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Analytical Methods

Analysis of selected veterinary antibiotics in fish by micellar liquid chromatography with fluorescence detection and validation in accordance with regulation 2002/657/EC

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ABSTRACT

A simple and sensitive method was optimised and validated for the simultaneous analysis of five quinolones (oxolinic acid, flumequine, enrofloxacin, difloxacin and sarafloxacin) in different fish muscles. Analytical separation was performed in less than 18 min using a C_{18} column with fluorescence detection and a micellar solution of 0.065 M sodium dodecyl sulphate, 12.5% propanol and 0.5% triethylamine buffered at pH 3 as the mobile phase. The method was fully validated in accordance with European Union Decision 2002/657/EC. Selectivity, linearity, decision limit, detection capability, detection and quantification limits, recoveries, and robustness were determined. Therefore, the micellar method was successfully applied to quantitatively determine quinolones in spiked muscle fishes, and the recoveries obtained were in the 87–110% range. High extraction efficiency for the quinolones was obtained without matrix interference in the extraction process and in the subsequent chromatographic determination. No organic solvent was used during the pretreatment step.

1. Introduction

Quinolones are among the most important antibacterial agents used in human medicine, and are active against both Gram-positive and Gram-negative bacteria through the inhibition of their DNA gyrase (AHFS, 1988). Their main use is in the treatment of human and veterinary diseases, and they prove very useful in preventing diseases in animals (Currie, Lynas, Kennedy, & McCaughey, 1998). They are well absorbed after oral administration and distribute extensively in tissues. Such characteristics make these drugs suitable to act as the therapy for a large number of infections on fish farms. These practices imply the drug residues persisting in edible tissue derived from treated animals and, therefore, maximum permitted levels have been set for most of them. Accordingly, their residues need to be controlled because there is concern about the possibility of exposure to low levels of these compounds, which may result in the development of resistance of human pathogens to antibiotics (Council Regulation (EEC) No 2377/90, 1990).

Studies have been published on different liquid chromatographic methods based on fluorescence and UV detection of quinolones in biological fluids (Espinosa-Mansilla, de la Pena, Gomez, & Lopez, 2006), food (Christodoulou, Samanidou, & Papadoyannis, 2007; Huang, Lin, Yu, & Feng, 2006; Karbiwnyk, Carr, Turnipseed, Andersen, & Miller, 2007; Marazuela & Moreno-Bondi, 2004; Pecorelli, Galarini, Bibi, Floridi, & Casciarri, 2003; Su, Chang, Chang, Chang, & Chou, 2003; Zhou et al., 2009) and environmental samples (Andreu, Blasco, & Pico, 2007; Golet, Strehler, Alder, & Giger, 2002). The latest generation of high performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) equipment allows the multiresidue determination of quinolones in different matrices (Diaz-Cruz & Barcelo, 2007; Hermo, Nemutlu, Kir, Barron, & Barbosa, 2008; Paschoal, Reyes, & Rath, 2009; Romero-Gonzalez, Lopez-Martinez, Gomez-Milan, Garrido-Frenich, & Martinez-Vidal, 2007; Samanidou, Evaggelopoulou, Trotzmuller, Guo, & Lankmayr, 2008). However, this equipment is very expensive and only a few laboratories can afford it. Other non-routine techniques such as terbium(III)-sensitised luminescence (Hernandez-Arteseros, Compano, Ferrer, & Prat, 2000), capillary electrophoresis (Barron, Jimenez-Lozano, Bailac, & Barbosa, 2002) or immunoaffinity chromatography (Holtzapple, Buckley, & Stanker, 1999) methods, have also been developed.

Furthermore, one of the main problems involved in multiresidue antibiotic analyses in real samples is that the procedures required for the extraction, cleanup and preconcentration of the matrix analytes before the instrumental analysis are tedious and complex. Micellar liquid chromatography (MLC) (Esteve-Romero, Carda-Broch, Gil-Agusti, Capella-Peiro, & Bose, 2005) is an attractive alternative to conventional HPLC methods that uses a surfactant solution above the critical micellar concentration instead of

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aqueous-organic solvents as mobile phases for the determination of compounds in a variety of matrices (e.g., physiological fluids and food) with direct injection. MLC allows the analysis of complex matrices, usually without the aid of extraction because micelles tend to bind proteins competitively, thereby releasing protein-bound drugs and proteins, thus considerably reducing the cost and analysis time. Proteins are solubilised in micellar media and washed harmlessly away to elute with the solvent front rather than precipitating into the column.

The aim of this work was to apply a multiresidue HPLC procedure with micellar mobile phases to simultaneously determine five quinolones (difloxacin, enrofloxacin, flumequine, oxolinic acid and sarafloxacin) in fish muscle samples. Through Council Regulation No 2377/90 (1990), the European Union has established maximum residue limits (MRLs) in samples of animal origin. According to this regulation, the MRLs of the studied quinolones in fish muscle are 300 μg/kg for difloxacin, 100 μg/kg for enrofloxacin, 600 μg/kg for flumequine, 100 μg/kg for oxolinic acid and 30 μg/kg for sarafloxacin. Therefore, analytical methods are required to check food samples before they are sent to markets. The method was validated in line with Commission Decision 2002/657/EC (2002) in terms of selectivity, linearity, decision limit, detection capability, precision, and robustness. The procedure developed herein could also prove useful to determine quinolones in fish samples.

2. Experimental

2.1. Reagents and materials

Oxolinic acid (OXO,>97%) and flumequine (FLU,>98%) were purchased from Sigma (St. Louis, MO, USA). Enrofloxacin (ENR,>98%), difloxacin (DIF, 99.8%) and sarafloxacin (SAR, 97.2%) were purchased from Fluka (Buchs SG, Switzerland). Sodium dodecyl sulphate (SDS, 99%) and sodium hydroxide (99%) were purchased from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate 1-hydrate (99%) and n-propanol (HPLC grade) were obtained from Scharlab (Barcelona, Spain). Hydrochloric acid (37–38%), methanol, ethanol (both HPLC grade) and triethylamine (99.5%) came from J.T. Baker (Deventer, the Netherlands). Ultrapure water was used throughout (Millipore S.A.S., Molsheim, France). Nylon filters were obtained from Sartorius-Stedim (Goettinge, Germany) and Micron Separations (Westboro, MA, USA).

2.2. Chromatographic conditions and instrumentation

The chromatographic system used was an Agilent Technologies Series 1100 (Palo Alto, CA, USA) equipped with a quaternary pump, thermostated autosampler tray and column compartments, and a fluorescence detector. The Agilent ChemStation (Rev. B.03.01) software was used for both instrumental control and chromatographic data acquisition. The Michrom software (Torres-Lapasió, 2000) was used for chromatographic data processing and optimisation studies.

The elution conditions were optimised. The experimental design consisting of five mobile phases (four located at the corners of a rectangular factor space and the fifth in its centre) buffered at pH 3 was used to examine the chromatographic behaviour of the 5 compounds. Thus, the quinolones were injected into the following mobile phases, SDS (M) / propanol (%, v/v): 0.05/2.5, 0.05/12.5, 0.1/7.5, 0.15/2.5, and 0.15/12.5, all containing 0.5% TEA. Furthermore, the model employed for these predictions was (Berthod & García-Álvarez-Coque, 2000):

$$k = \frac{K_{AS}\dfrac{1}{1 + K_{AD}\varphi}}{1 + K_{AM}\dfrac{1 + K_{MD}\varphi}{1 + K_{AD}\varphi}[M]}$$

where k is the retention factor, [M] and φ are the concentrations of the surfactant and modifier; K_{AS} and K_{AM} correspond to the equilibria between the solute in bulk water and the stationary phase or micelle, respectively; K_{AD} and K_{MD} measure the relative variation in the concentration of the solute in bulk water and micelles due to the presence of a modifier, as compared to a pure micellar solution (without a modifier).

The analytical separation was performed in a reversed-phase Kromasil C_{18} column (Scharlab) (150 mm × 4.6 mm, 5 μm particle size). A fluorescence detection programme was run at the following times and excitation/emission wavelengths (0–10 min: 260/366 nm when OXO and FLU were detected, 10–20 min: 280/450 nm when ENR, DIF and SAR were detected). The flow rate and injection volume were 1 mL/min and 20 μL, respectively. The chromatographic runs were carried out at room temperature.

The pH of the solutions was measured with a Crison potentiometer (Model micropH 2001, Barcelona) equipped with a combined Ag/AgCl/glass electrode. The analytical balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland). The vortex shaker and sonification unit were obtained from Selecta (Barcelona).

2.3. Preparation of standard and mobile phase solutions

The micellar mobile phases were prepared by weighing the appropriate amounts of SDS and sodium dihydrogen phosphate. These reagents were dissolved in ultrapure water and 0.5% (v/v) triethylamine was added, then the pH was adjusted to the desired value. Finally, propanol was added to achieve the desired concentration of the organic solvent and then water was added up to the mark-up of the volumetric flask.

Individual stock standard solutions at a concentration of 20 μg/mL for DIF, ENRO, FLU, OXO and SAR were prepared every 2 months. All stock solutions were prepared by dissolving pure substances in 5% ethanol with the aid of an ultrasonic bath, and they were finally filled up with micellar solution of 0.05 M SDS-pH 3. Stock solutions were stored at 4 °C. All working standards were prepared every day with the appropriate dilutions of the concentrated stock solutions. All solutions were filtered through 0.45 μm nylon membranes (Micron Separations) before analysis.

2.4. Preparation of fish muscle samples

Fish samples of gilthead (Greece), salmon (Norway), trout (Rioja, Spain), sea bass (Santa Pola, Spain), mussel (Galicia, Spain), prawn (Honduras), and turbot (Galicia) were purchased in a local market.

Extraction conditions in fish muscle samples were optimised as follows: fish samples were finely ground using a mincer (Model MZ10, Petra Electric, Burgau, Germany) at 5000 rpm for 5 min. Afterwards, 5 g of homogenised meat was mixed with 50 mL of 0.05 M SDS solution buffered at pH 3, and it was shaken continuously for 1 h. Then, the supernatant was filtered with a vacuum pump through 0.45 μm nylon membranes with a diameter of 47 mm (Sartorius-Stedim), and it was then placed directly into the autosampler vials.

2.5. Method validation

Method validation was performed to meet the criteria specified by European Commission Decision 2002/657/EC (2002).

To verify the absence of interfering endogenous compounds around the retention time of the analytes, 10 blank samples of different fishes were analysed. Stabilising agents (chloramphenicol and ethanol) and other quinolones used (ofloxacin and