Marine Pollution Bulletin 73 (2013) 154–160

Contents lists available at SciVerse ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Development and validation of a method for the simultaneous extraction and separate measurement of oxytetracycline, florfenicol, oxolinic acid and flumequine from marine sediments

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ARTICLE INFO

Keywords:
Marine sediment
HPLC
Oxytetracycline
Florfenicol
Oxolinic acid
Flumequine

ABSTRACT

A simple and rapid method for the detection and extraction of oxolinic acid, flumequine, florfenicol and oxytetracycline from marine sediments was developed and validated. The analytes were extracted from the marine sediment using a solution of oxalic acid diluted in methanol with sonication before detection by HPLC using a diode-array detector (florfenicol and oxytetracycline) and fluorescence (oxolinic acid and flumequine). The quantification limits (QL) were 100 ng/g for oxytetracycline and florfenicol and 5 ng/g for oxolinic acid and flumequine. The coefficients of variation of the repeatability and intermediate precision were less than 10% in all of the analytes. The calibration curves were linear between 50 and 500 ng/ml for oxytetracycline and florfenicol and 1 and 20 ng/ml for oxolinic acid and flumequine. The recuperation rate for the analytes was above 86%.

1. Introduction

Chemical substances are used to control fish diseases in aquaculture, with antibiotics being the most frequently used both prophylactically and metaphylactically (Lalumera et al., 2004; Nepejchalová et al., 2008; Smith, 2008). In aquaculture, and especially in salmon farming, antibiotics are mainly orally administered through feedstuffs (Alderman and Hastings, 1998; Hormazabal et al., 1996; Lalumera et al., 2004; Pouliquen and Morvan, 2005; Smith, 2008; Lu et al., 2009). The use of these substances during 2007–2010 in Chile showed a decreasing trend to 143.17 tons used during 2010 (Sernapesca, 2011a,b). During 2010 florfenicol was the main antibiotic used (52%) followed by oxytetracycline, flumequine and oxolinic acid (44%, 1% and 1%, respectively). It has been suggested that important amounts of these drugs are released into the marine environment as fecal, urinary excretion and uneaten food (Alderman and Hastings, 1998; Hormazabal et al., 1996; Lalumera et al., 2004; Nepejchalová et al., 2008; Pouliquen et al., 2005,2009). The potential but not yet proven impact of releasing high quantities of these substances is evidenced by the collateral effects such as the quantitative and qualitative modifications of the bacterial flora in the environment affecting the conservation of the marine biodiversity (Buschmann and Fortt, 2005), impacts on the food chain (Borja, 2002), development of antibacterial defenses in fish pathogens and transfer of bacterial resistance to human pathogens (Borja, 2002).

The purpose of this study was to implement and validate a methodology for the simultaneous detection of oxolinic acid (AO), flumequine (FMQ), oxytetracycline (OTC) and florfenicol (FF) in marine sediments.

2. Materials and methods

2.1. Sediment samples

Sediments samples were taken between May 2010 and September 2010 by staff of Instituto de Fomento Pesquero (IFOP). All analytical tests were conducted at IFOP Laboratories in Puerto Montt, Chile.

Blank marine sediments samples (with no antibiotic presence) were obtained from the sea bottom of different areas of Caleta Tortel (47°55′58″S, 74°38′21″W; 47°52′00″S, 74°38′02″W; 47°54′30″S, 74°30′30″W) and Melinka Norte (43°47′10″S, 73°52′39″W; 43°56′23″S, 73°58′24″W). These sites were chosen as to be far away from any fish farms thus avoiding the presence of the analytes of interest. The samples were obtained using cores of 110 mm from which a sub-sample of 3 cm from the uppermost layer of sediment was removed, according to the methodology described by Miranda et al. (2005). Only wet sediments were used during the development and validation of the methods.

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0025-326X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.marpolbul.2013.05.027

2.2. Chemicals

Methanol and acetonitrile (chromatographic grade), trifluoroacetic acid (TFA, spectroscopy grade), oxalic acid and sodium hydroxide (analytical grade) were obtained from Merck (Darmstadt, Germany). High purity water was prepared using the water purification system Easypure II (Barnstead). All certified standards of oxolinic acid, flumequine, florfenicol and oxytetracycline were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

2.3. Preparation of working standards used in HPLC detection

Standard solutions of 1 mg/ml of oxolinic acid and flumequine were prepared separately using sodium hydroxide 0.03 M. Oxytetracycline and florfenicol were dissolved using methanol. All solutions are maintained for 3 months at 4 °C. Furthermore, oxytetracycline standards were protected from light as it is a highly a photosensitive substance. Daily working solutions in water were prepared to 2 μg/ml for oxolinic acid and flumequine and 100 μg/ml for oxytetracycline and florfenicol. From these working solutions, a diluted water solutions were prepared to achieve 200 ng/ml (oxolinic acid and flumequine) and of 5 μg/ml (oxytetracycline and florfenicol).

2.4. Calibration curves

From the working solutions, external calibration curves of six points in three different days using ranges between 1–20 ng/ml (oxolinic acid and flumequine) and 50–500 ng/ml (florfenicol, oxytetracycline) were prepared on a volumetric basis in water.

2.5. Sample spiking

A diluted working solution was added to 3 g of blank sediment in a 50 ml conical centrifuge tube, in order to obtain the desired concentration of the analyte. Each tube of spiked sediment was capped and mixed on a vortex for 30 s and let to stand for at least 30 min before starting the extraction.

2.6. Extraction procedure

The same extraction process was carried out for all analytes. 3 g of wet blank sediment were weighed and transferred to a 50 ml tube. Then, 30 ml of 0.01 M of oxalic acid in methanol were added and the tube was shaken vigorously by hand and then using a vortex for 30 s. The tube was then placed in an ultrasonic bath for 30 min. The sample was centrifuged at 4000 rpm for 10 min and the supernatant was transferred to a 100 ml glass ball and evaporated using a rotary evaporator at 50 °C. After this, 3 ml solution of water/acetonitrile (75:25) was added for resuspension. The resuspended sample was centrifuged at 4000 rpm for 10 min. Finally, the supernatant was filtered into 1.8 ml HPLC vials using PVDF syringe filters of 0.22 μm pore and 3 mm diameter.

2.7. Instrumentation

A Shimadzu High Performance Liquid Chromatograph (HPLC) equipped with a Sil-10AF auto-sampler, LC-10AT quaternary pump, DGU-14A degasser, SCL-10A System Controller, RF 10AXL fluorescence detector, SPD-M20A UV/VIS diode array detector was used. The chromatographic separation was carried out using a Kromasil Phenyl Column (250 × 4.6 mm i.d., 5 μm). Class-VP software was used to analysis of HPLC runs associated with a personal computer. Elma Transonic 60H ultrasonic cleaning unit was used for extraction and degassing. A Sorvall ST16R centrifuge with a Thermo Scientific F15-6x100y rotor and a Büchi R-200 Rotavapor were also utilized for extraction.

2.8. HPLC analysis

The analytical HPLC column used was a 4.6 × 250 mm Kromasil Phenyl column. The mobile phase was 0.1% TFA in water, 0.1% TFA in methanol and 0.1% TFA in acetonitrile, of which the proportions were varied with or without gradient. Detection was carried out with a fluorescence detector (oxolinic acid and flumequine using an excitation wavelength of 325 nm and an emission wavelength of 365 nm) and diode array detector (oxytetracycline and florfenicol) using a monitoring wavelength of 355 nm and 220 nm, respectively. The volume of the injection was 50 μl. The optimized chromatographic conditions for the different analytes are summarized in Table 1.

2.9. Validation of methods

The methods were validated by evaluation of the selectivity, linearity, recuperation, repeatability, intermediate precision and quantification limit. The validation was based on the ICH guide (ICH Harmonized Tripartite Guideline, 2005) and the "Commission Decision 2002/657 of the European Community" (Commission Decision of 12 August 2002).

2.9.1. Linearity

The linearity was studied with a standard external calibration curve of 6 points as previously mentioned in Section 2.4. The linear equation of each analyte was calculated, as well as its determination coefficients, correlation (r^2) and significance value (p-value).

Table 1
Chromatographic conditions for the different analytes.

Analyte	Flow rate (ml/min)	Run time (min)	Detection	Injection (μl)	Oven $T°$ (°C)	Gradient (min)	%A	%B	%C
Florfenicol	1.0	22	DAD (220 nm)	50	40	0.1	85		15
						1.0	85		15
						15	50		50
						15.1	85		15
Oxytetracycline	1.0	22	DAD (355 nm)	50	40	Isocratic	65	30	5
Oxolinic acid and flumequine	1.5	22	Fl Ex (325 nm)	50	40	0.1	71	5	24
			Em (365 nm)			1.0	71	5	24
						10	49	5	46
						13	25	5	70
						15	25	5	70
						15.1	71	5	24

DAD: diode array detector, Fl: fluorescence detector, Ex: excitation wavelength, Em: emission wavelength, A: 0.1% TFA in water, B: 0.1% TFA in methanol, C: 0.1% TFA in acetonitrile.