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Determination of tetracyclines in pig and other meat samples using liquid chromatography coupled with diode array and tandem mass spectrometric detectors

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ABSTRACT

Two high performance liquid chromatographic methods (HPLC–DAD and LC–MS/MS) were developed to analyze tetracycline (TC) residues in pig meat (pork) samples. The method involved a sample preparation using a solid–liquid extraction (SLE) by McIlvaine buffer, followed by a solid-phase extraction (SPE) clean-up using Strata-XL cartridges. The developed sample clean-up resulted in a selective chromatogram in the HPLC–DAD separation and a reduced matrix effect (ME) in LC–MS/MS analysis. Moreover, HPLC columns packed with core–shell particles were tested for separation, which further enhanced the sensitivity and the selectivity of determinations. The validation of the methods for pig samples was carried out according to European Union 2002/657/EC decision. In addition, validation was also performed for bovine, chicken, and turkey meat samples using HPLC–DAD method. The performance characteristics of determinations were evaluated with both spiked and incurred samples, and were systematically compared. LC–MS/MS technique was found to be more accurate for spiked samples; however, HPLC–DAD method resulted in more reliable concentrations for incurred samples.

1. Introduction

Tetracyclines (TCs) are commonly used antibiotics in human and animal therapy. TCs are good alternative to macrolides and beta lactam antibiotics because they are effective against a wide range of Gram (+) and Gram (−) bacteria. The application of TCs in livestock led to the detection in foods of animal origin (Blasco, Di Corcia, & Picó, 2009; Cherlet, Schelkens, Croubels, & De Backer, 2003; Fritz & Zuo, 2007; Zhou et al., 2009). Currently, there is growing concern of antibiotic resistance that can be formed in infectious bacteria (Liu et al., 2012; Oberlé, Capdeville, Berthe, Budzinski, & Petit, 2012). Consequently, the European Union (EU), the United States of America (USA), and Russia have set maximum residue limit (MRL) concentrations for TCs in foods of animal origin. In the EU, four TCs have been authorized for using in animal therapy, namely oxytetracycline (OTC), tetracycline (TC), chlorotetracycline (CTC), and doxycyclines (DC). They all belong to group B compounds and share a MRL of 100 μg/kg in meat (Commission Regulation (EU) No. 37/2010). In Russia, the limit has been reduced to 10 μg/kg. In the USA, the MRL is 2000 μg/kg. The epimers of OTC, TC, and CTC have also been included in the regulations; hence, MRL is expressed as the sum of the parent compound and its epi forms (Commission Regulation (EU) No. 37/2010).

Numerous analytical methods have been applied to analyze TCs (Anderson, Rupp, & Wu, 2005; Oka, Ito, & Ikai, 1998; Oka, Ito, & Matsumoto, 2000; Önal, 2011). Screening detection of TCs in biological matrices can be done by using microbiological four-plate test or immunoassay (e.g. ELISA). ELISA has a good specificity only for TC and CTC (>70%). Chromatographic or electrophoretic determinations are usually needed to confirm and to quantify accurately TCs in samples. High or ultra high performance liquid chromatography (HPLC/UHPLC) has been considered as the preferable method for determining TCs in complex matrices (Önal, 2011). TCs have shown enhanced separation on fully porous C-18, C-8, phenyl, or polymeric based columns. After LC separation, the detection of TCs could be carried out using optical and mass spectrometric (MS) detectors. Among optical detectors, ultraviolet (UV) detectors are preferred due to their high sensitivity for TCs (Önal, 2011). Significantly, EU allows the confirmation of authorized substances with UV detection (Commission Decision, 2002/657/EC). However, this detection cannot be analyzed under 10 μg/kg as accurately as required.

The analytical limits can be enhanced using improved chromatographic separation and/or MS detection. For example, the application of core–shell HPLC column enables high sensitivity due to increased mass transfer, reduced longitudinal diffusion, and favorable eddy dispersion (Fekete, Oláh, & Fekete, 2012). In addition, LC separation

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coupled to MS detectors (e.g. single or triple quadrupole, time-of-flight analyzers) allowed the detection of TCs in sub-μg/kg level (Bousova, Senyuva, & Mittendorf, 2013; Jin et al., 2010; Önal, 2011). Accurate analysis of target compounds using MS detectors requires a careful sample preparation in order to avoid the matrix effect (ion suppression or ion enhancement) in the ion source (Matuszewski, Constanzer, & Chavez-Eng, 2003; Tölgyesi, Fekete, et al., 2012; Tölgyesi & Kunsági, 2013; Tölgyesi, Sharma, et al., 2012; Tölgyesi, Tölgyesi, Sharma, Sohn, & Fekete, 2010). In the last few years, our efforts have been to prepare samples for analyzing low levels of pharmaceuticals and other contaminants in biological samples using HPLC separation and tandem mass spectrometric (MS/MS) detection (Tölgyesi, Fekete, et al., 2012; Tölgyesi & Kunsági, 2013; Tölgyesi, Sharma, et al., 2012; Tölgyesi et al., 2010; Tölgyesi et al., 2013).

In the present paper, two HPLC methods (HPLC–DAD and LC–MS/MS) were developed for simultaneous analyses of OTC, TC, CTC, and DC using Strata-XL SPE cartridge for sample clean-up and HPLC columns packed with core–shell materials (Ascentis Express C-18 and Kinetex C-18) for chromatographic separations. Objectives of the study were: (i) optimization of the HPLC–DAD and LC–MS/MS methods to achieve adequate performance characteristics, (ii) validation of the developed methods in accordance with EU 2002/657/EC decision, (iii) application of the methods to real (incurred) samples, and (iv) the brief comparison of the developed techniques based on the observed/acquired results (e.g. matrix effect, accuracy, recovery, precision and analytical limits). Significantly, the success of the application of methods is proven by participating in an international proficiency test.

2. Experimental

2.1. Reagents, solutions, equipments, and samples

Standards of OTC hydrochloride, TC hydrochloride, CTC hydrochloride, DC hyclate, and methacycline hydrochloride (MC) were obtained from Sigma-Aldrich (Budapest, Hungary). MC was used as an internal standard in the LC–MS/MS analysis. Aminoglycosides (streptomycin, kanamycin, gentamycin) and lincomycin standards were purchased from Sigma-Aldrich (Budapest, Hungary). Both methanol and acetonitrile were of HPLC grades and were obtained from Lab-Scan (Budapest, Hungary). Suprapur formic acid (98–100%) and acetic acid (100%) were obtained from Merck (Budapest, Hungary). Trifluoroacetic acid (TFA), EDTA-Na × $2H_2O$, and Na_2PHO_4 × $2H_2O$ were obtained from Molar (Budapest, Hungary). Citric acid monohydrate was purchased from RK-TECH (Budapest, Hungary). McIlvaine buffer used for a sample extraction contained 37.2 g EDTA-Na × $2H_2O$, 10.9 g Na_2PHO_4 × $2H_2O$, and 12.9 g citric acid monohydrate; dissolved in 1000 mL of water (pH 4.5).

Stock solutions were individually prepared by dissolving 25 mg of standards (of accurate weight) in 25 mL methanol ("A" grade glass volumetric flask) to obtain a concentration of about 1 mg/mL. True concentrations of stock solutions were determined by considering the salt form of standards. A working standard solution containing 1 μg/mL concentration of each OTC, TC, CTC and DC was prepared by appropriately diluting the stock solutions with methanol. Stock and working standard solutions for the ISTD (MC) were prepared using the same procedure as was outlined above for the tetracycline standards. Working standard solutions were freshly prepared weekly and stored at −20 °C. Stock solutions were stored at −20 °C for up to three months.

Phenomenex Strata-XL SPE cartridges (6 mL, 200 mg, 100 μm), Kinetex C-18 HPLC column (150 mm × 4.6 mm, 2.6 μm), and HPLC vials were purchased from Gen-lab Ltd. (Budapest, Hungary). Supleco Ascentis Express C-18 HPLC column (150 mm × 4.6 mm, 2.7 μm) was obtained from Sigma-Aldrich (Budapest, Hungary). Merck Purospher C-18e (125 mm × 4.6 mm, 5 μm) and Varian OmniSpher C-18 (250 mm × 4.6 mm, 5 μm) columns were obtained from Merck (Budapest, Hungary) and BST Corp. (Budapest, Hungary), respectively. The meat samples for method development and validation were originated from the Hungarian residue control monitoring program (January 2012 to December 2012) and were stored at −20 °C until subjected to analysis. Proficiency test samples (incurred pig meats) were obtained from the ANSES European Union Reference Laboratory (EU-RL, Fougeres, France). A pig liver certified reference material (CRM) containing incurred CTC (580 ± 110 μg/kg) was purchased from the Joint Research Centre Institute for Reference Materials and Measurements (JRC IRMM, Geel, Belgium).

2.2. Instruments

A Sigma 3–18 K centrifuge (Osterode am Harz, Germany), a Janke & Kunkel IKA KS125 shaker (Staufen, Germany), and a TurboVap LV evaporator (Hopkinton, MA, USA) were used during the sample preparation. HPLC-DAD instrument was a HP 1100 LC system, equipped with a G1322A degasser, a G1311A quaternary pump, a G1313A autosampler, a G1316A column thermostat, and a G1315A DAD detector (Agilent Technologies, Waldbronn, Germany). Agilent Technologies ChemStation A.10.02 (1757) software was used for data acquisition and evaluation. The LC–MS/MS system consisted of an Agilent Technologies 1200 binary pump HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat), coupled to an Agilent 6410A triple quadrupole mass spectrometer, which was equipped with an Agilent G1978B multimode ion source (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and evaluation were performed using Agilent Mass Hunter B.01.04 software.

2.3. Sample preparation

Five gram samples were weighed into 50 mL polypropylene centrifuge tubes and 10 mL McIlvaine buffer was added to the samples before vortex-mixing for 30 s. During the method development, 2% acetic acid in water (v/v, pH 2) was also tested as a sample extraction medium. The tubes were then sealed and shaken at ambient temperature for 30 min (700/min), followed by centrifugation at 25 °C for 10 min using 15,652 × *g* speed (10,000 rpm). After centrifugation, the supernatants were collected in glass tubes. Extraction was repeated one more time and the upper layers were pooled and homogenized. Ten mL extracts were transferred onto Strata-XL cartridges, which were previously conditioned with 6 mL methanol, 6 mL water, and 6 mL McIlvaine buffer, respectively. Extracts were passed through SPE columns drop wise and cartridges were washed with 6 mL McIlvaine buffer and 6 mL water. SPE columns were vacuum dried for 10 min before eluting the target compounds with 6 mL methanol containing 0.02 M oxalic acid into glass receiving tubes. Sample elution with 5% (v/v) acetic acid in methanol, 100% acetonitrile, 5% (v/v) acetic acid in acetonitrile, 1% (v/v) TFA in acetonitrile were also tested during method development. In the case of samples prepared for LC–MS/MS analysis, ISTD (MC, 100 μg/kg) was also added to the receiving tubes. Samples were evaporated to 50–100 μL at 45 °C under a gentle stream of nitrogen. The final volume of samples was adjusted to 500 μL with 0.01 M aqueous oxalic acid and homogenized with vortex-mixing for 20 s. As the last step, samples were filtered through 0.45 μm Phenex nylon filters (Gen-lab Ltd., Budapest, Hungary) into 2.0 mL HPLC vials.

2.4. HPLC–DAD conditions

Tetracyclines were separated on an Ascentis Express C-18 (Fused-core®) HPLC column using a linear gradient elution. The initial mobile phase consisted of 0.01 M aqueous oxalic acid solution–acetonitrile (90/10, v/v, pH 2). The acetonitrile composition of the mobile phase was 10% at 0 min, 15% at 8 min, 25% at 15 min, 25% at 20 min, and 100% at 21 min. The stop and post times were 28 and 10 min, respectively. Injection volume was 20 μL and the flow rate was adjusted at 0.8 mL/min. Detection wavelength was set at 365 nm