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Journal of Chromatography B, 857 (2007) 142–148
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous determination of selected veterinary antibiotics in gilthead seabream (*Sparus Aurata*) by liquid chromatography–mass spectrometry

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Received 12 April 2007; accepted 5 July 2007
Available online 23 July 2007

Abstract

A method was optimised and validated for simultaneous monitoring of several drugs of different classes of antibiotics such as quinolones (oxilinic acid and flumequine), tetracyclines (oxytetracycline), sulfonamides (sulfadiazine) and trimethoprim in fish muscle and skin. The method is based on solid–liquid extraction without further sample clean up followed by liquid chromatography–mass spectrometry (LC–MS) determination with electrospray ion source (ESI) in positive mode. The limits of quantification (LOQs) were lower than 20 μg/kg for all compounds and repeatability, expressed as relative standard deviations (RSD), were lower than 15%. Therefore, the LC–MS method was successfully applied for the quantitative determination of antibiotics in gilthead sea bream muscle and skin and oxytetracycline in medicated fishes.

Keywords: Multiclass; Antibiotics; Liquid chromatography; Mass spectrometry; Gilthead sea bream

1. Introduction

Aquaculture production has notably increased in the last decades, mainly thanks to intensive farming. Together with market globalisation, this gives rise to the spreading of several fish diseases, increasing the demand for veterinary drugs for aquatic species. Many classes of antibiotics are commonly used in aquaculture worldwide to treat infections caused by a variety of bacterial pathogens of fish [1]. Thus, antibacterial agents used for treatment of fish diseases, include sulfodiazine, oxytetracycline, and oxolinic acid among others [2]. However, the potential hazards associated with the presence of these products in edible tissues from aquaculture include allergies, toxic effects, acquisition of drug resistance in pathogens in the human body [3] as well as their potential carcinogenic character [4]. So there is a global concern about the consumption of low levels of antimicrobial residues in aquatic foods and the effects of these residues on human health. In this sense, the European Union has established maximum residue limits (MRLs) for these compounds in food-producing animal tissues in order to ensure human food safety [5].

These limits require the development of sensitive and specific methods for the determination of antibiotic residues in food. Several published papers have proposed different methods based on immunoassay techniques [6,7]. These methods generally do not distinguish among members of a given class of antibiotics, provide only semiquantitative measurements of residues and sometimes give rise to false positives [8]. Nevertheless they are still used because of their simplicity and low-cost although other techniques must be used in order to confirm the results obtained by bioassay techniques. For this reason, other techniques, such as chromatography or electrophoresis [9], have been proposed to overcome these shortcomings, and liquid chromatography (LC) is the most frequently approach used. In relation to detection methods, they are diverse and include UV [10,11] or fluorescence detection [12,13]. However, Public Health Agencies, based on European Union guidelines, rely on the detection by mass spectrometry (MS) for confirmation of antibiotics in foodstuffs [14], considering that it provides more reliable identification and confirmation of these analytes than conventional detectors. Bearing in mind that most antibiotics are thermally labile and low-volatile compounds, liquid

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doi:10.1016/j.jchromb.2007.07.011

chromatography coupled to MS (LC–MS) and tandem mass spectrometry (LC–MS/MS) have become the most popular techniques for the determination of these analytes during the last few years [3,8,15–19], using electrospray (ESI) or atmospheric pressure chemical ionisation (APCI) sources [20,21]. In this sense, LC coupled to ESI has become a very valuable technique for multiresidue analysis, because it is more sensitive, selective and allow rapid and multiresidue determination in complex matrices, providing structural information [22].

Thus, many LC–MS methods have been developed for antibiotic groups such as sulfonamides [20,23], tetracyclines [24,25] and quinolones [22,26], although there is still a challenge to develop multiresidue methods, which are relatively scarce [27,28].

Furthermore, one of the main problems involved in multiresidue antibiotic analysis in real samples is the tediousness and complexity of the procedures required for the extraction, cleanup and preconcentration of the matrix analytes before instrumental analysis. Most of the extraction methods are time consuming and costly, involving extraction techniques such as liquid-liquid extraction [29], solid phase extraction (SPE) [30,31], matrix solid phase dispersion (MSPD) [32], and pressurized liquid extraction [33], including several steps such as elution, evaporation and sample resuspension, and clean-up [4]. Besides, the effective extraction and analysis of multiple classes of compounds is still a significant challenge in multi-class residue method development since the wide range of polarities, solubilities and pK_as of antibiotics, so new extraction procedures should be developed.

The aim of this work was to develop a method for the simultaneous determination of selected veterinary antibiotics, including 2 fluoroquinolones (oxolinic acid and flumequine), one tetracycline (oxytetracycline) and one sulfonamide (sulfadiazine), that are widely used in veterinary medicine as well as trimethoprim, a dihydrofolate reductase inhibitor, which is commonly used in the combination with sulfonamides as potentiator. The method involves a simple and rapid extraction procedure and the determination of several antibiotics in gilthead seabream (S*parus aurata*) from fish farm by LC–MS, considering the use of single quadrupole allows the method to be more widely adapted, since LC–MS is currently more common than LC–tandem MS in many routine laboratories.

2. Experimental

2.1. Chemicals and reagents

Oxytetracycline hydrochoride (>98.5%), flumequine (>99.0%), sulfadiazine (>99.5%), trimethoprim (>99.5%) and oxolinic acid (>98.0%) were all from Dr. Ehrenstorfer (Augsburg, Germany). Stock standards solutions of individual compounds (with concentrations between 200 and 300 mg/L) were prepared by exact weighing of the powder and dissolved in 100 mL of methanol (HPLC grade, Panreac, Barcelona, Spain), which were then stored at −20 °C in the dark. A multicompound working standard solution at a concentration of 10 mg/L of each compound was prepared by appropriate dilutions of the stock solutions with methanol and stored in screw-capped glass tubes at −20 °C in the dark. This solution was stable for 3 weeks, after which it was replaced by a new fresh solution.

One molar citrate acid solution at pH 4 was prepared by dissolving citric acid (Panreac) in water and pH was adjusted with NaOH 1 M (Panreac). EDTA–Na_2 was obtained from Merck (Darmstadt, Germany) and acetonitrile was purchased from Panreac. Other reagents were of analytical reagent grade. Cartridges Oasis HLB 200 mg from Waters (Milford, Massachusetts) and 500 mg C_{18} Sep-Pak cartridges (Milford, MA, USA) were used for cleanup during optimisation of the extraction procedure. Ultrapure water was obtained from a Milli-Q Gradient water system (Millipore, Bedford, USA).

2.2. Apparatus

The HPLC system was an Alliance 2695 equipped with an autosampler, degasser and heater column purchased by Waters (Mildford, Massachussetts, USA). The mass spectrometer system was a ZQ 2000 single quadrupole from Waters-Micromass (Manchester, UK). Data was collected by MassLynx 4.0 software in a personal computer. An Atlantis dC_{18} 150 mm × 2.00 mm i.d. 5 μm (Waters) was used for all separations. The C_{18} column was equilibrated at 30 °C with a mobile phase consisting of 90% of eluent A (0.1% aqueous solution of formic acid) and 10% of eluent B (methanol) at a flow rate of 0.3 mL/min, using the following gradient profile: 90% eluent A for 3 min; then, the percentage of eluent A was decreased linearly to 45% in 9 min, maintained at this composition for 3 min; later, eluent A was decreased again linearly to 10% in 3 min, keeping this composition for 5 min; finally, eluent A was restored to 90% in 2 min and maintained at this composition for 5 min.

Analytes were detected with ESI in positive mode. The source and desolvation temperatures were 120 and 350 °C, respectively, and the flow rates for desolvation and cone gas were 350 and 50 L/h, respectively, from a generator N_2 Flo purchased from Claind (Lenno, Italy). Capillary voltage was set to 3.5 kV.

All pH measurements were made with a Crison Basic 20 pH-meter (Insulab, Valencia, Spain) equipped with a combined AgCl–glass electrode assembly. A high-speed homogenizer Polytron PT2100 (Kinematica A.G., Littan/Luzern, Switzerland), a P-selecta Centromix mod S-549 centrifuge (Selecta, Barcelona, Spain), a kitchen blender Braun MX32 (Barcelona, Spain) and a rotary evaporator R-114 (Büchi, Flawil, Switzerland) were used to process samples. An analytical balance AB204-S from Mettler Toledo (Greifensee, Switzerland) was also used.

2.3. Extraction procedure

The sample was edible muscle and skin from gilthead seabream (*sparus aurata*). The tissue samples were minced and homogenized using a kitchen blender. To 1 g of sample, 10 mL of acetonitrile, 1 mL of 1 M citric acid (pH 4.0) and 0.5 mL of 0.5 M EDTA–Na_2 solution were added and homogenised using Polytron for 3 min. The sample was centrifuged at 3000 rpm for