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High-performance liquid chromatographic determination of oxolinic acid in the plasma of seabass (*Dicentrarchus labrax*) anaesthetized with 2-phenoxyethanol

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Abstract

A high-performance liquid chromatographic (HPLC) method for the determination of oxolinic acid (OA) in the plasma of seabass (*Dicentrarchus labrax*) anaesthetized with 2-phenoxyethanol is described. The samples were extracted and cleaned up by a solid-phase extraction procedure using C_{18} extraction cartridges. After the eluent was evaporated, the dry residue was dissolved in 1/15 *M* phosphate buffer. OA was determined by using an isocratic HPLC method with UV detection at 340 nm. Seabass drug-free plasma samples were spiked with OA at 0.2, 1.0, 5.0 and 25.0 μg/ml. Validation of the method showed good precision and accuracy. The mean recovery was 92.2%, with a relative standard deviation lower than 5%. The quantification limit was 0.2 μg/ml. The method was tested on 300 plasma samples of OA-treated seabass. © 1997 Elsevier Science B.V.

Keywords: Oxolinic acid; 2-Phenoxyethanol

1. Introduction

The quinolone oxolinic acid [5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylic acid (OA)] is a chemotherapeutic agent widely used in fish farming for the treatment and prevention of bacterial infections in fish. Its extensive use makes the development of analytical methods for pharmacokinetic and environmental impact studies necessary. Many methods based on high-performance liquid chromatography (HPLC) for the determination of OA in fish serum and tissues have been published [1–10], but HPLC methods for the determination of OA in fish plasma are few [11–13]. One of the three methods [11] used a solid-phase extraction procedure (SPE), and another one [12] used a deproteinization and liquid–liquid extraction procedure (LLE) for the determination of OA. In these two cases, OA was detected with a fluorescence detector. In the third method [13], dialysis and a trace enrichment system were used to clean up samples before HPLC analysis with UV detection. All these published studies have used salmon plasma.

The purpose of this study was to describe a simple and adequate HPLC method for the determination of OA in plasma samples of seabass anaesthetized with 2-phenoxyethanol. This method was needed to study

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the pharmacokinetics of OA in seabass, which must be anaesthetized to collect blood samples.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and methanol (Merck, Darmstadt, Germany) were of HPLC grade. Orthophosphoric acid (85%), potassium dihydrogenphosphate, sodium hydrogenphosphate and 1/3 *M* sodium hydroxide (Merck) were analytical-grade reagents. Dimethylformamide (HPLC grade) was obtained from Carlo Erba (Milan, Italy). 2-Phenoxyethanol was a commercial solution containing 1.11 g of pure reagent per ml (Aquaveto, La Loupe, France). The water used in buffers and eluents was purified with a Nanopure system (Barnsted, IA, USA). Oxolinic acid (OA) and nalidixic acid (NA) were purchased from Sigma (St. Louis, MO, USA).

2.2. Apparatus

A Merck-Hitachi HPLC system was used, consisting of an L-6200A solvent-delivery pump, an AS-2000 autosampler, an UV–Vis L-4250 detector, a T-6300 oven and a D-6000 interface. The system was driven by a Deskpro-386S/20 computer (Compaq, Houston, TX, USA) using the D-6000 HPLC-Manager software (Merck).

The analytical cartridge (LiChroCart 125×4 mm I.D.) and the guard cartridge (LiChroCart 4×4 mm I.D.) were packed with 5 μm LiChroSpher 100 RP-18 end-capped (C_{18}E) sorbent (Merck).

Centrifugations were performed with a Sigma Model 88479 centrifuge (Bioblock, Illkirch, France). Solvents were filtered with a Sartorius solvent filtration system (Grosseron, Saint-Herblain, France).

Solid-phase extraction used a Vac Elut™ vacuum manifold system (Analytichem International, Harbor City, CA, USA) receiving ten Bond-Elut extraction cartridges (Analytichem International) packed with 100 mg of octadecylsilica (C_{18}). Evaporation using a stream of nitrogen was used to dry eluates (Bioblock).

2.3. Standard solutions

Stocks solution of OA and NA (internal standard) were prepared at a concentration of 1000 μg/ml in 0.03 *M* sodium hydroxide solution and these were stored at 4°C. When stored under these conditions, they were stable for one month. A range of working standard solutions were prepared daily by diluting stock solutions with 1/15 *M* phosphate buffer (pH 8.0). At the beginning of each day, a 50-μl volume of these standard solutions was injected onto the HPLC cartridge.

2.4. Analytical sample preparation

Frozen plasma samples were thawed at room temperature for 1 h and centrifuged at 500 *g* for 5 min in order to remove fibrin. Drug-free plasma samples (475 μl) from untreated seabass were spiked with OA (25 μl) at predetermined concentrations. A 200-μl volume of this OA-spiked plasma was immediately added to 200 μl of a 10 μg/ml NA working solution and kept for 1 h. Just before the extraction, 3 ml of 1/15 *M* phosphate buffer were added to this OA and NA spiked plasma. These prepared samples were vortex-mixed for approximately 1 min.

2.5. Extraction and clean-up procedure

The solid-phase extraction cartridges were activated with methanol (2×3 ml) and 0.05% orthophosphoric acid solution (3 ml). After the prepared plasma samples had been passed under low vacuum, the extraction cartridges were flushed with water (1 ml) and 0.05% orthophosphoric acid solution (0.5 ml) and then dried out by increasing the vacuum. OA and NA were eluted with 6×250 μl of acetonitrile, taking care to break the vacuum between each of these six elution fractions. The combined eluates were evaporated to dryness under a stream of nitrogen in a dry bath at 40±2°C. Residues were reconstituted with 200 μl of 1/15 *M* phosphate buffer (pH 8.0). Then, the solutions were vortex-mixed, briefly sonicated and, after a 30-min resting period, once again vortex-mixed. A 50-μl volume was injected onto the chromatographic cartridge.

2.6. Operating conditions

In a first assay, the chromatographic conditions were the same as those described for the determination of OA in sediment [14]. In a second assay, the mobile phase was acetonitrile–0.02 *M* orthophosphoric acid (pH 2.3)–dimethylformamide (10:60:30, v/v). Acetonitrile and 0.02 *M* orthophosphoric acid were separately filtered through Whatman 47 mm, 0.20 μm nylon filters (Maidstone, UK). Then, orthophosphoric acid was sonicated for 15 min. Dimethylformamide was used without any preparation.

The analytical cartridge was conditioned by flushing it with acetonitrile–water (50:50, v/v; 0.2 ml/min for 30 min); acetonitrile–0.02 *M* orthophosphoric acid (50:50, v/v; 0.2 ml/min for 30 min); mobile phase (0.2 ml/min for 30 min; 0.4 ml/min for 20 min and 0.8 ml/min until the baseline was stable).

The cartridge was placed in the oven at 24°C, the flow-rate of the mobile phase was 0.8 ml/min, the UV detector measured peak areas at 340 nm and 50-μl samples were injected onto the cartridge at intervals of 12 min. The guard cartridge was changed after about 250 sample injections.

2.7. Calculations and validation of the analytical method

Standard calibration curves for OA (0, 0.2, 1.0, 5.0, 25.0 μg/ml) were obtained by analysing three replicates of each spiked plasma for three days. They were drawn by plotting the known OA concentrations against the ratio of OA to NA peak areas and calculated by the least-squares linear regression procedure.

The equations of the calibration curves were $y = bx + a$. In this model, the independent variable (x) represented the concentration of OA in the samples, the dependent variable (y) represented the detector response (areas ratios), b was the slope and a the y-intercept. A Cochran test was used to compare the homogeneity of the experimental error variances.

The limit of quantification (LOQ) was defined as the concentration of the lowest standard which presented the desired precision (5 or 10% R.S.D.) [15].

The extraction recovery was defined as the response of a processed spiked matrix standard and was expressed as a percentage of the response of pure standard solutions that had not been subjected to sample pretreatment [15]. It was expressed as a ratio of slopes measured at several OA concentrations. Errors of the analytical measurements were determined by precision and accuracy. Precision was estimated by the relative standard deviation (R.S.D.) of the measured concentrations of replicate samples [16]. Accuracy was estimated by the bias, i.e. the percentage difference between the calculated mean values and the theoretical concentrations [16].

3. Results and discussion

3.1. Liquid chromatography and extraction conditions

OA is a lipophilic molecule, characterized by a carboxylic acid function, a ketone function and a quinoleine group. Moderately concentrated solutions were prepared because of its poor solubility in many common solvents and because of its intramolecular bonding [17]. NA is more hydrophobic than OA. They will be isolated from seabass plasma using C_{18} reversed-phase cartridges before their determination in plasma samples using HPLC methods.

The chromatographic conditions described by Pouliquen et al. [14] were modified because of the presence of the anaesthetic (2-phenoxyethanol) in the plasma. When the previously described HPLC conditions were used to determine OA in spiked plasma from anaesthetized seabass, a peak was obtained that was higher than the one obtained with the OA working standard solution and it had the same retention time. When the concentration of 2-phenoxyethanol was high compared with the OA concentration (6.66 and 0.45 μg/ml, respectively), only one peak was obtained at the OA retention time. A low 2-phenoxyethanol concentration in the presence of a high OA concentration (0.66 and 49.50 μg/ml, respectively) allowed us to observe two peaks near the retention time of OA (Fig. 1). Variations in the composition of the mobile phase [acetonitrile–0.02 *M* orthophosphoric acid solution (35:65, v/v, instead of 24:76, v/v)] did not improve the resolution between OA and 2-phenoxyethanol.

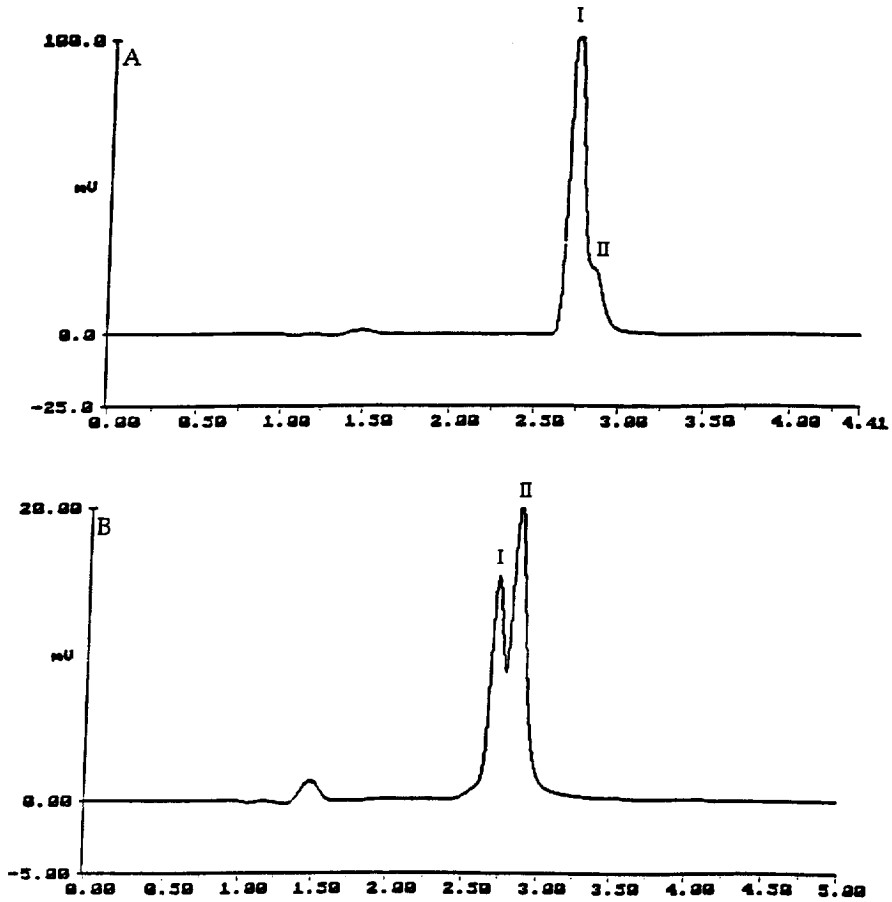

Fig. 1. Chromatograms of oxolinic acid and 2-phenoxyethanol solutions: (A) High concentration of 2-phenoxyethanol compared with OA concentration, (B) low concentration of 2-phenoxyethanol compared with OA concentration. Peak I=2-phenoxyethanol and peak II=oxolinic acid. HPLC conditions: Analytical column, $C_{18}E$, 5 μm; mobile phase, acetonitrile–0.02 *M* orthophosphoric acid, pH 2.3 (35:65, v/v); flow-rate, 1 ml/min; UV detection, 262 nm; injection volume, 50 μl.

Dimethylformamide was used to reduce the peak tailing and to increase the number of theoretical plates (*N*). Less peak tailing was observed but *N* did not increase significantly (from 3298 to 3555). Using a C_2, a C_8 or a C_{18} analytical cartridge gave similar results. Peak heights and retention times were almost similar with C_8 and C_{18} cartridges. C_2 cartridges gave the shortest retention time but the lowest peak height. Finally, a $C_{18}E$ analytical column was chosen in order to improve the peak shape.

A test was performed using a fluorescence detector (F-1000 Merck-Hitachi, Darmstadt, Germany) instead of an UV detector. The fluorescence detector was operated at an excitation wavelength of 320 nm and an emission wavelength of 350 nm. The peaks obtained were twenty times higher but the background noise was significant. OA and 2-phenoxyethanol could not be separated using fluorescence detection.

The detection wavelength of 262 nm was changed because interference by 2-phenoxyethanol was always observed at the retention time of OA. A wavelength of 340 nm was chosen because of its correspondence with the second absorption maximum of OA and with the zero absorption of 2-phenoxyethanol. Under these conditions of wavelength and analytical cartridge, the highest efficiency was obtained with a mobile phase of acetonitrile–

0.02 *M* orthophosphoric acid–dimethylformamide (10:60:30, v/v). A flow-rate of 0.8 ml/min was chosen to maintain the pressure near 8–9 MPa. The retention times of OA and NA were 5.91 (R.S.D.= 0.40%) and 9.87 min (R.S.D.=1.11%), respectively, using a working standard solution, and 5.77 (R.S.D.=1.74%) and 9.66 min (R.S.D.=1.29%) using a spiked plasma sample. At the end of each day, the analytical column was regenerated using acetonitrile–water (50:50, v/v) for 120 min at a flow-rate of 0.3 ml/min.

The solid-phase extraction procedure used was a modification of that provided in a previous study [11]. The volume of the plasma sample was reduced to 200 μl (blank plasma or spiked plasma with OA and NA) and the analytes were eluted using 6×250 ml of acetonitrile. Moreover, after evaporation of the solvent, the residue was redissolved in 200 μl of 1/15 *M* phosphate buffer before being injected into the HPLC system. The recovery increased from 80 to 92% as a result of the above modifications.

3.2. Selectivity and specificity

The response of the antibiotic OA and of the internal standard NA in the matrix was compared with the response of a solution containing these antibiotics to determine the specificity. Blank samples were included in the calibration curves to ensure that no interfering components were co-eluted with OA and NA.

Fig. 2 shows typical chromatograms of a working standard solution and of an extracted plasma sample containing OA and NA. Fig. 3 shows chromatograms

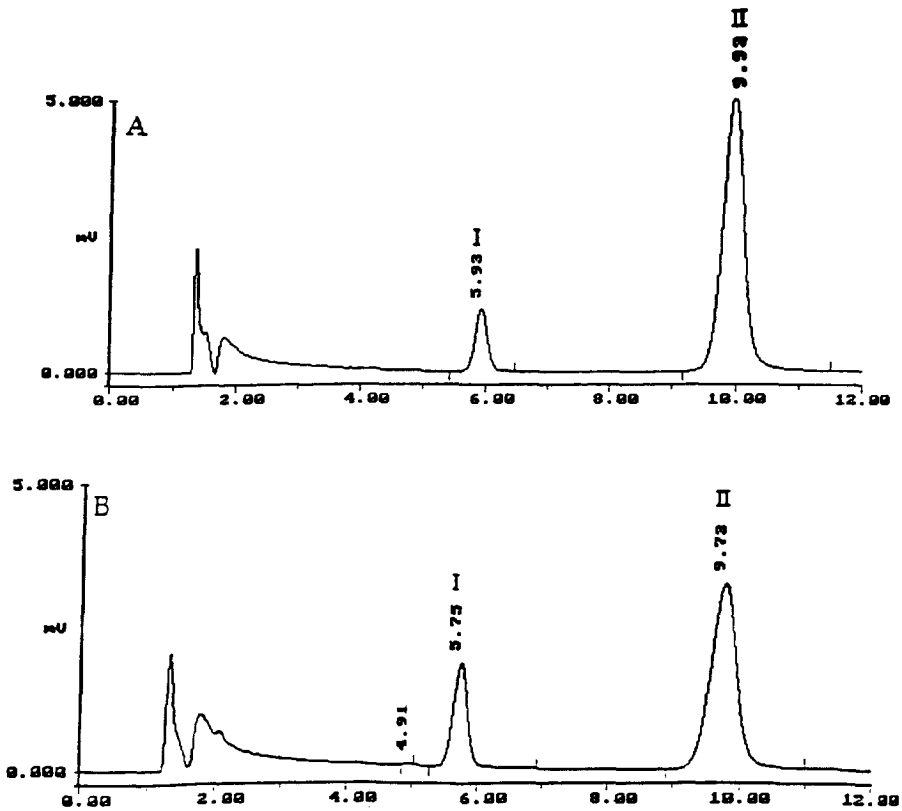

Fig. 2. (A) Chromatogram of pure oxolinic acid (0.5 μg/ml) and nalidixic acid (10 μg/ml). (B) Chromatogram of extracted seabass plasma spiked with oxolinic acid (1 μg/ml) and nalidixic acid (10 μg/ml). Peak I=oxolinic acid and peak II=nalidixic acid. HPLC conditions: Analytical column, $C_{18}E$, 5 μm; mobile phase, acetonitrile–0.02 *M* orthophosphoric acid solution, pH 2.3–dimethylformamide (10:60:30, v/v); flow-rate, 0.8 ml/min; UV detection, 340 nm; injection volume, 50 μl.

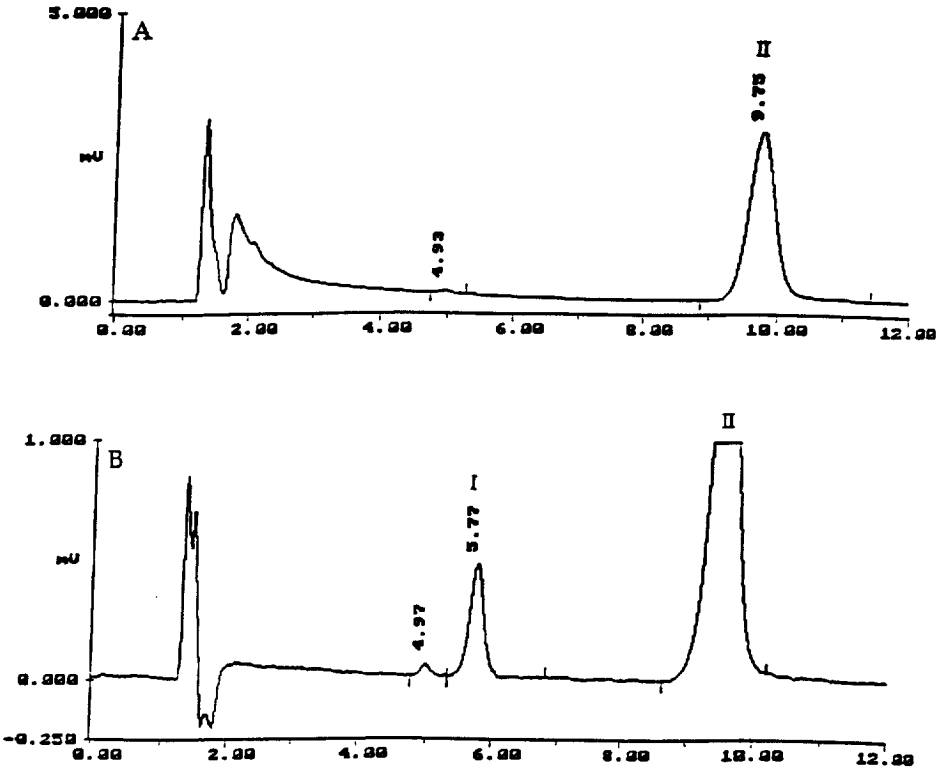

Fig. 3. (A) Chromatogram of extracted seabass blank plasma spiked with the internal standard (NA, 10 μg/ml). (B) Chromatogram of extracted seabass plasma after intravenous administration of 10 mg of oxolinic acid per kg body weight. Peak I=oxolinic acid and peak II=nalidixic acid. HPLC conditions: Analytical column, $C_{18}E$, 5 μm; mobile phase, acetonitrile–0.02 *M* orthophosphoric acid solution, pH 2.3–dimethylformamide (10:60:30, v/v); flow-rate, 0.8 ml/min; UV detection, 340 nm; injection volume, 50 μl.

of a blank seabass plasma sample that was spiked only with the internal standard, and of a seabass plasma sample that was collected after intravascular injection of 10 mg of OA per kg body weight. Chromatograms of spiked plasma showed an interference near 4.9 min (standard deviation=0.014). Chromatograms of treated seabass plasma samples showed the same peak. The resolution factor (R_s) was calculated to be 1.8 using seabass plasma spiked with OA and NA and was 1.7 with treated seabass plasma. In each case, the quality of the separation was good. A *t*-test showed that the *y*-intercept was not significantly different from zero (Table 1).

3.3. Linearity and correlation

The calibration curves plotted as peak-area ratio of OA to NA against concentration of OA were found to be linear from 0.2 to 25 μg/ml ($y=0.28x+0.038$; $r=0.985$). The values of the correlation coefficients (0.982 to 0.999) indicates that a relationship exists between the concentration and the response. The existence of significative slopes was determined by a Fischer test: The calculated *F*-ratios (mean−square of regression/mean−square of residual) were always higher than the critical value [F (0.05; 1; 10)=4.96] at the 0.05 level (Table 1). Using another method, a Student's *t*-test, with values above the theoretical value [t (0.05; 11)=2.201], showed the significance of the correlation between the concentration and the response (Table 1).

3.4. Accuracy and precision

Accuracy was determined by the most widely used approach based on the recovery. The acceptance

Table 1
Linearity and correlation of the calibration curves obtained from seabass plasma spiked with oxolinic acid from 0.2 to 25 μg/ml

	Day 1 (n=12)	Day 2 (n=12)	Day 3 (n=12)
Slope	0.3045	0.2609	0.2734
Intercept (constant)	0.0459	0.0250	0.0477
Correlation coefficient	0.982	0.999	0.997
Existence of slope (Fischer test, F)	497[a]	185 413[a]	3497[a]
Correlation validity (Student's test, t)	22.30[a]	140.00[a]	59.13[a]
Comparison of the constant from zero (Student's test, t)	0.252[b]	1.025[b]	0.810[b]

$y=bx+a$; y=ratio of oxolinic acid to nalidixic acid peak areas; x=oxolinic acid concentration (μg/ml); b=slope; a=y-intercept.
Theoretical values: F (0.05, 1, 10)=4.96; t (0.05, 10)=2.201.
Abbreviations: n=total number of data.
Index [a]=significative values and [b]=non-significative values at the 0.05 level.

criterion for this parameter is set at a bias value lower than 10 [16] or 15% [18]. Each concentration within the range was used and the bias values are shown in Table 2. Only the bias value for the lowest concentration was above the acceptable limits.

Within-day and between-day precisions were calculated for highest, lowest and median concentrations. R.S.D. values for the within-day precision did not vary greatly (6.47 to 8.48%) (Table 3). Those for the between-day precision varied from 10.53 to 17.05% (Table 3). Assay results with R.S.D. values under 10% may be considered as acceptable [16]. As for accuracy, the value for the lowest concentration was above the acceptable limit [16]. A narrower range may improve the bias and reproducibility for extreme values.

3.5. Recovery

Experiments were performed at the different concentrations within the range. The analytical recovery data are shown in Table 4. The mean recovery

Table 2
Accuracy data obtained from seabass plasma spiked with oxolinic acid from 0.2 to 25 μg/ml

Mean calculated concentration (μg/ml)	Theoretical concentration (μg/ml)	Bias (%)
0.23	0.20	15.56
0.99	1.00	0.62
5.05	5.00	1.00
23.52	25.00	5.90

Table 3
Precision data obtained from seabass plasma spiked with low, medium and high concentrations of oxolinic acid

	Spiked concentration (μg/ml)		
	0.2	5	25
Repeatability			
R.S.D. (%)	8.48	6.47	8.49
Reproducibility			
R.S.D. (%)	17.05	10.53	10.91
n	3	3	3
k	3	3	3
Cochran value	0.741[b]	0.627[b]	0.887[a]

Abbreviations: R.S.D.=relative standard deviation; k=number of assay groups; n=number of replicates per group.
Theoretical values: Cochran (0.05, 3, 2)=0.87.
Index [a]=significant values and [b]=non-significant values at the 0.05 level.

Table 4
Extraction recoveries of oxolinic acid from seabass plasma spiked with oxolinic acid from 0.2 to 25 μg/ml

	Recovery (%)	R.S.D. (%)
Day 1	98.72	
Day 2	89.35	
Day 3	88.48	
Mean	92.18	3.16

Abbreviation: R.S.D.=relative standard deviation. Values obtained by the slopes method.

obtained by the slopes method was 92.18% (R.S.D.= 13.16%).

3.6. Limits of detection and quantification

The detection limit (LOD) is the lowest analyte concentration that produces a response that is detectable above the noise level of the HPLC system. Typically, the LOD is calculated as the mean blank signal plus three times the standard deviation [18] or $3\ S_b/S$ [15], where S is the sensitivity (slope of the calibration curve), S_b represents the standard deviation of the blank measurement and 3 is a factor for a 0.999 level of confidence. The LOD was calculated to be 0.014 or 0.02 μg/ml, depending on the calculation method used. These values were lower than the one reported for salmon plasma, i.e. 0.05 μg/ml obtained using UV detection [13], and are obviously higher than those obtained for salmon plasma using fluorescence detection, i.e. 0.005 and 0.003 μg/ml [11,12]. The quantification limit (LOQ) is the lowest amount of analyte that can be quantitatively determined with defined precision and accuracy under the experimental conditions. The R.S.D. was acceptable when its value ranged from 10 to 20% [15]. A concentration of 0.2 μg/ml was the concentration level that gave a R.S.D. that was close to but lower than 20% when a precision study was performed (Fig. 4).

4. Conclusion

Our study has shown that OA can be extracted from anaesthetized seabass plasma and determined using HPLC with UV detection. A validation study had demonstrated that the analytical method was reliable for pharmacokinetic and environmental impact studies. This method allowed the measurement of OA concentration in 30 samples a day without any difficulty.

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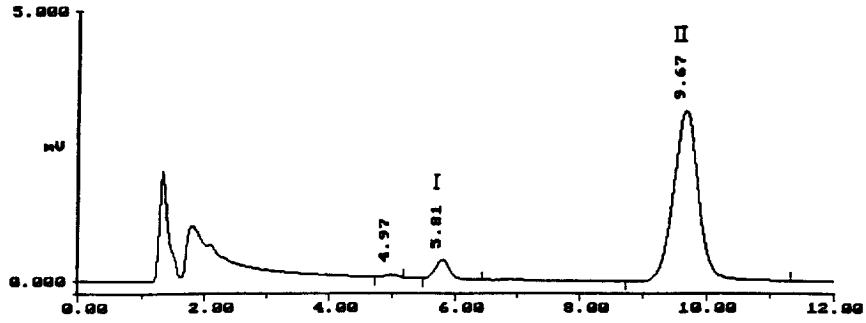

Fig. 4. Chromatogram of extracted seabass plasma spiked with oxolinic acid at the level of the LOQ (0.2 μg/ml) and nalidixic acid (10 μg/ml). Peak I=oxolinic acid and peak II=nalidixic acid. HPLC conditions: Analytical column, $C_{18}E$, 5 μm; mobile phase, acetonitrile–0.02 *M* orthophosphoric acid solution, pH 2.3–dimethylformamide (10:60:30, v/v); flow-rate, 0.8 ml/min; UV detection, 340 nm; injection volume, 50 μl.

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