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Determination of oxolinic acid in faeces and urine of turbot (*Scophthalmus maximus*) by high-performance liquid chromatography using fluorescence detection

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Abstract

A procedure for the determination of oxolinic acid (OA) in faeces and urine of turbot (*Scophthalmus maximus*), using reversed-phase high-performance liquid chromatography is described. Liquid chromatography was performed on a 5-μm PuroSpher RP-18E® cartridge using acetonitrile and 0.001 *M* aqueous orthophosphoric acid solution as mobile phase, with fluorescence detection. After the addition of an internal standard, oxolinic acid was extracted by using a liquid–liquid extraction procedure. Linearity and precision were checked over the concentration ranges 1.0–1000 μg/g (faeces) and 0.06–10.00 μg/ml (urine). Limits of detection of OA in faeces and urine were 0.20 μg/g and 0.02 μg/ml respectively. Mean extraction recoveries of OA from faeces and urine were 102.0 and 91.6% respectively.

Keywords: *Scophthalmus maximus*; Turbot; Oxolinic acid

1. Introduction

Because of its high potency against Gram-negative bacteria, oxolinic acid (OA) is a common antibacterial agent used against bacterial infections in marine fish farming. The drug is usually administered to fish mixed with feed at a dosage rate of 10–20 mg per kg of biomass per day for 8–10 days.

The first sign of an infectious and systemic disease in fish is usually a reduced feed intake. Furthermore, OA is very poorly absorbed through the digestive tract of marine fish [1]. Therefore, a large amount of OA presumably reaches the marine environment of the fish farms, especially by way of faeces and urine. The determination of this amount of OA is important in order to have a better knowledge of the fate and effects of OA in the marine environment.

Many methods using high-performance liquid chromatography (HPLC) with octylsilane or octadecylsilane stationary phases for the determination of OA in fish tissues have been published [2–16]. None of the methods have described the analysis of OA in urine and only one method has been described for the analysis of OA in faeces [1]: in this report, the extraction and HPLC procedures were not fully

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described and the precision and limits of detection and quantification were unknown.

The purpose of the present work was to develop a precise and accurate HPLC method for the determination of OA in faeces and urine of turbot (*Scophthalmus maximus*) in order to apply this method to experimental studies.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (Merck, Darmstadt, Germany) was of HPLC-grade. Chloroform (BDH Chemicals, Toronto, Canada), methanol, orthophosphoric acid 85%, 0.1 *M* sodium hydroxide solution, 1 *M* chlorhydric acid solution (Merck) and zinc sulphate heptahydrate (Panreac, Barcelona, Spain) were analytical-grade reagents. The water used in buffers and eluents was deionised with a Nanopure apparatus (Barnstead, Dubuque, IO, USA). OA, nalidixic acid (NA) and flumequine (FLU) were purchased as pure standards from Sigma (St. Louis, MO, USA).

2.2. Apparatus

A Merck HPLC system was used consisting of a solvent-delivery pump L-6200 A, an autosampler AS 2000, a fluorescence detector F-1000 and a Deskpro 386S/20 computer (Compaq, Houston, TX, USA). Peak heights and concentrations were calculated with the HPLC Manager Software System D-6000 (Merck). The analytical cartridge, a 5-μm PuroSpher RP-18E® 125×4.6 mm I.D. (Merck), was equipped with a 5-μm PuroSpher RP-18E® guard cartridge, 20×4.6 mm I.D.

2.3. Chromatographic conditions

The mobile phase (pH 3.4) consisted of acetonitrile and 0.001 *M* aqueous orthophosphoric acid solution (33:67 v/v) and was degassed for 30 min before use (Transonic 570, Elma, Munich, Germany).

The cartridge temperature was set at 24°C. The operating flow-rate was 0.6 ml/min. The fluorescence detector was set at an excitation wavelength of 330 nm and an emission wavelength of 360 nm. The sample volume injected onto the HPLC system was 10 μl. The guard cartridge was removed at intervals of 500 sample injections.

The analytical cartridge was conditioned prior to use by flushing with: (1) a mixture of mobile phase:acetonitrile:water (50:25:25 v/v) at a flow-rate of 0.4 ml/min for 15 min; (2) mobile phase at a flow-rate of 0.4 ml/min for 15 min; (3) mobile phase at a flow-rate of 0.6 ml/min for 15 min. Moreover, the analytical and guard cartridges were flushed for 2 h after each day of operation with a mixture of acetonitrile:water (50:50 v/v) at a flow-rate of 0.2 ml/min.

2.4. Standard solutions

Stock solutions of OA, NA and FLU were prepared in a 0.03 *M* aqueous sodium hydroxide solution at a concentration of 5 (OA) or 1 (OA, NA, FLU) mg/ml and were stable for one month when stored at 4°C. Working standard solutions were prepared by dilution in deionised water before use.

2.5. Extraction procedure

Faeces samples (0.5 g) were diluted in water (dilution factor from 1:20 to 1:500). A 200-μl volume of the diluted faeces samples was spiked with FLU as internal standard (40 μl of a working standard solution containing 100 μg FLU/ml). Diluted and spiked faeces samples were extracted with 20 μl of a 10% aqueous zinc sulphate solution, 200 μl of a 0.1 *M* aqueous sodium hydroxide solution and 600 μl of acetonitrile. After homogenization for 20 min at ambient temperature (Rotator Drive, Heidoiph, Keiiheim, Germany) and centrifugation at 4000 *g* for 10 min (Jouan GR 4-22, Saint-Herblain, France), the supernatant was evaporated to dryness in a sand bath under nitrogen stream (40°C). The dry extract was dissolved in 4.0 ml of mobile phase, vortexed for 1 min and sonicated for 1 min (Transonic 570). A 200-μl volume of the supernatant was transferred into a 0.2-ml vial before injection onto the HPLC system.

Urine samples from turbot were centrifuged at 1000 *g* for 10 min (Jouan GR 4-22). A 100-μl volume of the supernatant was diluted in water and

then was spiked with NA as internal standard (40 μl of a working standard solution containing 10 μg NA/ml). Samples were extracted two times with 1 ml of a 0.1 *M* aqueous sodium hydroxide solution and then another 1 ml of the same solution. After homogenization for 10 min at ambient temperature (Rotator Drive) and centrifugation at 2000 *g* for 10 min (Jouan GR 4-22), the supernatants were combined. Two ml of a 1 *M* aqueous chlorhydric acid solution were added to the supernatants and then extracted with 2 ml of chloroform. After homogenization for 10 min (Rotator Drive) and centrifugation at 2000 *g* for 10 min (Jouan GR 4-22), the organic phase was evaporated to dryness in a sand bath under nitrogen stream (40°C). The dry extract was dissolved in 200 μl of mobile phase, vortexed for 2 min, sonicated for 1 min (Transonic 570) and centrifuged at 2000 *g* for 10 min (Jouan GR 4-22). The supernatant was transferred into a 0.2-ml vial before injection onto the HPLC system.

2.6. Validation assay

The calibration curves for OA were made by spiking samples (1 g faeces and 1 ml urine) with standard solutions of OA to yield 1.0, 2.0, 250.0 and 1000.0 μg/g and 0.06, 0.40, 2.50 and 10.00 μg/ml, respectively. The spiked samples were extracted using the above procedures. Each level was assayed in four replicates for three days [17].

The analyses of spiked samples were compared with those of standard solutions to calculate the extraction recoveries [17]. For the determination of OA in faeces, standard solutions contained OA at a concentration of 1.0, 2.0, 250.0 and 1000.0 μg/ml and FLU at a concentration of 1 μl/ml. For the determination of OA in urine, standard solutions contained OA at a concentration of 0.06, 0.40, 2.50 and 10.00 μg/ml and NA at a concentration of 2 μg/ml.

The calibration curves were drawn by plotting the ratio of the peak heights of OA (mV) to the peak heights of the internal standard (mV) against the ratio of the known concentrations of OA (μg/ml or μg/g) to the known concentrations of the internal standard (μg/ml or μg/g). They were used to study linearity, regression and precision. The limits of detection and quantitation were calculated as the smallest concentrations giving a signal-to-noise ratio greater than 3 and 10, respectively [17].

3. Results and discussion

3.1. Chromatographic conditions

HPLC reversed-phases have often been used for determining quinolone antibiotics in fish tissues and gave good performance in terms of plate numbers [2–4,10,11,13,16,18–20]. In our experiment, using a PuroSpher 100-RP18E® prepacked cartridge instead of a LiChroSpher 100-RP18E® (Merck) prepacked cartridge narrowed the OA, NA and FLU peaks and improved capacity factors, symmetry factors, theoretical plate counts and resolutions (Table 1). This is in accordance with previous reports about the importance of using endcapped materials in order to reduce the interactions of quinolones with residual silanol groups of the stationary phase [18–21].

All the previous workers using reversed-phase systems added an organic modifier to the predominantly aqueous eluent [1–5,8,10–13]. Comparisons of three organic modifiers — methanol, tetrahydrofuran and acetonitrile — indicated that peaks were more symmetrical by using acetonitrile than methanol (data not shown) and that the presence of tetrahydrofuran in the mobile phase did not reduce

Table 1
Results of the chromatographic performance of the HPLC system by using either a PuroSpher 100-RP18E or a LiChroSpher 100-RP18E as stationary phase[a]

	Capacity factor	Symmetry factor	Theoretical plate count	System resolution
PuroSpher 100-RP18E				
Oxolinic acid	4.71	1.11	6250	
Nalidixic acid	13.12	1.07	10714	14.91[b]
Flumequine	21.39	1.10	7564	20.13[c]
LiChroSpher 100-RP18E				
Oxolinic acid	3.72	2.00	1563	
Nalidixic acid	8.68	4.25	1293	5.85[b]
Flumequine	16.54	3.48	1368	8.02[c]

[a] For more details, see Section 3.1.
[b] Between oxolinic acid and nalidixic acid.
[c] Between oxolinic acid and flumequine.

peak tailing and did not increase the theoretical plate counts of the cartridge, when using a PuroSpher 100-RP18E® prepacked cartridge [21].

OA, NA and FLU show strong fluorescence in aqueous acid solutions [2,7,12–15]. Orthophosphoric acid was used as aqueous eluent in order to make use of the increased specificity and sensitivity given by the fluorescence detection and to improve the peak shape by masking the residual silanol groups of the stationary phase [2,5,7,9,10,12–15].

The optimum mobile phase had a pH of 3.4 and was 67% of 0.001 *M* aqueous orthophosphoric acid solution and 33% of acetonitrile.

Under the operating conditions, OA, NA and FLU were eluted in 5.6, 9.7 and 12.1 min respectively (Figs. 1B and D). No peaks interfering with the elution of OA, NA and FLU were noted on the chromatograms.

The analytical and guard cartridges were flushed for 2 h after each day of operation with a mixture of acetonitrile:water. Under these conditions, the cartridge was used for more than 2000 injections without any significant change in its performances and the guard cartridge was only removed every 500 injections.

3.2. Extraction and recoveries

The oral bioavailability of OA in turbot was about 30% [22]. So, about 70% of the OA dose that was orally administered to turbot were not absorbed and were eliminated in faeces. Because of high expected OA concentrations in faeces [1], samples should always be diluted before extraction.

When OA was extracted from fish plasma and serum, better recoveries were often obtained by using a solid-phase extraction procedure rather than a liquid-phase extraction procedure [20,23]. In a preliminary study, a solid-phase extraction procedure was optimized in fish faeces and urine.

Many solid-phase extraction cartridges were used: Bond Elut® Octyl, Octadecyl (Analytichem International, Harbor City, CA, USA) and Isolute SAX® Ion Exchange (Touzart and Matignon, Courtaboeuf, France). Whatever the cartridge and the solvents (methanol, acetonitrile, orthophosphoric acid solution and water) may be, the recoveries were always below 35% and the chromatograms of a blank turbot faeces or urine extract sometimes showed a peak at the retention times of OA, NA or FLU. These results indicated that the solid-phase extraction procedure was not well suited to the analysis of OA in turbot faeces and urine, especially because of interactions between some turbot faeces and urine components and the stationary phase of the solid-phase extraction cartridges. So, a liquid-phase extraction procedure was optimized in order to extract OA from turbot faeces and urine.

For faeces, an extraction procedure using zinc sulphate, sodium hydroxide and acetonitrile gave the best recoveries: 102% for OA (Table 2), 100% for FLU and only 40% for NA. So, FLU was selected as internal standard in faeces. The recoveries of OA from turbot faeces were above those obtained in rainbow trout faeces (89.7–98.1%) [1].

The extraction procedure developed for faeces was not suitable for urine because the extraction recoveries were below 40%. For urine, an extraction procedure using, first sodium hydroxide (OA, NA and FLU were soluble in aqueous alkaline solutions), second chlorhydric acid and chloroform (these antibiotics were soluble in organic solvents but not in aqueous acid solution) gave the best recoveries: 91.6% for OA (Table 2), 90% for NA and only 50% for FLU. So, NA was selected as internal standard in urine.

Fig. 1 shows chromatograms of blank and spiked samples. The resulting extracts were free from interference, indicating that satisfactory purifications could be achieved by the present methods.

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

A linearity and regression study was performed for each calibration curve separately [17]. Slopes and intercepts of the calibration curves were not significantly different between the three days at the 0.05 level (data not shown); therefore, a mean calibration curve was determined (Table 2). The high values of the correlation coefficients (0.997 and 0.978) indicated good correlations between OA 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 0.05 level (data not shown).

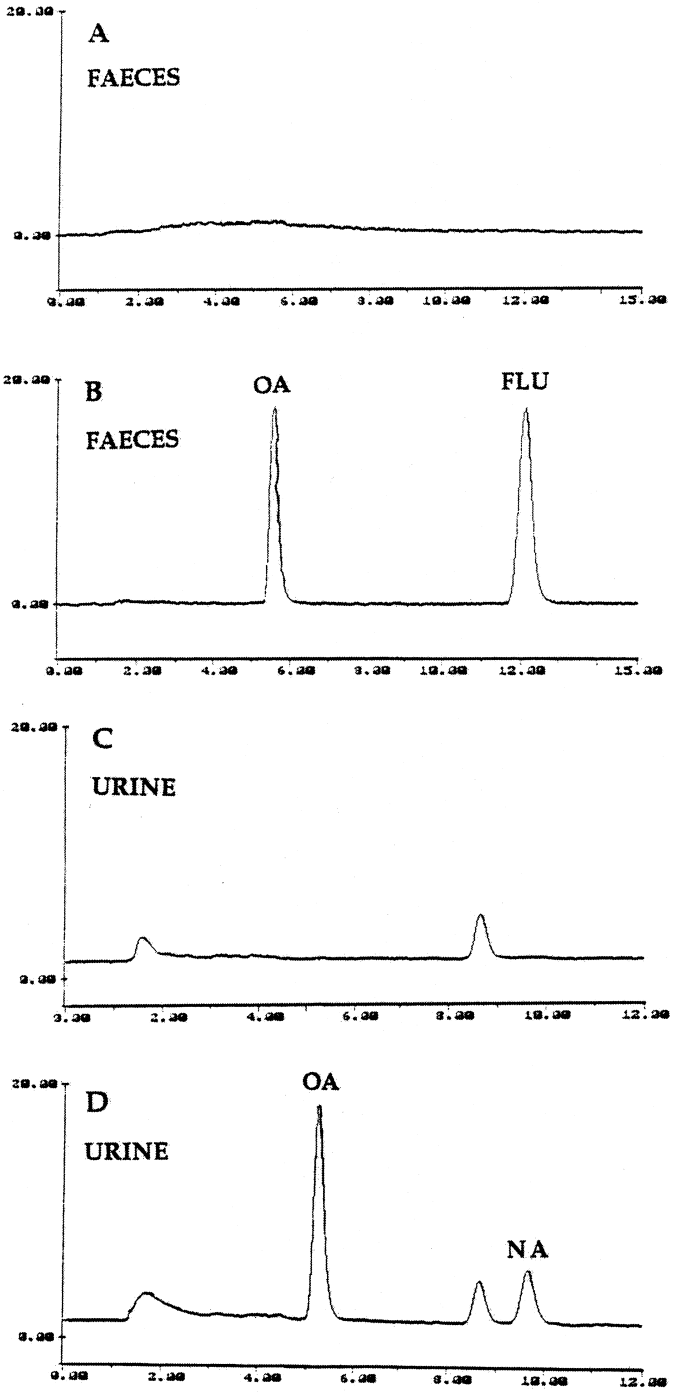

Fig. 1. HPLC chromatograms (peak height in mV in terms of time in min). (A) Blank faeces sample; (B) faeces sample spiked with oxolinic acid (OA) and flumequine (FLU) as the internal standard (1 and 1 μg/g respectively); (C) blank urine sample; (D) urine sample spiked with oxolinic acid (OA) and nalidixic acid (NA) as the internal standard (5 and 2 μg/ml respectively). Conditions: mobile phase, acetonitrile: 0.001 *M* aqueous orthophosphoric acid solution (33: 67 v/v), pH 3.4; cartridge 125×4.6 mm, C18E (5 μm); flow-rate, 0.6 ml/min; excitation wavelength 330 nm and emission wavelength 360 nm; injection volume, 10 μl.

The relative standard deviations of within-run and run-to-run precision were between 3.8 and 9.7% for turbot faeces and between 10.2 and 14.2% for turbot urine (Table 3).

The limits of detection and quantitation of OA in turbot faeces were 0.20 and 1.00 μg/g respectively (Table 2). Those of OA in turbot urine were 0.02 and 0.06 μg/ml respectively (Table 2). These limits of quantitation were accepted (RSD <20.0% with n= 12) because the mean values of the drug peak heights were significantly different from the intercepts at the 0.05 level and greater than three standard deviations [17,24,25]. The limits of detection and quantitation of OA in turbot faeces were high but the OA concentrations in fish faeces after an oral administration of the drug were also high [1].

4. Conclusion

The described method provides a selective, reliable and precise mean for the determination of OA in faeces and urine of turbot. The method does not require complex extraction or derivatization techniques. An analyst familiar with the method could easily process twenty samples a day. The method is

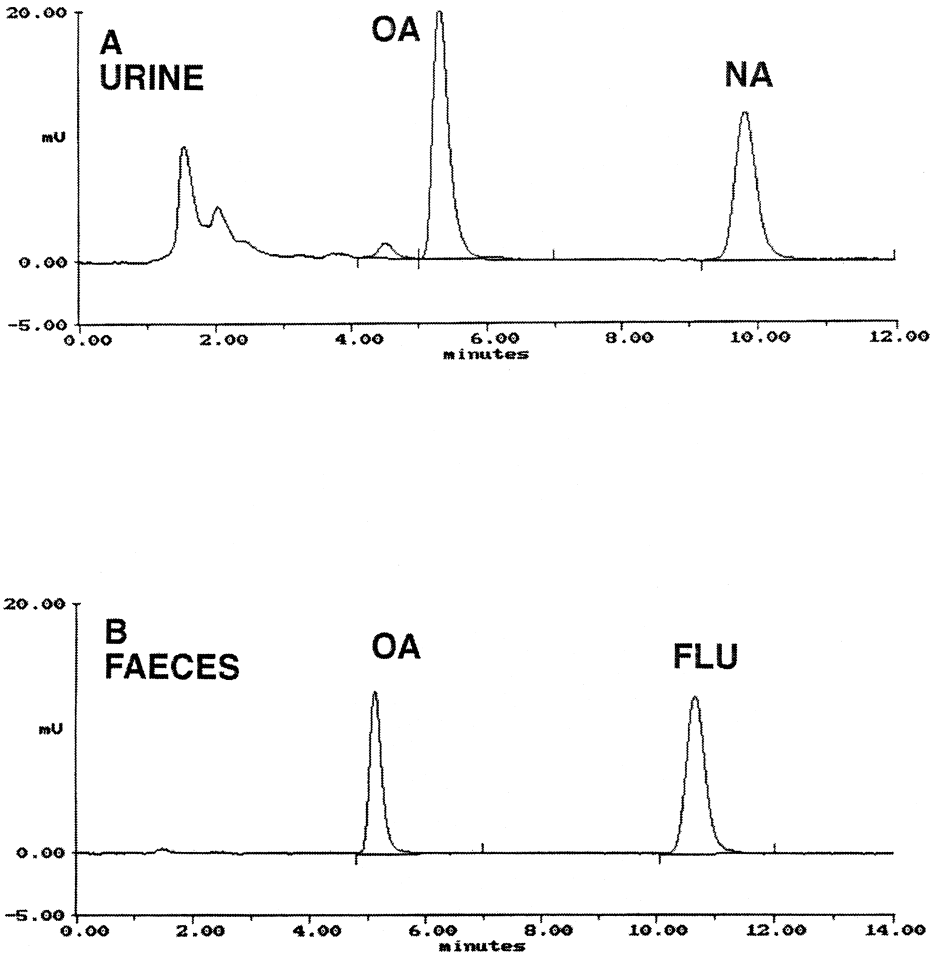

Fig. 2. HPLC chromatograms (peak height in mV in terms of time in min). (A) Urine extract from turbot treated by oxolinic acid at a single oral dose of 36 mg/kg (concentration in urine 7 h after the single oral dose: 1.5 μg/ml); (B) faeces extract from turbot treated by oxolinic acid at a single oral dose of 36 mg/kg (concentration in faeces 4 h after the single oral dose: 222.5 μg/g); conditions: see Fig. 1; OA=oxolinic acid, NA=nalidixic acid, FLU=flumequine.

Table 2
Linearity, regression, extraction recoveries, limits of detection and quantitation: data from the validation assay of the determination of oxolinic acid in faeces and urine of turbot[a]

	Faeces	Urine
Linearity and regression		
Concentration range	1–1000 μg/g	0.06–10 μg/ml
Slope[b]	2.088	2.139
Intercept[b]	−0.041	−0.171
Correlation coefficient[b]	0.997	0.978
Extraction recoveries		
Mean (%)[b]	102.0	91.6
Standard deviation (%)[b]	12.5	13.4
Limit of detection	0.2 μg/g	0.02 μg/ml
Limit of quantitation	1.0 μg/g	0.06 μg/ml

[a] For more details, see Section 3.2.
[b] $n=48$.

Table 3
Within-run and run-to-run precision: data from the validation assay of the determination of oxolinic acid in faeces and urine of turbot[a]

	Within-run RSD[b] (%)	Run-to-run RSD[b] (%)
Faeces μg/g		
Spiked level 1	9.7	9.7
Spiked level 2	7.7	7.7
Spiked level 250	3.8	3.9
Spiked level 1000	6.0	6.0
Urine μg/ml		
Spiked level 0.06	13.6	14.2
Spiked level 0.40	13.9	13.9
Spiked level 2.50	13.3	13.3
Spiked level 10.00	10.2	10.2

[a] For more details, see text.
[b] RSD: Relative standard deviation ($n=12$).

suitable for kinetics studies of OA in turbot (Fig. 2), and perhaps in another fish species.

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