High-performance liquid chromatographic determination of oxolinic acid and flumequine in the live fish feed *Artemia*

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Abstract

A high-performance liquid chromatography (HPLC) analytical method for the determination of oxolinic acid and flumequine in *Artemia* nauplii is described. The samples were extracted and cleaned up by a solid-phase extraction (SPE) procedure using SPE C_{18} cartridges. Oxolinic acid and flumequine were determined by reversed-phase HPLC using a mobile phase of methanol–0.1 M phosphate buffer, pH 3 (45:55, v/v) and a UV detection wavelength of 254 nm. Calibration curves were linear for oxolinic acid in the range of 0.2–50 µg/g ($r^2 = 0.9998$) and for flumequine in the range of 0.3–50 µg/g ($r^2 = 0.9994$). Mean recoveries amounted to 100.8% and 98.4% for oxolinic acid and flumequine, respectively. The quantification limit was 0.2 µg/g for oxolinic acid and 0.3 µg/g for flumequine. Quantitative data from an in vivo feeding study indicated excellent uptake of both drugs by *Artemia* nauplii. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Artemia; Oxolinic acid; Flumequine

1. Introduction

Quinolones are synthetic antibacterial agents, which are especially active against Gram-negative bacteria and they are used both in human [1] and in veterinary medicine [2–4]. Bacterial diseases of fish pose a major problem in intensive culture systems and they have been treated with various chemotherapeutics, such as potentiated sulfonamides and, more recently, with the quinolones oxolinic acid and flumequine [5,6]. An efficient method for the administration of therapeutics to fish larvae consists of using *Artemia* nauplii, which are their live feed, supplemented with the therapeutic agents. This method proved quite effective in administering potentiated sulfonamides or tetracyclines to fish larvae with minimal leakage of the administered drugs to the environment [7–9].

Several methods have been reported for the determination of quinolones in samples of fish or fish feed, using either bioassays [10,11], high-performance liquid chromatography (HPLC) [12–18], or gas chromatography [19]. These methods have been used for the determination of either oxolinic acid [12,14,17,18] or flumequine [1,3,4] or both [6,13,15,16] and they apply tissue specific liquid–liquid extraction [13,16] or solid-phase extraction (SPE) procedures [12,14,18]. Moreover, the methods

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previously described for the simultaneous determination of flumequine and oxolinic acid in fish tissues [5,16], plasma [13] or silage [6] employed the use of fluorescence detection [6,13,15,16], gradient elution [13] or even column-switching [15]. In addition, the liquid–liquid extraction protocols that were used [6,13,15,16], produced poor results in the case of *Artemia* samples. There is no analytical method available for the determination of quinolones in the live fish feed *Artemia* nauplii. In fact, the only method that was previously used [11], was a non-specific radial-diffusion bioassay that employed no extraction procedure and was of moderate sensitivity.

In this paper, a simple, rapid and sensitive analytical method is described for the simultaneous determination of flumequine and oxolinic acid in *Artemia* nauplii, using SPE with reversed-phase chromatography and UV detection at 254 nm.

2. Experimental

2.1. Chemicals and reagents

Methanol (Malinckrodt Baker, Deventer, The Netherlands) and *n*-hexane (Lab Scan, Dublin, Ireland) were of HPLC grade. Sodium dihydrogenphosphate, sodium hydrogenphosphate and sodium hydroxide (E. Merck, Darmstadt, Germany) were analytical-grade reagents. Oxolinic acid and flumequine were purchased from Sigma (St. Louis, MO, USA).

2.2. Apparatus

The HPLC system consisted of a Marathon isocratic pump (Rigas Labs., Thessaloniki, Greece), a Rheodyne 7125 valve injector with a 100-μl loop, an SSI Model 500 UV–Vis variable-wavelength detector and a Hewlett-Packard HP 3396 Series II integrator. The analytical column was Hypersil octadecylsilane ODS 150×4.6 mm I.D., 5 μm particle size, protected with a guard column 10×4.6 mm I.D., packed with the same material (Alltech, Deerfield, IL, USA). Centrifugation was performed with an Eppendorff bench centrifuge. Solvents were filtered with an Alltech HPLC solvent filtration system using Alltech membrane filters (of a diameter of 47 mm and a pore size of 0.45 μm). The SPE cartridges were C_{18} Extract-Clean from Alltech.

2.3. Standard solutions

Stock solutions of oxolinic acid and of flumequine were prepared at a concentration of 1 mg/ml in 0.1 M sodium hydroxide and they were stored at 4°C in dark flasks. Under these storage conditions they were stable for 1 month. Oxolinic acid was used as the internal standard for the determination of flumequine and vice versa. A range of working standard solutions was prepared daily by diluting stock solutions with double distilled water.

2.4. Analytical procedure

Frozen samples of *Artemia nauplii* (0.5 g) were homogenized in 3 ml 0.1 M sodium dihydrogenphosphate buffer. The pH values of the homogenization buffer ranged from 2 to 9 and the recoveries of both oxolinic acid and flumequine were determined in all cases. The homogenate was centrifuged, the sediments were reconstituted in another 3 ml of the homogenization buffer and recentrifuged. The aqueous supernates were combined and used for liquid–liquid extraction or for SPE. Liquid–liquid extraction was performed using 2×5 ml *n*-hexane and the organic phase was discarded. For SPE, the SPE cartridges were preconditioned with 5 ml methanol, 5 ml water and 5 ml 0.1 M phosphate buffer of the same pH value as the homogenization buffer, each time. After application of the samples the cartridges were flushed with 2×5 ml *n*-hexane and they were dried out. Oxolinic acid and flumequine were eluted with 5 or 10 ml of 0.1 M phosphate buffer, pH 3–methanol (1:9, v/v). The methanolic phase was evaporated under vacuum, the aquatic phase (1 ml) was collected and 100 μl were injected onto the chromatographic column.

2.5. Operating conditions

In a first set of experiments the mobile phase consisted of 0.1 M phosphate buffer, pH 3–methanol at 65:35, 60:40, 55:45, 50:50, 45:55 (v/v). The values of the capacity factor (*k*) were determined and the presence of interferences was recorded in each

case. The hold-up time (t_0) was determined by injecting 50 μl of a NaNO$_2$ solution (1 mg/ml) which is not retained on the column and is eluted in the void volume of mobile phase.

In the second set of experiments the mobile phase was 0.1 *M* phosphate buffer–methanol (55:45, v/v), while pH values ranging from 2 to 6 were tested and the capacity factors were determined for each pH value of the mobile phase.

The analytical cartridge was conditioned by flushing with methanol–water (20:80, v/v) at 1 ml/min for 30 min and then the mobile phase was introduced. The cartridge was at ambient temperature, the flow-rate of the mobile phase was 1 ml/min, the UV detector operated at a wavelength of 254 nm, peak areas were integrated and 50-μl samples were injected onto the cartridge at intervals of 30 min. The guard cartridge was changed after about 200 sample injections.

2.6. Calculations and validation of the analytical method

Calibration curves were prepared from *Artemia* nauplii samples (0.5 g) to which 100 μl of the internal standard solution (100 μg/ml) and 50 μl of the appropriate drug standard solutions were added to give a final concentration of 1.0, 5.0, 10.0, 20.0 and 50.0 μg/g of tissue. As mentioned earlier, oxolinic acid was used as the internal standard for flumequine determination and vice versa.

The calibration curves were constructed and linear regression equations were obtained by plotting the ratios of oxolinic acid or flumequine peak areas to the peak areas of the internal standard, against the known concentrations of oxolinic acid or flumequine. The extraction recovery was calculated as the percentage of the ratio of the peak area of the drugs in a processed spiked sample standard to the peak area of the same drug in a pure standards solution [18]. Precision was estimated by the relative standard deviation (RSD) of the measured concentrations of replicate samples and accuracy was estimated as the bias (found concentration−theoretical concentration)/theoretical concentration×100 [20]. The limit of detection (LOD) was determined as the lowest analyte concentration detectable above the noise level of the system, with $S/N=3$. The limit of

quantitation (LOQ) was defined as the lowest concentration of a processed standard that presented a precision of 10–20% RSD [18].

2.7. Feeding experiment

Artemia cysts (GSL, EG grade) were incubated in artificial seawater (salinity 35 g/l, pH 8.75), under continuous aeration and illumination (2000 lux) as previously described [7–9]. After 24 h instar I *Artemia* nauplii were collected and transferred in clean artificial seawater at a density of 100 individuals/ml. Then, two rations of an emulsion (Selco, INVE Aquaculture *Artemia* Systems, Belgium) in which either oxolinic acid (5, 10 or 20%, w/w) or flumequine (20, 30, or 40%, w/w) were administered to the nauplii. The first ration was administered 2 h after the onset of incubation, the second ration 8 h after the onset of incubation and the duration of the enrichment period was 24 h. Blank *Artemia* samples were fed with the emulsion in which no drugs were added. The nauplii were collected after 24 h of enrichment, they were thoroughly washed, and stored at −40°C until used for further analysis.

3. Results and discussion

3.1. Liquid chromatography conditions

The effects of the percentage of methanol and of the pH of the buffer in the mobile phase on the capacity factor of oxolinic acid and flumequine are shown in Table 1. Methanol levels in mobile phase, that were higher than 50%, caused the drug peaks to coelute with endogenous compounds in *Artemia* nauplii tissues, while a methanol content lower than 45% resulted in long retention times that exceeded 20 min. As for the effect of the pH of the mobile phase, at pH 2 the baseline was receding even after 2 h of equilibration while the pH values of 4, 5 and 6 gave rise to severe peak tailing.

Previous investigators employed acidic mobile phases, of a pH value 2 to 3, and a content of organic modifiers ranging from 10 to 30% for the simultaneous determination of oxolinic acid and flumequine in fish tissues [5,15,16], fish silage [6] and in

Table 1
Effect of (A) the percentage of methanol and (B) the pH of the mobile phase on the capacity factor of oxolinic acid and flumequine

	Capacity factor (k)	
	Oxolinic acid	Flumequine
(A) Methanol (%)		
35	5.65±0.1	15.77±0.7
40	4.62±0.8	10.36±0.3
45	2.66±0.1	6.57±0.1
50	1.63±0.1	3.87±0.1
55	0.94±0.3	3.05±0.3
(B) pH of mobile phase		
2	2.84±0.2	6.69±0.3
3	2.66±0.1	6.57±0.1
4	2.64±0.2	6.50±0.1
5	2.60±0.2	6.48±0.2
6	1.92±0.1	5.57±0.1

mussels [21]. However, the use of a higher content of organic modifier in our studies is in accordance with that reported recently either for the determination of flumequine [4] or of oxolinic acid [18].

It appears that the problem of tailing peaks at pH values higher than 3 was also encountered in the analysis of oxolinic acid in fish tissues [14]. Peak tailing and the presence of a shoulder is also evident in our chromatograms possibly due to column overload. A change of the column resulted in the improvement of the peak shape of flumequine and only a peak tailing remained. The use of tetrahydrofuran in the mobile phase also greatly improves the peak shape (data not shown) but it also results in the coelution of oxolinic acid with matrix components. In our studies the best separation and peak shape in the determination of oxolinic acid and flumequine in *Artemia* nauplii samples were obtained with the use of the mobile phase methanol–0.1 *M* phosphate buffer (45:55, v/v) at pH 3 (Figs. 1 and 2, respectively). Under these conditions the capacity factor for oxolinic acid was 2.66±0.12, for flumequine 6.57±0.07, while the asymmetry factors were 0.90 and 0.86 for oxolinic acid and flumequine, respectively. The retention times were 5.94 min for oxolinic acid and 12.4 min for flumequine, with a hold-up time (t_0) of 1.58 min, while no interferences from the sample matrix were observed.

3.2. Extraction conditions and recovery

The recoveries of oxolinic acid and flumequine from spiked *Artemia* samples using different pH values of the phosphate buffer used for the extraction are shown in Table 2. Best recoveries were obtained for both drugs at pH 3. Two extraction cycles each with 3 ml of phosphate buffer were required, as the second extraction step was found to improve recovery by 8% for oxolinic acid and by 10% for flumequine. Multiple extraction cycles have also been reported to improve recovery of oxolinic acid from fish tissue [14]. The extraction of the sample lipids with hexane appeared to be a crucial and pH related step in the extraction protocol. At high pH values, *Artemia* lipids, which are mainly carotenoids and pigments, were not dissolved in hexane but remained in the aquatic phase and coeluted with the drugs from the SPE cartridge, resulting in adverse interferences in the final chromatograms. An attempt to remove these matrix constituents by filtration through a 0.2-μm syringe filter resulted in low extraction recoveries, suggesting possible interactions between the matrix lipids and the drugs. At low pH values (pH<3) clogging of the SPE cartridges was observed.

In addition to SPE, liquid–liquid extraction was also tested for its efficacy. Hexane was first added during the centrifuging of the homogenized tissue samples. To this end two extractions were performed during the two sequential centrifuging steps, each employing 3 ml of hexane, the aqueous phase was collected and the recovery was found to be 32.1±8.1% (*n*=4) for oxolinic acid and 56.1±3.4% (*n*=4) for flumequine. Then hexane was added in the aqueous supernate that resulted from the centrifuging step, two extractions employing 5 ml hexane each were performed, and the recovery was 53.8±4.6% for oxolinic acid and 54.5±4.5% for flumequine. Finally the extraction of lipids with hexane on the SPE cartridge was tested. The cartridge was equilibrated, the sample was applied, then 2×5 ml hexane was added on the SPE cartridge and the drugs were eluted. This approach resulted in the highest recoveries that amounted to 100.8±6.3% for oxolinic acid and to 98.9±5.3% for flumequine. It should also be noted that the elution of the drugs from the SPE cartridge with 10 ml of phosphate buffer, pH 3–
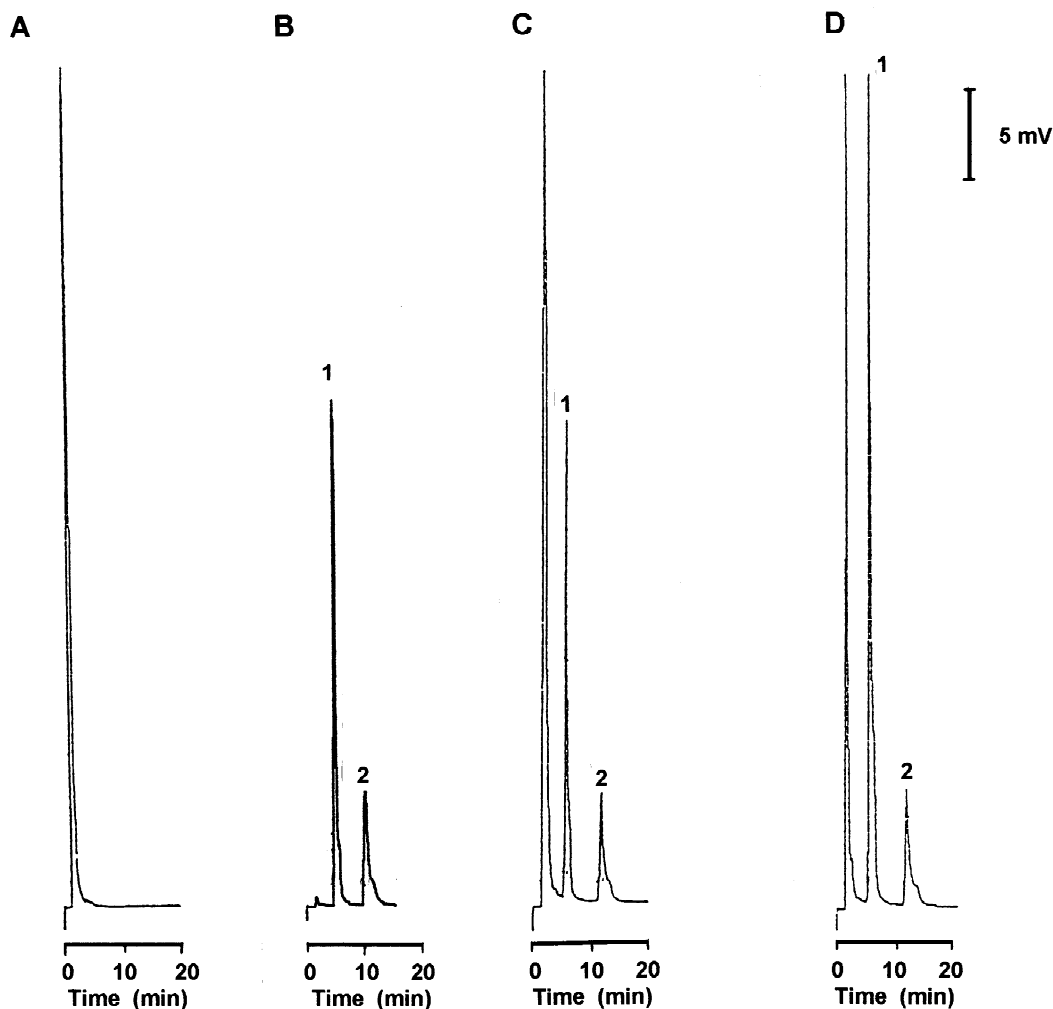
Fig. 1. Representative chromatograms for oxolinic acid series: (A) a blank *Artemia* nauplii extract, (B) a working standard solution containing 5 µg/ml oxolinic acid and 10 µg/ml flumequine, (C) an extracted *Artemia* sample spiked with 5 µg/ml oxolinic acid and 10 µg/ml flumequine, (D) an extracted sample of *Artemia* nauplii fed with an emulsion containing 20% (w/w) oxolinic acid and spiked with 10 µg/ml of the internal standard flumequine. Chromatographic conditions: Hypersil-ODS 5 µm (150 mm×4.6 mm I.D.) column, mobile phase 0.1 *M* phosphate buffer, pH 3–methanol (55:45), flow-rate 1.0 ml/min, UV detection at 254 nm. Peaks: 1=oxolinic acid, 2=flumequine.

methanol (1:9, v/v) improved recovery by 17% in comparison with the use of 5 ml of the same elution mixture.

3.3. Specificity and selectivity

Specificity was determined by comparing the response of oxolinic acid and flumequine in the tissue matrix with the response of the same components in a solution containing the pure analytes. Blank *Artemia* samples were also included in the calibration curves to ensure the absence of matrix peaks that might coelute with either of the two drugs. Typical chromatograms of a blank *Artemia* sample, of a working standard solution, of an extracted spiked *Artemia* sample and of an *Artemia* sample
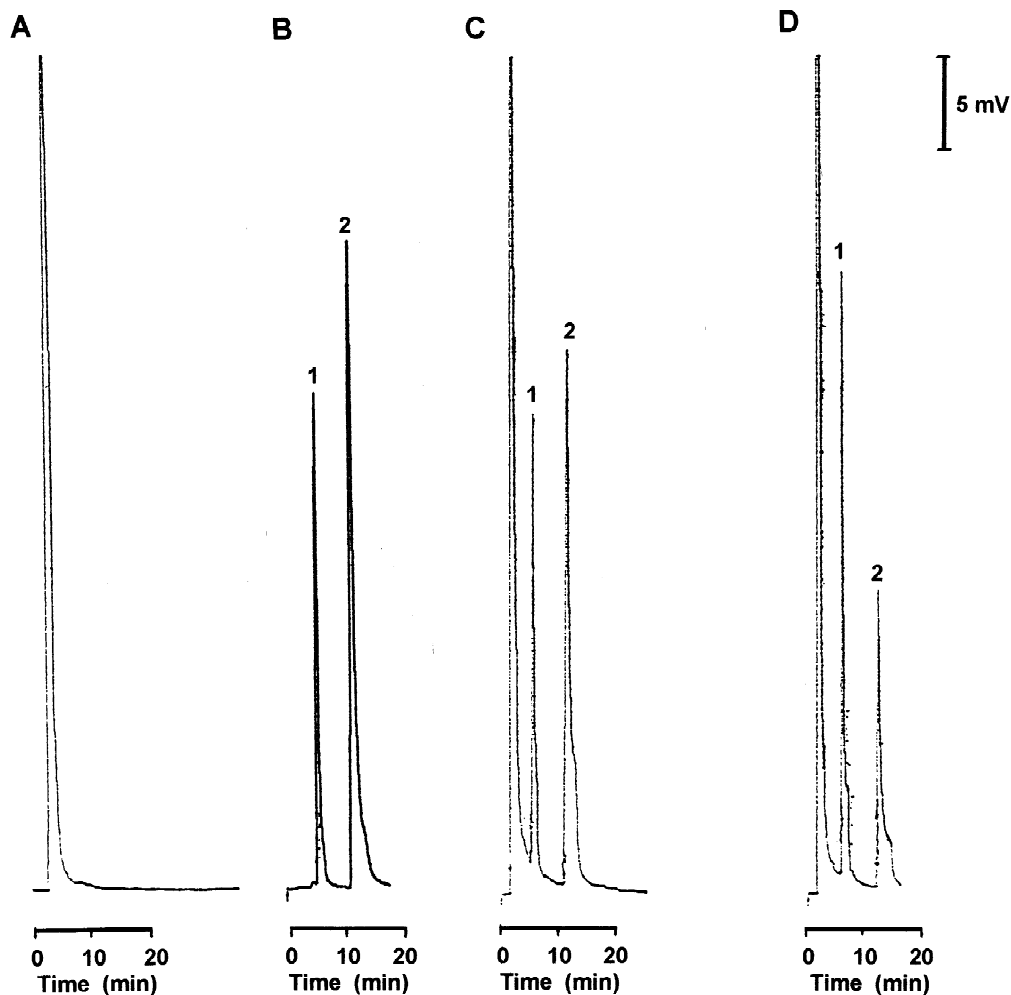
Fig. 2. Representative chromatograms for flumequine series: (A) a blank *Artemia* nauplii extract, (B) a working standard solution containing 25 μg/ml flumequine and 5 μg/ml of oxolinic acid, (C) an extracted *Artemia* sample spiked with 25 μg/ml flumequine and 5 μg/ml of oxolinic acid, (D) an extracted sample of *Artemia* nauplii fed with an emulsion containing 40% (w/w) flumequine and spiked with 5 μg/ml of the internal standard oxolinic acid. Chromatographic conditions: as in Fig. 1. Peaks: 1=oxolinic acid, 2=flumequine.

fed with the drugs, are given for oxolinic acid in Fig. 1 and for flumequine in Fig. 2. No interferences were recorded in blank *Artemia* samples proving that the extraction protocol that was followed was quite efficient. Good resolution of the two analytes was achieved in all of the samples, as is concluded from the calculated resolution (R_s) value, which amounted to 1.90 for spiked *Artemia* samples, to 1.84 for *Artemia* fed with oxolinic acid and to 1.71 for *Artemia* fed with flumequine.

3.4. Linearity and correlation

The linearity of the standard curves was investigated in the range of 0.1 to 50 μg for oxolinic acid and 0.15 to 50 μg for flumequine. This corresponds to concentrations of 0.2 to 100 and 0.3 to 100 μg/g tissue, respectively. The calibration curve for oxolinic acid was plotted as the peak area ratio of oxolinic acid to flumequine against the concentration of oxolinic acid. The calibration curve for

Table 2

Effect of extraction buffer pH on the recovery of oxolinic acid and flumequine from spiked *Artemia* samples[a]

pH of extraction buffer (0.1 *M* sodium dihydrogenphosphate)	Recovery (%) (mean±SD)	
	Oxolinic acid (n=4)	Flumequine (n=4)
2	29.9±0.2	58.9±0.2
3	100.8±6.3	98.9±5.3
4	83.2±0.6	64.5±0.1
5	77.0±0.1	66.1±4.6
6	71.6±0.1	70.3±9.8
7	47.9±2.6	57.7±1.9
8	44.9±2.2	70.8±1.3
9	29.5±0.4	65.7±0.1

[a] *Artemia* samples (0.5 g) were spiked with oxolinic acid and flumequine (each at a concentration of 10 µg/ml which corresponds to 20 µg/g).

flumequine was plotted as the ratio of the peak area of flumequine to oxolinic acid against the concentration of flumequine. Equations of the standard curves obtained by analyzing spiked samples were $y=0.005+0.200x$ ($r=0.9999$) for oxolinic acid and $y=-0.006+0.100x$ ($r=0.9997$) for flumequine. The corresponding equations for the standard curves obtained by direct injection of the pure analytes were $y=0.004+0.196x$ ($r=0.9998$) for oxolinic acid and $y=-0.004+0.104x$ ($r=0.9991$) for flumequine. The fact that there is good agreement in the slopes of the two sets of standard curves for each drug suggests a similar behavior of the two analytes in the course of sample pretreatment.

3.5. Accuracy, precision and recovery

The determination of accuracy was based on the recovery of known amounts of analyte spiked into the sample matrix [20]. The acceptance criterion for this parameter is set at a value lower than 10% [18]. From the accuracy data shown in Table 3, it is concluded that accuracy was good at all concentrations tested, for both analytes.

Table 3

Accuracy data obtained from *Artemia* samples spiked with oxolinic acid or flumequine from 0.2 to 50 µg/g

Nominal concentration (µg/g)	Mean calculated concentration (µg/g) (mean±SD)	Accuracy (%)
Oxolinic acid		
0.2	0.2±0.03	6.0
1.0	1.1±0.1	8.0
5.0	4.7±0.2	−5.6
10.0	9.7±0.3	−3.1
20.0	18.6±0.4	−7.0
50.0	50.8±2.8	1.6
Flumequine		
0.3	0.3±0.04	3.3
1.0	1.0±0.04	0.8
5.0	4.8±0.1	3.6
10.0	9.9±0.4	0.6
20.0	19.0±0.5	5.0
50.0	46.3±0.9	7.4

Table 4
Precision data obtained from spiked *Artemia* samples[a]

	Oxolinic acid concentration (μg/g)					
	50	20	10	5	1.0	0.2
N	8	8	8	12	9	10
Mean±SD (μg/g)	50.8±2.8	18.6±0.4	9.7±0.3	4.7±0.2	1.1±0.1	0.2±0.02
Repeatability RSD (%)	3.7	2.0	2.9	4.0	4.6	4.8
Reproducibility RSD (%)	5.6	1.9	3.4	4.3	10.4	13.4
	Flumequine concentration (μg/g)					
	50	20	10	5	1.0	0.3
N	8	8	12	8	8	10
Mean±SD (μg/g)	46.3±0.9	19.0±0.5	9.9±0.4	4.8±0.1	1.0±0.04	0.3±0.04
Repeatability RSD (%)	1.7	3.2	1.7	3.1	3.9	4.7
Reproducibility RSD (%)	1.9	2.9	3.9	2.7	3.9	13.6

[a] Repeatability RSD (%) corresponds to intra-day precision and reproducibility RSD (%) to inter-day precision of HPLC analysis. N represents the number of analyses during the study of precision.

Within- and between-day precision data are given in Table 4. Acceptable RSD values (lower than 10%) were obtained for all concentrations and for both analytes, with the exception of the lowest concentration tested, where reproducibility appeared slightly elevated amounting to 13.4% for oxolinic acid and to 13.6% for flumequine.

The analytical recovery data are shown in Table 5.

Table 5
Recovery rates of oxolinic acid and flumequine in spiked *Artemia* samples ($n=6$)

Oxolinic acid concentration (μg/g)	Recovery (mean±SD)	RSD (%)
0.2	105.5±13.6	12.9
1.0	110.6±11.6	10.4
5.0	93.1±4.1	4.3
10.0	96.8±3.8	3.9
20.0	99.8±1.5	1.5
50.0	98.9±2.1	2.2
Flumequine concentration (μg/g)		
0.3	101.6±10.9	10.7
1.0	100.7±5.1	5.0
5.0	96.8±1.7	1.8
10.0	102.0±2.4	2.3
20.0	94.6±2.6	2.8
50.0	92.4±1.9	1.1

Mean recovery amounted to 100.8±6.3% (RSD= 4.9%) for oxolinic acid and to 98.9±5.4% (RSD= 3.9%) for flumequine.

3.6. Limits of detection and quantification

The detection limit is defined as the lowest concentration of the analyte, that produces a response detectable above the noise level of the system, which typically is three times the noise level [20]. The LOD was calculated to be 0.012 μg/g for oxolinic acid and 0.037 μg/g for flumequine. These values are in accordance with those reported when UV detection is employed [18], but higher than the values obtained using fluorescence detection [13].

The LOQ in *Artemia* samples was taken as the lowest concentration of the analyte, which is measured with a defined precision and accuracy under the experimental conditions used. Precision was considered acceptable when its RSD value ranged from 10 to 20% [18]. The LOQ was determined to be 0.2 μg/g for oxolinic acid and 0.3 μg/g for flumequine. The intra-assay ($n=10$) and inter-assay ($n=8$) RSDs were 4.8 and 13.4% for oxolinic acid and 4.7 and 13.6% for flumequine, respectively (Table 4). As the inter-assay RSD was lower than 15% for both analytes, the mean values of the analyte peak heights were significantly different ($P<0.05$) from the intercepts and they were greater than

three standard deviations [21], these LOQs were accepted.

3.7. Animal study

The bioencapsulation study was used to determine the efficacy of *Artemia* nauplii enriched with oxolinic acid or flumequine, to serve as carriers of these antimicrobials to fish larvae, minimizing the risk of leaching or decomposition of the drugs in the environment. The nauplii appeared to incorporate the administered drugs and the use of increasing concentrations of oxolinic acid or flumequine in the enrichment medium resulted in an increase of the levels of these drugs in the animals (Fig. 3). Previous studies employing the use of potentiated sulfonamides [7,9] or tetracyclines [8] have also proven the efficacy of enriched *Artemia* nauplii to act as carriers of therapeutics to fish larvae. The levels of oxolinic acid and flumequine in *Artemia* nauplii that were determined in the present study amounted to 2496 ± 247 μg oxolinic acid per g of dry weight of the nauplii and to 450 ± 15 μg flumequine per g dry weight of the nauplii. These levels of oxolinic acid are quite higher than those reported previously in a study that employed a bioassay for the determination of oxolinic acid in *Artemia* [11]. This might be due to the better incorporation of the drugs in the enrichment emulsion that we used for animal studies as well as to the more efficient and specific method

of determination of the drugs in *Artemia*, that is presented in this study. The flumequine levels in the enriched *Artemia* nauplii were quite lower than those of oxolinic acid, although higher concentrations of the drug in the enrichment emulsion were used (Fig. 3). However, preliminary experiments with seabass larvae challenged with pathogenic bacteria and treated with *Artemia* nauplii enriched with either oxolinic acid or flumequine, showed an excellent increase in the survival of infected fish.

4. Conclusion

Using the combination of SPE with a simple HPLC system and UV detection, both oxolinic acid and flumequine can be determined with high precision and extraction recovery. This method allowed the measurement of oxolinic acid or flumequine concentration in 20 samples per day and due to the high extraction efficiency at least 300 injections were performed on each analytical column prior to chromatographic deterioration. However for best peak shape a smaller number of injections is recommended. The validation study demonstrated that the analytical method is selective and reliable for pharmacokinetic studies and environmental impact assessment of the bioencapsulation technique.
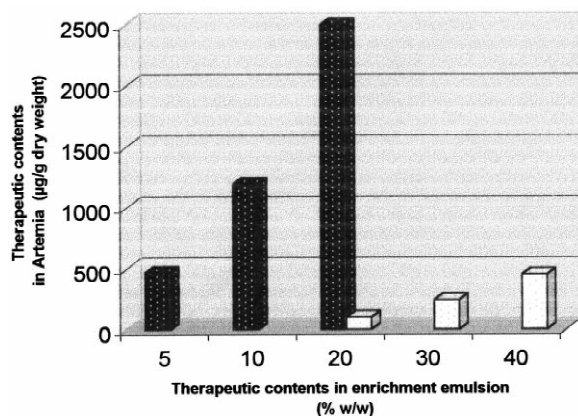
Acknowledgements

Fig. 3. Levels of oxolinic acid (■) and flumequine (□) in *Artemia* nauplii fed with emulsions containing different concentrations of each drug. For experimental conditions see text.

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