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Rapid and simple determination of oxolinic acid and oxytetracycline in the shell of the blue mussel (*Mytilus edulis*) by high-performance liquid chromatography

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Abstract

A simple procedure for the determination of oxolinic acid (OA) and oxytetracycline (OTC), two antibacterial agents, in the shell of the blue mussel (*Mytilus edulis*), using reversed-phase high-performance liquid chromatography is described. Liquid chromatography was performed on a 5-μm LiChroSpher 100 RP-18E column using acetonitrile and a 0.02 *M* orthophosphoric acid solution as the mobile phase, with ultraviolet detection. After roughly grinding the shell, drugs were extracted using a methanolic oxalic acid solution. Linearity and precision were checked over the concentration range 0.04–0.32 μg/g. Limits of detection of OA and OTC were 0.012 and 0.008 μg/g, respectively. Mean extraction recoveries of OA and OTC from mussel shell were 72.9 and 65.4%, respectively. To demonstrate the usefulness of the analytical procedure, an experimental study was performed in blue mussels exposed to the drugs for eight days. © 1997 Elsevier Science B.V.

Keywords: Oxolinic acid; Oxytetracycline

1. Introduction

Because of their wide antibacterial spectrum and high potency, oxolinic acid (OA) and oxytetracycline (OTC) are common antibacterial agents used against bacterial infections in fish farming. The drugs are administered to fish mixed with feed at a dosage rate of 10–20 mg (OA) or 50–100 mg (OTC) per kg of biomass per day for eight–ten days. The first sign of an infectious and systemic disease in fish is usually a reduced feed intake. Furthermore, OA and OTC are very poorly absorbed through the intestinal tract of fish [1]. Therefore, a large part of the medicated feed presumably reaches the environment of the fish farms.

Experimental studies showed the potential contamination of marine shellfish tissues by OA and OTC [2–6]. No report has previously been published about the potential contamination of the shell of shellfish by these drugs, although OTC was used for marking the skeleton of aquatic species [7–11] and tetracycline could be incorporated into the shell of some marine bivalves [12].

Some methods using high-performance liquid

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chromatography (HPLC) with octylsilane or octadecylsilane columns for the determination of OA and OTC in shellfish tissues have been published [3,13,14]. No method for the analysis of OA and OTC in the shellfish shell has been described.

The purpose of this work was to develop a simple, rapid and accurate HPLC method for the determination of OA and OTC in the shell of the blue mussel (*Mytilus edulis*) and to apply this method to an experimental study.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (BDH, Toronto, Canada) and methanol (Carlo Erba, Milan, Italy) were of HPLC grade. Orthophosphoric acid, oxalic acid, perchloric acid (Merck, Darmstadt, Germany) and sodium hydroxide (Panreac, Barcelona, Spain) were analytical-grade reagents. The water used in buffers and eluents was distilled and purified with an Elgastat Spectrum RO2 (Elga, Buckinghamshire, UK). Oxolinic acid and oxytetracycline were purchased as pure standards from Sigma (St. Louis, MO, USA).

2.2. HPLC apparatus

The HPLC system consisted of a chromatograph Varian 5000 equipped with a Valco injection valve (Varian, Palo Alto, CA, USA), an L 4250 variable-wavelength absorbance detector and a D 2500 integrator (Merck). The data were handled with a computer Deskpro 386S/20 Mod 40 3.5 (Compaq, Houston, TX, USA) equipped with the HPLC Manager Software System (Merck). The analytical column, a 5-μm LiChroSpher 100 RP-18E, 125×4.6 mm I.D. (Merck), was equipped with a 5-μm LiChroSpher 100 RP-18E guard column, 4.6×4 mm I.D.

2.3. Chromatographic conditions

The mobile phase was acetonitrile–0.02 *M* orthophosphoric acid (24:76, v/v), pH 2.3. Each solvent was filtered using a Sartorius HPLC solvent filtration system (Gottingen, Germany) with 47 mm, 0.22 μm nylon filters (MSI, Westboro, MA, USA).

The chromatographic experiments were performed at ambient temperature (20±2°C). The operating flow-rates were 1.0 (OA) or 1.2 ml/min (OTC) and the UV detector was set at 262 (OA) or 355 nm (OTC). The sample volume injected onto the analytical column was 100 μl. The guard column was replaced at intervals of 100 to 150 sample injections.

The analytical column was conditioned prior to use by flushing it with acetonitrile–water 75:25, v/v (2 h), 50:50, v/v (2 h), 40:60, v/v (2 h), 30:70, v/v (2 h) and mobile phase (5 h) at a flow-rate of 0.2 ml/min. Moreover, the column was rinsed for 2 h after each day of operation using acetonitrile–water (24:76, v/v) at a flow-rate of 0.2 ml/min.

2.4. Preparation of standard solutions

Stock standard solutions (1 mg/ml) were prepared in 0.03 *M* aqueous sodium hydroxide (OA) or in methanol (OTC). They were stable for one month when stored at +4 (OA) or −20°C (OTC). Working standard solutions were prepared by diluting aliquots of the stock standard solutions with water (OA) or 0.1 *M* perchloric acid (OTC) immediately before use. All of these solutions were protected from light throughout the analyses (preparation in a dark room and storage of the flasks in a tightly shut polystyrene box). Aliquots of the working standard solutions (0.100 and 0.400 μg/ml) were injected onto the HPLC column at the beginning of each day of operation. The calibration graphs were based on the measurement of peak heights.

2.5. Sample preparation

The shells of the blue mussel (*Mytilus edulis*) were carefully cleared from flesh, rinsed with distilled and purified water and allowed to dry in a dark room for one night. Shells were broken using a cutting pliers and were reduced to a powder using a coffee-mill for 30 s.

A 5-g sample was extracted twice with 15 ml of a 0.013 *M* methanolic oxalic acid solution (pH 1.4). After homogenization for 15 min (Rotator Drive, Heidolph, Keilheim, Germany) and centrifugation at 10 000 *g* for 5 min at +4°C (Jouan Model MR 1822

centrifuge, Saint-Herblain, France), the supernatants were combined and filtered through 70 mm Whatman No. 541 filter paper (Maidstone, UK). The filtrate was evaporated to dryness at +40°C using a rotary evaporator (Büchi 461, Flavil, Switzerland). Prior to analysis, the extract was dissolved in 1.0 ml of water (OA) or 0.1 *M* perchloric acid (OTC) and centrifuged at 10 000 *g* for 5 min at +4°C (Jouan Model MR 1822 centrifuge).

2.6. Validation assay

Standard calibration curves for OA and OTC (concentration range: 0.04, 0.08, 0.16 and 0.32 μg/g) were obtained by analysing four replicates of each spiked 5.0 g sample for five days. Spiked samples were obtained by mixing OA or OTC powder with shell powder; four successive dilutions with homogenization for 1 h (Rotator Drive, Heidolph) were required to obtain the desired drug concentration and the best homogenization of the powders. Standard calibration curves were drawn by plotting the known drug concentrations against the peak heights. They were used to study linearity, regression, precision and extraction recoveries (peak heights obtained by chromatographing spiked and extracted shells versus peak heights obtained by chromatographing the working standard solutions). The limits of detection and quantitation were defined as the smallest concentrations that gave signal-to-noise ratios greater than three and ten, respectively. The precision of the limit of quantitation was determined by spiking shell samples at this limit and analysing five replicates for two days. The limit of quantitation was accepted only if the mean value of the drug peak heights was significantly different from the intercept at the $P=0.05$ level and greater than three standard deviations [15,16].

2.7. Experimental conditions and device

The experiment was carried out at the IFREMER Laboratory (Noirmoutier, France) in four, 2.4 m^3 tanks, called A, B, C and D. Each tank was filled with 2000 l of seawater that had been filtered through a 150-μm membrane. Then, 135 blue mussels [individual weight of the mussels was 15.7±2.1 g (mean±standard deviation, $n=100$)] were placed in each tank. The temperature, pH and salinity of the seawater were 12.0±0.5°C, 8.0±0.2 and 33.0±0.5‰ (mean±standard deviation, $n=15$), respectively. Tanks were covered with a black plastic cover and supplied with air in order to maintain the dissolved oxygen concentration in seawater at saturation level.

After acclimatization of the bivalves to the conditions in the tanks for five days, under running filtered seawater (1.4 l/min), the seawater circulation system was shut off. Antibacterial agents in their pure form (OA or OTC hydrochloride) were dissolved in 5 l of filtered seawater and added to the seawater in each of the four tanks to give a test concentration of 1.50 mg/l [5,17,18]. OA was dissolved in tanks A and B and OTC in tanks C and D. After two days exposure, the seawater was drained and the tanks were rinsed and again filled with 2000 l of filtered seawater, into which the test antibacterial agent was added. All of these manipulations were repeated on the second, fourth and sixth days of contamination. On the eighth day, the seawater was drained and the tanks were rinsed and again filled with 2000 l of filtered seawater. Then, the seawater circulation system was reestablished and the seawater flow-rate was 1.4 l/min for fourteen days.

Blue mussels were sampled during the contamination (eight days) and decontamination (fourteen days) periods. They were dissected and the shells were immediately stored in black polyethylene bags and maintained at −20°C until the analyses were carried out. A mixture of the five shells picked from each tank on each sampling day was used for the determination of OA and OTC concentrations.

3. Results and discussion

3.1. Chromatographic conditions

The choice of the stationary and mobile phases has been discussed elsewhere [13,14].

OTC and OA were eluted in 2.2 and 6.3 min, respectively. The analytical column was used for more than 1500 sample injections without any change in its performance; the final number of theoretical plates was 2650 compared with an initial value of 2800. No additional peaks that could

possibly result from impurities or degradation products and interfere with the OA or OTC peaks were noted on the chromatograms (Fig. 1). The guard column was replaced after 100 to 150 injections because of its saturation with shell components, which resulted in a drifting baseline.

3.2. Extraction and recoveries

Extraction of organic compounds from animal calcified tissues (shell, tooth, claw, hoof, etc.) was a difficult challenge [19]. On the one hand, preliminary treatments, either chemical, enzymatic or physical (heating, microwave or ultra-sound), were required in order to improve the subsequent extraction procedure. On the other hand, preliminary treatments were often restricted because most organic compounds were unstable. In the present work, preliminary treatment of the mussel shell was reduced to rough grinding because of the instability of OA and especially OTC to light and heating [20].

The shell powder was a very adsorbent and absorbent matrix because of its low water content (about 0.5%). Spiked samples could be obtained by adding a high volume of a working standard solution to the shell powder. This spiking procedure induced

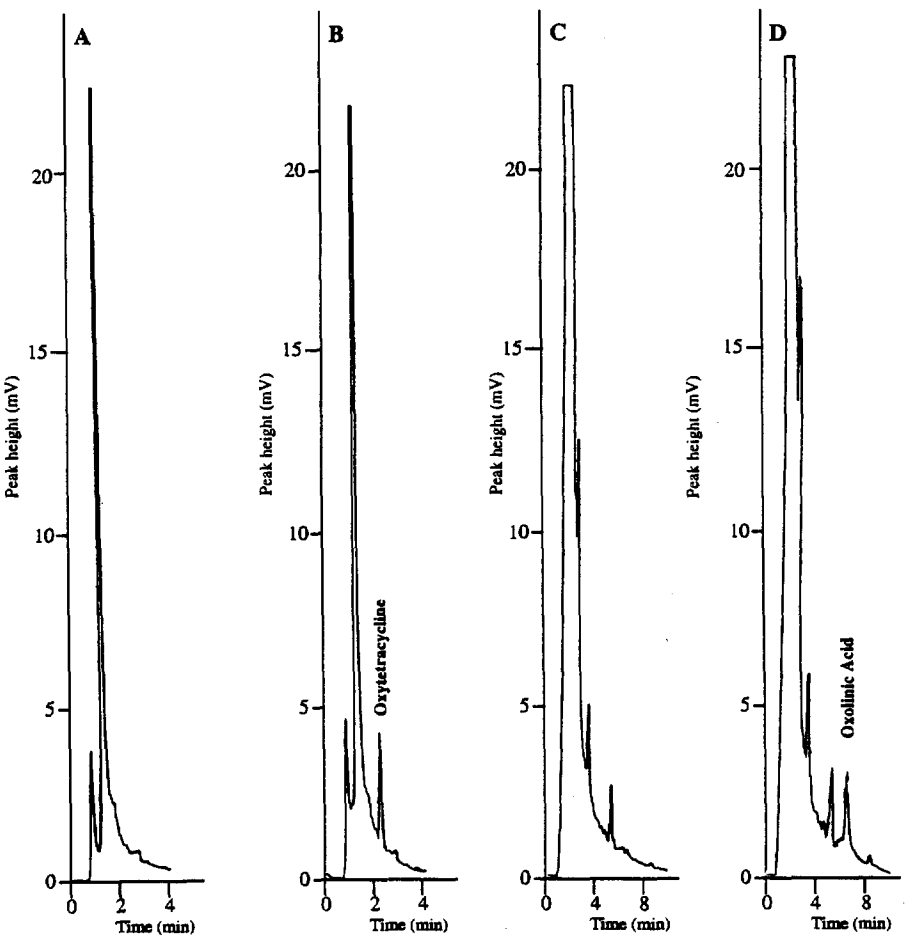

Fig. 1. HPLC chromatograms (A) blank mussel shell sample; (B) mussel shell sample containing oxytetracycline (OTC) at a concentration of 0.32 μg/g; (C) blank mussel shell sample; (D) mussel shell sample containing oxolinic acid (OA) at a concentration of 0.08 μg/g. Conditions: mobile phase, acetonitrile–0.02 *M* orthophosphoric acid solution (24:76, v/v), pH 2.3; column, 125×4 mm, C_{18E} (5 μm); flow-rate, 1.2 (OTC) or 1.0 ml/min (OA); wavelength, 355 (OTC) or 262 nm (OA); injection volume, 100 μl.

an homogeneous distribution of the drug into the matrix but also an increase of the water content of the matrix. So, spiked samples were obtained by mixing the shell powder with the drug powder and by carrying out four successive dilutions, so that homogenization of the powders was optimal.

Extraction of OA and OTC from animal tissues was often difficult [13,14] because these drugs formed complexes with many cations (Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, etc.). For example, calcium made up 55% of the mussel shell's weight. The extraction solvent used was methanol, although OA and OTC were more soluble in *N,N*-dimethylformamide and dimethylsulfoxide. Methanol was preferred to the other two organic solvents because of its higher volatility. Acidification of the methanol increased both the solubility of the drugs in the solvent and the desorption of the drugs from the matrix [13–17]. Extraction recoveries were better using citric, oxalic or succinic acids rather than chlorohydric, sulfuric or trichloroacetic acids (data not shown). The best extraction recoveries were obtained using a 0.013 *M* methanolic oxalic acid solution because of the buffering and chelating properties of oxalic acid. Adding disodium ethylenediaminetetraacetate, another chelating agent, to this solution did not increase the extraction recoveries. Using solid-phase extraction cartridges was not necessary because the filtered extract did not give additional chromatographic peaks that could interfere with the OA and OTC peaks (Fig. 1).

Under these conditions, the mean extraction recoveries of OA and OTC from mussel shell were 72.9 (R.S.D.=11.4%, n=75) and 65.4% (R.S.D.= 10.6%, n=75) respectively, over the concentration range 0.04–0.32 μg/g. These mean recoveries were slightly lower than those obtained in mussel tissues [13,14].

3.3. Linearity, regression, precision and limits of detection and quantitation

A linearity and regression study was performed separately for each calibration curve. Slopes and intercepts of the calibration curves were not significantly different over the five days at the P=0.05 level (data not shown); therefore, a mean calibration curve was determined (Table 1). The high values of the correlation coefficients (0.986 to 0.997) indicated good correlations between drug concentrations and peak heights. Moreover, the F-values for the linearity test were higher than the critical value and, consequently, the slopes were significantly different from 0 at the P=0.05 level.

Table 1
Linearity and regression data for the calibration graphs obtained from mussel shell (5 g samples) spiked with oxolinic acid or oxytetracycline from 0.04 to 0.32 μg/g

	Oxolinic acid (n=75)	Oxytetracycline (n=75)
Slope	23 534	10 264
Intercept	302	82
Correlation coefficient	0.998	0.990
Linearity test (F-test)	1752[a]	2312[a]

$y=ax+b$; y=drug peak height; x=drug concentration (μg/g); a=slope; b=intercept.
[a]Slope is significantly different from 0 at the 0.05 level.

The relative standard deviations of within-run and run-to-run precision for spiked mussel shell were between 4.0 and 12.1% (Table 2).

The limits of detection and quantitation of OA in mussel shell were 0.012 and 0.040 μg/g, respectively and those of OTC were 0.008 and 0.025 μg/g, respectively. These limits of quantitation were accepted (relative standard deviation <20.0% with n= 10) because the mean values of the drug peak heights were significantly different from the intercepts at the P=0.05 level and were greater than three standard deviations [15,16]. The limits of detection and quantitation of OA in mussel shell were almost the same as those reported in mussel tissues [17], whereas those of OTC were lower than those reported in mussel tissues [14].

Table 2
Precision data obtained from mussel shell (5 g samples) spiked with oxolinic acid or oxytetracycline from 0.04 to 0.32 μg/g

	0.04 μg/g (n=18)	0.08 μg/g (n=19)	0.16 μg/g (n=20)	0.32 μg/g (n=18)
R.S.D.[a] of within-run precision (%)				
Oxolinic acid	12.1	10.7	6.8	3.4
Oxytetracycline	4.0	7.8	6.7	9.2
R.S.D.[a] of run-to-run precision				
Oxolinic acid	12.1	10.7	6.8	8.6
Oxytetracycline	4.0	8.5	8.0	9.6

[a]Relative standard deviation.

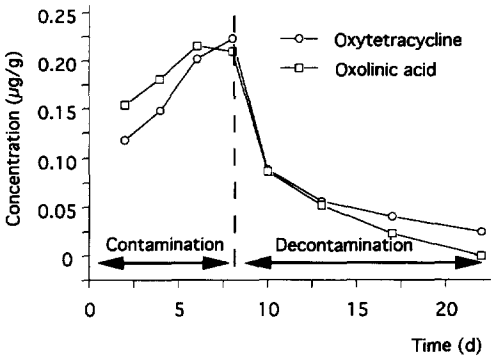

Fig. 2. Kinetics of oxolinic acid and oxytetracycline in blue mussel shell after an eight-day exposure of mussel to the drugs. Each point represents the average concentration of the samples from two tanks on a sampling day. For further details, see text.

3.4. Experimental study

The experimental study (Fig. 2) clearly indicated that OA and OTC could contaminate the mussel shell. The kinetics of OA and OTC in mussel shell were not significantly different. The drug concentrations in mussel shell increased, reaching a peak at the end of the contamination period. They quickly decreased during the first two days of the decontamination period and then decreased more slowly until the end of the decontamination period. The drug concentrations in mussel shell must be compared to those in mussel tissues (gills, muscles, digestive gland, mantle and foot). All of these results will be reported in another publication.

4. Conclusion

The described method provides a selective, reliable and precise method for the rapid determination of OA and OTC in the blue mussel shell. The method does not require time-consuming, complex extraction or derivatization techniques. An analyst familiar with the method could easily process twenty samples a day. The method is suitable for kinetics and residue studies on OA and OTC in the shell of the blue mussel, and perhaps in other shellfish species.

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