 0.03–10 μg kg⁻¹, respectively. Detailed linearity, limit of detection, and quantitation results are summarized in Table 2. The LOQs of all of the analytes were far below the MRLs regulated by China and the EU and fit for trace analysis of the veterinary antibiotic residues in milk.

The method was validated by the spiking 38 veterinary drugs at three different concentrations in raw milk blank samples, each in triplicate (0.03, 0.05, and 0.1 μg kg⁻¹ for group 1 compounds; 0.1, 0.15, and 0.3 μg kg⁻¹ for group 2; 0.3, 0.5, and 1 μg kg⁻¹ for group 3; 1, 1.5, and 3 μg kg⁻¹ for group 4; 3, 5, and 10 μg kg⁻¹ for group 5; 15, 25, and 30 μg kg⁻¹ for group 6). Accuracy and precision were evaluated by means of recovery experiments at the spiking levels. The results were satisfactory for all analytes, with recovery rates ranging 68–118% (Table 2). The specificity of the method was studied by analyzing blank samples. No peaks were observed at the same retention times of the target veterinary antibiotics, indicating that there was no matrix compound-induced false signal in the samples.

The precision of the method was proved in terms of repeatability (intra-day and inter-day precision), expressed as the relative standard deviations (RSD) values of the set of replicate analyses at the spiked levels. Table 2 shows the coefficients of variation for the determination of the 38 veterinary antibiotics in raw milk. All of the intra-day and inter-day RSD values were lower than 15%, indicating good repeatability and reliability of the proposed method.

3.4 Application of the method to real samples

A total of 25 raw milk samples collected from local dairy farms in Beijing were analyzed. Traces of four veterinary antibiotic residues below allowable levels were detected in three samples: 2.58 μg kg⁻¹ of flumequine (sample No. 3), 1.77 μg kg⁻¹ of sulfapyridine and 4.2 μg kg⁻¹ of sulfamethoxazole (sample No. 8), and 11.25 μg kg⁻¹ of lincomycin (sample No. 11).

4. Conclusion

A selective and rapid method was developed and validated for the simultaneous detection of 38 veterinary antibiotics, from six different classes (14 β-lactams, eight quinolones, eight sulfonamides, four tetracyclines, three macrolides and one lincosamide) in raw milk samples. The method was satisfactory in terms of linearity, recovery, precision, and analytical limits under the requirements of the European Communities Decision 2002/657/EC. The method enables the determination of the target veterinary antibiotic residues in the low ng/g range and is qualified to carry out risk warnings for veterinary antibiotic residues in raw milk.

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