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Determination of veterinary antibiotics in bovine urine by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A follow-up of antibiotics (tetracyclines, fluoroquinolones, cephalosporins, penicillins and amphenicols) in the bovine urine is important for two reasons: to understand if they are still present in organism, and whether their occurrence in urine might be considered as an environmental risk. A validated HPLC–MS/MS method (Decision 2002/657/EC) for antibiotics determination in bovine urine was developed. CCα and CCβ were in the range of 0.58–0.83 and 0.55–1.1 ng mL^{-1}, respectively. Recoveries were 92–108%, with inter-day repeatability below 12%. Analysis of bovine urine revealed frequent presence of tetracyclines, which was related with animal's age. The cause, most presumably, might be found in different therapeutic protocols applied for veal calves and young bulls enrolled in this study. Most abundant was oxytetracycline with highest level in veal calves (1718 ng mL^{-1}) vs. young bulls (2.8 ng mL^{-1}). Our results indicate the necessity of antibiotics monitoring in bovine urine before animals undergo further processing in the food industry.

1. Introduction

Antibiotics constitute an important group of pharmaceuticals that have been widely used in veterinary medicinal practices to treat a wide range of diseases. Only 20% of antibiotics are used to medicate sick animals, while 80% are used as production tools: either to prevent diseases that arise from the way animals are treated during breeding (so-called "production diseases"), or for growth-promotion purposes. The widespread exploitation of antibiotics in the past has favoured the growth of resistant microorganisms, resulting in ever widening antimicrobial resistance, an important human health issue. On Dec. 11th, 2013, the U.S. Food and Drug Administration (FDA) announced important steps to ensure the judicious use of antibiotics in food animals, as one approach to addressing antimicrobial resistance in human medicine (FDA, implementing plan). European Union (EU) national and international authorities emphasise the need for environmental and health risk assessment for chemicals with antimicrobial effects (Kools, Moltmann, & Knacker, 2008; Serratosa et al., 2006). As a result, new strategies to reduce antibiotic utilisation in animal husbandry have been proposed (Trevisi et al., 2014). The increasing awareness of food safety with respect to antimicrobial resistance (European Community, 2005a) has resulted in the banning of any antibiotic with growth-promoting activity: antibiotics are only allowed to be added to animal feed for therapeutic purposes (European Community, Regulation 1831/2003/EC). This decision was based on opinions from the Scientific Steering Committee, which recommended the progressive phasing-out of antibiotics used for growth stimulation, while still preserving animal health and animal welfare (European Community, 2005b).

Generally, the food animal industry has grown into an integrated production system where large quantities of antibiotics are administered to the animals for therapeutic or sub-therapeutic purposes. This may lead to accumulation of residues in food matrices as milk (Zhan et al., 2012) or meat (Stubbings & Bigwood, 2009). These residues may include the non-altered parent compounds as well as metabolites, and may have direct or indirect toxic effects on consumers. Logically, these compounds are excreted by the animals and end up in the urine and faeces. This, consequently, carries substantial environmental problems, as during the maturation process, the animal dejections become manure, which is frequently used in agriculture.

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To minimise the exposure of antibiotics to humans and safeguard public health, European legislation (Commission Regulation ECC/2377/90 and 37/2010) has established corresponding tolerance levels, termed as maximum residual limits or levels (MRLs), for controlling the use of antibiotics in food-producing animals. Also, the Italian National Residue Control Plan (NRCP, 2014) is very precise: samples taken at slaughterhouse are screened for the presence of residues/metabolites on the bases of MRLs. Analysis of positive screening tests for these residues in animal products must adhere to legislation laid out in Council Directive 96/23/EC and Commission Decision 2002/657/EC, whereby suitable confirmatory methods are based on chromatographic analysis and mass spectrometric detection.

Despite the above mentioned research that deals with the determination of antibiotics in food, manure (Panseri et al., 2013), soil (Carballo, Barreiro, Scharf, & Gans, 2007) and waste water (Babic, Asperger, Mutavdzic Horvat, & Kastelan-Macan, 2006), the data on the animal's urine content of the most frequently exploited antibiotics in veterinary medicine are sporadic. To the best of our knowledge, no method has previously been reported for simultaneous screening of major antibiotics groups in bovine urine as a starting matrix, although reports on detection of antibiotics in general in bovine urine are already available (Heller, Smith, & Chiesa, 2006; Kondo, Morikawa, & Tateyama, 1989). In addition, reports on multiclass analysis of antibiotics in human urine has been published recently (Cazorla-Reyes, Romero-González, Frenich, Rodríguez Maresca, & Martínez Vidal, 2014; Wang, Wang, Zhou, & Jiang, 2014) as well as determination of some individual groups e.g. tetracyclines (Jin et al., 2010).

Urine analysis is a useful alternative to improve the effectiveness of surveillance plans, as it offers several advantages compared to the analysis of other biological samples (liver, kidney, blood, muscles, etc.). Urine collection, similarly to hair sampling (Fernández et al., 2014) is a non-invasive procedure and offers a possibility to evaluate drug withdrawal time after eventual inevitable treatment of sick animals.

The antibiotics considered in this investigation were selected using the following criteria: documented frequent utilisation, lower degree of metabolism in animals' bodies, and environmental traits. Additionally, the exemplification of different classes of antibiotics was aimed at covering a wide-ranging assortment of substances with antimicrobial activity used in Italian animal husbandry. Therefore, our antibiotics of interest were amoxicillin and ampicillin (penicillins), chlortetracycline, doxycycline, oxytetracycline, tetracyclines (tetracyclines), ciprofloxacin, enrofloxacin, lomefloxacin, marbofloxacin (fluoroquinolones), cephalexin, cefquinome (cephalosporins), florfenicol, florfenicol amine (amphenicols APHs) and streptomycin (aminoglycoside).

The simultaneous determination of these compounds is especially difficult because of large differences in their physicochemical properties, such as polarity, solubility, pK_a, and stability. Many liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods have been employed for a multiclass determination of antibiotics in various matrices including foodstuffs and environmental samples, relying on different purification strategies (Boix et al., 2014). There are various factors which need to be taken into consideration during development of a method that would be capable of analysing the wide range of compounds to the required level (e.g. pH, extraction methods, mobile phase composition, mass spectrometry acquisition parameters).

This paper reports the results of our work on multi-residue analysis using LC–MS/MS to determine the concentrations of target antibiotics, with a single SPE pre-treatment, chromatographic separation and mass detection method. The method was developed in order to test eventual presence of antibiotics residues in bovine urines collected at a slaughterhouse.

2. Materials and methods

2.1. Chemicals and reagents

All solvents were of HPLC or analytical grade and purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Formic acid 98–100% and hydrochloric acid 37% were obtained from Riedel-de Haën (Sigma–Aldrich, St. Louis, MO, USA). Water was purified by a Milli-Q system. The chemicals for the preparation of artificial urine were from Sigma–Aldrich (St. Louis, MO, USA). The extraction cartridges (Oasis HLB 3 cc, 60 mg) were provided by Waters (Milford, MA, USA). Amoxicillin, ampicillin, cefalexin, cefquinome sulphate, florfenicol, florfenicol amine, lomefloxacin hydrochloride, ciprofloxacin, enrofloxacin, marbofloxacin, tetracycline hydrochloride, doxycycline hyclate, chlortetracycline hydrochloride, oxytetracycline (as European Pharmacopoeia Reference Standard), streptomycin solution (1 mg mL^{-1} in 1 mM EDTA) and sulfameter (internal standard IS) were purchased from Fluka (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Artificial urine preparation

Artificial urine was prepared in our laboratory for the validation studies, as described by Fabregat, Pozo, Marcos, Segura, and Ventura (2013). Briefly, 0.1 g of lactic acid, 0.4 g of citric acid, 2.1 g of sodium bicarbonate, 10 g of urea, 0.07 g of uric acid, 0.8 g of creatinine, 0.37 g of calcium chloride·2H_2O, 5.2 g of sodium chloride, 0.0012 g of iron(II) sulphate·7H_2O, 0.49 g of magnesium sulphate·7H_2O, 3.2 g of sodium sulphate·10H_2O, 0.95 g of potassium dihydrogen phosphate, 1.2 g of dipotassium hydrogen phosphate, and 1.3 g of ammonium chloride were dissolved in 1 L of ultrapure water.

2.3. Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared in methanol and kept at −40 °C. Working solutions, containing each of the studied analytes at the concentrations of 10 and 100 ng mL^{-1}, were prepared daily. Each working solution was maintained at 4 °C during the method validation procedures.

2.4. Sample collection

In order to verify the developed method in actual conditions, 39 urine samples were collected at the slaughterhouse before processing. The samples arrived from three different slaughterhouses and were randomly collected from Friesian veal calves (6 and 11 months old) and Limousine young bulls (18 months old). Following collection they were immediately frozen (−20 °C) and taken to laboratory. During the transportation, the samples remained frozen using the dry ice. Upon the arrival in laboratory the samples were finally stored at −40 °C until the analysis was performed.

2.5. Sample extraction

Urine samples (5.5 mL) were centrifuged 5 min at 2500*g* at 4 °C. Five mL of supernatant was spiked with the internal standard to the final concentration of 2 ng mL^{-1}. The compounds of interest were extracted by using the Oasis HLB Cartridges under vacuum. The SPE cartridges were preconditioned with 3 mL of methanol, 3 mL of 0.5 M HCl and 3 mL of Milli-Q water. The samples were loaded, and after all the urines passed through the SPE, the cartridges were washed with 3 mL of water and 3 mL of methanol: water (20:80, v/v). Finally, samples were eluted using 5 mL of