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# Determination of oxolinic acid in the bryophyte *Fontinalis antipyretica* by liquid chromatography with fluorescence detection

Raphaël Delépée*, Hervé Pouliquen

*Ecole Nationale Vétérinaire de Nantes, UMR Chimiothérapie Aquacole et Environnement, B.P. 40706, 44307 Nantes, Cedex 03, France*

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**Abstract**

A large amount of oxolinic acid administered in freshwater fish farms reaches the environment. In order to allow environmental monitoring, an HPLC method to determine oxolinic acid in the bryophyte *Fontinalis antipyretica* was developed. Nalidixic acid was used as an internal standard. Oxolinic and nalidixic acids were extracted by cracking the bryophytes in liquid nitrogen with 0.1 *M* acid oxalic solution in ethyl acetate and a liquid–liquid clean up procedure was then performed. Mobile phase was a 0.02 *M* orthophosphoric acid aqueous solution–acetonitrile mixture (70:30, v/v). The stationary phase was 5 μm PuroSpher RP18e and quinolones were detected by fluorescence. The linearity, accuracy and precision of this method were demonstrated by a validation assay. The limits of detection and quantitation were 1 and 10 ng/g respectively. The linearity range was 10 to 500 ng/g. This method was applied to a 25 days experimental study performed with the bryophyte *Fontinalis antipyretica*. © 2002 Elsevier Science B.V. All rights reserved.

*Keywords:* Quinolones; Bryophytes; *Fontinalis antipyretica*; Oxolinic acid

## 1. Introduction

Fish farming, and particularly freshwater fish farming, has spectacularly grown during the last few decades. Because of fish concentration and poor water quality, bacterial diseases become an important problem and antibacterial agents are consequently used in order to keep good productivity. Oxolinic acid (OA) is commonly used because of its high potency against Gram-negative bacteria. As a consequence of oral administration as medicated pelleted feed, a large amount of the drug reaches the environment. Furthermore, OA is very poorly absorbed through the digestive tract, the oral bioavailability varies between 13.6 and 38.1% according to different authors [1,2]. Finally about 60 to 85% of OA reach the environment. In these conditions it is necessary to monitor OA amounts in the freshwater environment. Many papers describe analytical methods to determine OA residues in sediment [3–6] or water [6] but none determine OA in plants. We chose *Fontinalis antipyretica* because it is a widespread bryophyte in Europe [7].

The purpose of the present work was to develop a precise and accurate HPLC method for the determi-

*Corresponding author. Tel.: +33-240-687-630; fax: +33-240-687-778.

*E-mail addresses:* rdelepee@vet-nantes.fr (R. Delépée), pouliquen@vet-nantes.fr (H. Pouliquen).

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nation of OA residues in *Fontinalis antipyretica* and to apply this method to an experimental study.

## 2. Experimental

### 2.1. Chemicals and reagents

*Fontinalis antipyretica* samples were collected in Chézine river (Loire-Atlantique, France, 1°37′W–47°14′N). Acetonitrile (Merck, Darmstadt, Germany) and ethyl acetate (BDH Chemicals, Toronto, Canada) were HPLC-grade. Chloroform (BDH), orthophosphoric acid 85% (Merck), oxalic acid dihydrate (Sigma, St. Louis, MO, USA), glacial acetic acid, sodium hydroxide pellets (Panreac, Barcelona, Spain) were analytical-grade reagents. The water used in buffers and eluents were deionized with a Milli-Q academic apparatus (Millipore, Bedford, MA, USA). OA and nalidixic acid (NA) were purchased as pure standards from Sigma.

### 2.2. Apparatus

The liquid chromatograph was a Gilson 307 (Villiers le Bel, France) with a fluorescence programmable detector Jasco FP-1520 (Tokyo, Japan). The samples were injected in the chromatograph with a Gilson 234 auto-sampler. The separation was performed on a 5 μm PuroSpher RP-18 end-capped (e) 125 × 4.6 mm I.D. (Merck) cartridge preceded by a 5 μm PuroSpher RP-18e 125 × 4.6 mm I.D. guard cartridge in a Shimadzu CTO-10-AS-VP (Kyoto, Japan) column oven. Peak heights were calculated with a Merck D2500 integrator.

### 2.3. Chromatographic conditions

The mobile phase, a 0.02 *M* orthophosphoric acid aqueous solution–acetonitrile mixture (70:30, v/v), was degassed for 15 min before use (Sonicater 88155, Bioblock Scientific, Illkirch, France). The operating flow-rate was 1.0 ml/min and the cartridge temperature was set to 27 °C. Wavelength settings were excitation, 325 nm; emission, 365 nm; and injection volume was 20 μl. After each day of operation the analytical and guard cartridges were flushed for 30 min with a mixture of acetonitrile–water (50:50, v/v).

### 2.4. Standard solutions

Stock standard solutions of OA and NA (1 mg/ml) were prepared twice in a 0.03 *M* sodium hydroxide aqueous solution at a concentration of 1 mg/ml. These solutions were stable for 1 month when stored at 4 °C. Working standard solutions were prepared before use by diluting the stock solutions with deionized water.

### 2.5. Extraction and clean-up procedure

Bryophyte samples (2.0 g) were spiked with NA as an internal standard (50 μl of a working standard solution containing 5 μg NA/ml) and 25 ml of a 0.1 *M* oxalic acid solution prepared in ethyl acetate were added. The mixture was frozen in liquid nitrogen for about 10 s and crushed with an homogenizer (Ultra-Turrax, Bioblock Scientific). The mixture obtained was homogenized for 10 min at ambient temperature (Rotator Drive, Heidolph, Schwabach, Germany) and centrifuged twice at 8000 *g* for 5 min at room temperature (Jouan GR 4-22, St. Herblain, France). Supernatants were combined and extracted twice with 5 ml of a 1 *M* sodium hydroxide aqueous solution by a 5 min homogenization (Rotator Drive) and a 5 min centrifugation at 8000 *g* at room temperature (Jouan GR 4-22). Aqueous supernatants were combined, acidified with glacial acetic acid (2 ml), shaken vigorously (Top-mix 94323, Heidolph) and extracted 2 times by 5 and 3 ml of chloroform. After a 5 min homogenization (Rotator Drive) and a 5 min centrifugation at 8000 *g* at room temperature (Jouan GR 4-22), the combined organic phases were evaporated to dryness under a nitrogen stream (35 °C). The dry extract was dissolved in 1 ml of mobile phase, shaken intensively 30 s (Top-mix 94323), sonicated for 5 min (sonicater 18155) and transferred into a 0.5 ml vial before injection into the HPLC system.

### 2.6. Validation assay

The calibration curves were made by spiking samples (2 g bryophytes) with standard solutions of

OA to yield 10.0, 25.0, 50.0, 125.0, 250.0 and 500.0 ng/g. A blank sample was also extracted to check specificity. After a 30 min contact time, samples were extracted using the above procedure. Each level was assayed once a day for 4 days. The calibration curves were drawn by plotting the ratio of the OA peak height (μV) to the NA (internal standard) peak height (μV) against the known OA concentration (ng/g or ng/ml). A regression calculation was performed and the bias was calculated for each fortification level as following:

$$\text{bias} = \frac{\text{calculated conc.} - \text{theoretical conc.}}{\text{theoretical conc.}} \times 100 \quad (1)$$

Recoveries were determined from the comparison between spiked samples and standard solutions containing 20.0, 50.0, 100.0, 250.0, 500.0 and 1000.0 ng/g OA and 250.0 ng/g NA. They were calculated by comparing individual peak heights of spiked samples with peak heights of corresponding standard solutions.

Accuracy and precision were estimated by spiking samples (quality controls, QCs) with standard solutions of OA to yield 25.0 (about 2 times the smallest level), 200.0 and 400.0 ng/g (about 80% of the highest level). The standard solutions used to spike these samples were prepared from a different stock solution than the one used for the calibration curves. QCs were extracted using the above procedure. Each level was assayed 3 times a day for 4 days. Concentrations were calculated by OA/NA peaks heights ratio. Accuracy was evaluated by calculated bias [Eq. (1)]. Precision was studied by calculating the relative standard deviations of repeatability and intermediary precision. The limit of detection (LOD) was determined using the intercept ($\bar{a}$) and the mean square error (MSE) of the mean regression curve by Eq. (2).

$$\text{LOD} = \bar{a} + 3 \times \sqrt{\text{MSE}} \quad (2)$$

### 2.7. Experimental conditions

A semi-static experimental study was performed in order to estimate the contamination of the bryophyte *Fontinalis antipyretica* in freshwater. This experiment used 200 l tanks; 2 of them contained OA (100 ng/g) and one of them did not contained OA. The experiments period was divided into 2 periods: A 10 days exposure period during which water contained OA and a 15 days post-exposure period during which water did not contain OA. During the experiment, the water was completely changed every 2 days. *Fontinalis antipyretica* samples were collected at days 0, 2, 4, 6, 10, 11, 12, 13, 16, 20, 25 and analyzed by the above method.

## 3. Results and discussion

### 3.1. Chromatographic conditions

Reversed-phase HPLC has often been used to determine quinolone antibiotics in various matrices [8–11] and end-capping stationary phase improved peak symmetry and capacity factor [10,12]. The 5 μm PuroSpher RP-18e cartridge was consequently chosen and allowed suitable chromatographic separation. Moreover, OA and NA show strong fluorescence in aqueous acid solutions [12–15] so orthophosphoric acid was used as the aqueous eluent in order to make use of the increased specificity and sensitivity given by fluorescence detection and to improve the peak shape by masking the residual silanol groups of the stationary phase. Extraction and clean-up procedure (see Section 3.2) was effective enough so as to have no peak interfering with OA or NA on the chromatogram (Fig. 1A). Under these conditions, the mobile phase composition and the flow-rate were optimized in order to obtain a good compromise between resolution and analysis time. The optimum was 67% of 0.02 *M* orthophosphoric acid aqueous solution and 33% of acetonitrile and flow-rate was set to 1.0 ml/min. Under these conditions, the operating system resolution was 10.03 and OA and NA were eluted in 3.9 and 7.5 min respectively (Fig. 1B).

### 3.2. Extraction and recoveries

No method for the determination of quinolones (or antibacterials in general) in plants was available.

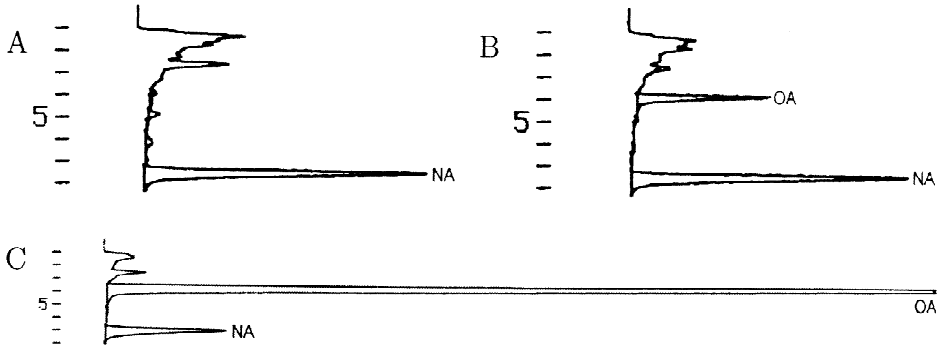


Fig. 1. HPLC chromatograms. (A) *Fontinalis antipyretica* blank sample with nalidixic acid (NA) as internal standard (125 ng/g); (B) *Fontinalis antipyretica* sample spiked with oxolinic acid (OA) and NA (50 and 125 ng/g respectively); (C) *Fontinalis antipyretica* sample from the experimentation (day 25), this sample was spiked with NA (125 ng/g) and OA concentration was 1700 ng/g. Retention times of OA and NA were 3.9 and 7.5 min respectively. For HPLC conditions see Section 2.3.

Few authors studied polychlorinated biphenyls in aquatic macrophytes [16] or polycyclic aromatic hydrocarbons in bryophytes [17]. They used solid/liquid Soxhlet extraction [16] or liquid nitrogen cracking extraction [17]. Many solid/liquid extractions were tried: Crushing with sand, soxhlet extraction, sodium hydroxide hydrolysis and liquid nitrogen cracking. Recoveries were under 50% with sand crushing and soxhlet (with water–methanol–chloroform, 1.3:8.2:90.5, w/w/w) (data not shown). Sodium hydroxide hydrolysis extracts contained a gel making the clean-up procedure very difficult. Liquid nitrogen cracking allowed good recoveries if a chelatant agent was added to the extraction solvent. Two chelatant agents were assayed and 0.1 *M* oxalic acid allowed better recoveries than $Na_2EDTA$. Ethyl acetate was used because it was not freezing during the liquid nitrogen cracking and did not extract gelling components.

The main challenge of the clean-up procedure was to remove a large amount of the pigments of the bryophyte. The liquid-phase clean-up procedure using quinolones acid-base properties (solubility in organic solvents in acidic conditions and solubility in basic aqueous solutions) allowed better recoveries than solid-phase extraction and chromatograms were free from any interference (Fig. 1A). The first liquid/liquid clean-up step from ethyl acetate to sodium hydroxide removed hydrophilic pigments (i.e. carotenoids). The second-step from acidic aqueous solution to chloroform removed polar pigments. The homogeneity of the experimental standard deviations of recoveries of each level was checked. Mean recovery was 68.3% for OA and 73.4% for NA (Table 1).

### *3.3. Linearity, regression, accuracy, precision, limits of detection and quantitation*

A linearity and regression study was performed for each calibration curve separately. Slopes and intercepts were not significantly different between the 4

Table 1
Recoveries of OA and NA from *Fontinalis antipyretica*

| | OA | NA |
|---|---|---|
| Mean recoveries (%) ($n=4$) | | |
| Spiked 10 ng/g | 70.0 | 73.8 |
| Spiked 25 ng/g | 71.6 | 74.0 |
| Spiked 50 ng/g | 68.7 | 73.9 |
| Spiked 125 ng/g | 71.1 | 76.2 |
| Spiked 250 ng/g | 61.9 | 67.9 |
| Spiked 500 ng/g | 66.7 | 74.5 |
| Statistical tests | | |
| Cochran test ($n=6$) | 0.262 | 0.227 |
| Critical value of C[a] | 0.616 | 0.616 |
| Mean recovery ($n=24$) | 68.3 | 73.4 |
| Standard deviation | 5.3 | 3.8 |

[a] $P = 0.95$.

Table 2
Precision and accuracy data from the validation assay for the determination of OA in *Fontinalis antipyretica*

| Spiked (ng/g) | Maximum bias (%) | Repeatability RSD[a] (%) | Intermediary precision RSD[a] (%) |
|---|---|---|---|
| 25 | +13.9 | 3.6 | 6.5 |
| 200 | +8.8 | 3.0 | 4.0 |
| 250 | +14.9 | 1.6 | 7.2 |

[a] Relative standard deviation ($n=12$).

days at the 0.05 level (data not shown). Bias between calculated and theoretical concentrations were calculated for each level of the calibration curve. Each day individual bias should be between −15 and +15% [18]. A high value of the correlation coefficient (0.998) and the acceptable bias (always under 12.5%, data not shown) indicated a good correlation between OA and NA peaks height ratios and OA concentrations. 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). The response was then linear between 10 and 500 ng/g, the mean slope and intercept were 0.0281 and 0.0180 respectively ($n=28$).

QCs bias were always in the ±15% range. Repeatability and intermediary precision relative standard deviations were all below 15% (Table 2). QCs will be used in the routine analysis to validate each day of operation.

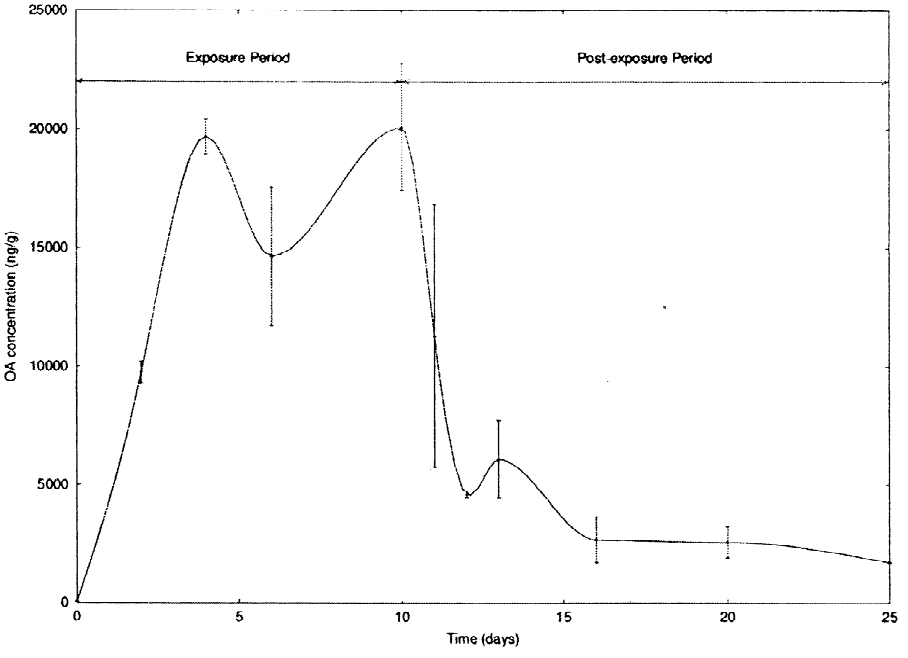


Fig. 2. Uptake and decline of OA in *Fontinalis antipyretica* tissues after contact with water containing OA at a concentration of 100 ng/ml. Each data point represents the average of the two tanks, the upper point is the maximum concentration and the lower point is the minimum concentration. For further details see text.

LOQ was 10 ng/g because this concentration was the lowest acceptable level of the calibration curve [18]. MSE was 0.054 so LOD [calculated with Eq. (2)] was 1 ng/g.

### *3.4. Experimental conditions*

The results of the experimental study are shown in Fig. 2. The OA concentration in *Fontinalis antipyretica* increased to reach a peak on the last day of the exposure period. After the end of the exposure period OA concentration decreased quickly during the first 2 days and more slowly between the second and the fifteenth days of the post-exposure period. OA concentration was still high (1721 ng/g) after 15 days (see Fig. 1C for the chromatogram). OA concentration in some samples was above the linearity range ($>500$ ng/g). In this case we assumed the linearity was a function of the OA quantity and not of the OA concentration; so *Fontinalis antipyretica* samples were then weighted in order to extract between 20 and 1000 ng of the OA. The OA concentrations in the reference tank were always below the limit of detection of the method.

## 4. Conclusion

The statistical validation showed that the method described here was selective, reliable, precise and allowed monitoring of OA in the freshwater bryophyte *Fontinalis antipyretica*. This method allowed analysis of 24 samples a day. In conclusion, the proposed HPLC method is an efficient and reliable means for environmental OA residue studies in the bryophyte *Fontinalis antipyretica*.

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