

Angelina Pena<sup>1</sup>  
Daniela Chmielova<sup>2</sup>  
Celeste M. Lino<sup>1</sup>  
Petr Solich<sup>2</sup>

<sup>1</sup>Group of Bromatology, Center of Pharmaceutical Studies, University of Coimbra, Coimbra, Portugal

<sup>2</sup>Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Hradec Kralove, Czech Republic

## Short Communication

# Determination of fluoroquinolone antibiotics in surface waters from Mondego River by high performance liquid chromatography using a monolithic column

A novel LC–fluorescence detection method based on the use of a monolithic column for the determination of norfloxacin, ciprofloxacin, and enrofloxacin antibiotic residues in environmental waters was developed. Fluoroquinolones (FQs) were isocratically eluted using a mobile phase consisting of 0.025 M phosphoric acid solution at pH 3.0 with tetrabutylammonium and methanol (960:40, v/v) through a Chromolith Performance RP-18e column (100 × 4.6 mm) at a flow rate of 2.5 mL/min and detected at excitation and emission wavelengths of 278 and 450 nm, respectively. After acidification and addition of EDTA, water samples were extracted using an Oasis HLB cartridge. Linearity was evaluated in the range of 0.05 to 1 µg/mL and correlation coefficients of 0.9945 for norfloxacin, 0.9974 for ciprofloxacin, and 0.9982 for enrofloxacin were found. The limit of quantification was 25 ng/L for the three FQs. The recovery of FQs spiked into river water samples at 25, 50, and 100 ng/L fortification levels ranged from 76.5 to 91.0% for norfloxacin, 78.5 to 97.2% for ciprofloxacin, and 79.4 to 93.6% for enrofloxacin. This method was successfully applied to the analysis of water samples from the Mondego River, and ciprofloxacin and enrofloxacin residues were detected in eight water samples.

**Keywords:** Environment / Fluoroquinolones / Monolithic column / Pharmaceuticals / Surface waters

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## 1 Introduction

Pharmaceutical compounds are widespread contaminants of the aquatic environment. Since traditionally they have not been viewed as environmental contaminants, the study of their presence in the environment is in some ways a new area of research which has taken off in recent years. Our current knowledge indicates that residues of pharmaceuticals at trace quantities are widespread in aquatic systems [1].

Antibiotics constitute a large group of pharmaceuticals, which are widely administered in human and veterinary medicine. The extensive use of these antibiotics may result in their presence in the environment.

Antibiotics are believed to be of greatest concern among all pharmaceuticals due to the potential risk of antibiotic resistance. Studies in the United States of America and Europe have detected antibiotic resistant bacteria in drinking water supplies [2, 3].

According to previous studies and publications, one of the most prevalent groups of antibiotics found in the environment, and particularly in surface waters, is that of the widely used, highly potent fluoroquinolones (FQs) [4–6].

Administered FQs are largely excreted as unchanged compounds in urine, and consequently discharged into hospital sewage or municipal wastewater [7].

Unfortunately, sewage water treatment plants are not able to completely remove these compounds and thus significant quantities of the active compound are transported into environmental aquatic systems [7]. Further input possibly also comes from the use of sewage sludge and livestock manure as fertilizers on agricultural crops, favouring the accumulation of these antimicrobials in soils, because of their strong sorption properties. The subsequent surface run-off, leaching to deeper soil layers

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**Correspondence:** Dr. Angelina Pena, Group of Bromatology, Center of Pharmaceutical Studies, University of Coimbra, Coimbra, Portugal  
**E-mail:** apena@ci.uc.pt  
**Fax:** +351 39 829725

**Abbreviation:** CIPRO, ciprofloxacin; ENRO, enrofloxacin; FD, fluorescence detection; FQ, fluoroquinolones; NOR, norfloxacin

and finally to ground water, or drift during manure spreading, results in accumulation in environmental waters [8].

Therefore, more monitoring and surveillance studies are needed at local level to determine exactly how the antibiotics make their way into public waterways, and to obtain a better understanding of the transport and environmental fate of antibiotics.

Currently, different methods are available for the determination of FQs in environmental water samples. Water samples are analyzed after solid-phase extraction by HPLC with fluorescence (FD), mass spectrometric, or tandem mass spectrometric detection [9–11].

Although several studies have been published on the application of monolithic columns in antibiotics analysis, it is still a relatively new area, despite a marked increase in the last three years [12–14].

The present work deals with the development of novel HPLC methodology for determination of FQ antibiotics, namely norfloxacin (NOR), ciprofloxacin (CIPRO), and enrofloxacin (ENRO), in environmental waters using a Chromolith RP-18e silica monolithic column and fluorescence detection. The method was successfully applied to the monitoring of twenty-two water samples from the Mondego River, since it receives the effluents of wastewater treatment plants corresponding to 210 000 population equivalents, and including four central hospitals, several clinics, and livestock industries. So far no work has been published on the environmental analysis of pharmaceuticals using monolithic columns.

## 2 Experimental

### 2.1 Reagents

Standards of norfloxacin, ciprofloxacin, and enrofloxacin were purchased from Sigma–Aldrich (Steinheim, Germany). These FQs were of >98% purity. HPLC grade methanol was supplied by Carlo Erba (Milan, Italy). The followed reagents were purchased: phosphoric acid RPE-ACS (from Carlo Erba, Milan, Italy), sulphuric acid 95–97% Reagente “Baker Analyzed” (J.T. Baker, Deventer, The Netherlands), tetrabutylammonium (Sigma–Aldrich, Steinheim, Germany), and EDTA (Merck, Germany). Water was of HPLC grade. The cartridges used for SPE were Oasis HLB 6 cc/200 mg (Waters Corp., Millford, MA).

### 2.2 Apparatus and chromatographic conditions

The HPLC system used here consists of two Model 307 pumps (Gilson Medical Electronics, France), a Model 7125 injector (Rheodyne, Cotati, California, USA), and a fluorimeter detector (LabAlliance, France) operated at an excitation wavelength of 278 nm and an emission wavelength of 450 nm. The spectral bandwidth was 10 nm for

both excitation and emission. The results were recorded on an SP 4270 integrator (Hewlett Packard, Philadelphia, USA).

The chromatographic separation was accomplished in 14 min by isocratic elution with a mixture consisting of 0.025 M phosphoric acid, adjusted to pH 3.0 with tetrabutylammonium (TBA) and methanol (960:40) through a monolithic Chromolith Performance RP-18e column (100 × 4.6 mm) with a 10 mm monolithic precolumn from Merck (Darmstadt, Germany). The HPLC system was operated at room temperature and the flow rate was 2.5 mL/min.

## 2.3 Method and sample preparation

### 2.3.1 Sample collection and preparation

A total of twenty-two surface river water samples were collected in 2 L amber glass bottles at different points (on the banks) of the Mondego River near Coimbra during a three-month winter period, from November 2006 to January 2007. The water samples were first vacuum filtered through glass fibre filter (Schleicher & Schuell) to prevent clogging of the 0.2 µm membrane filter (Schleicher & Schuell) used in the next filtration step, and stored in the dark at 4 °C.

### 2.3.2 Standard fluoroquinolone solutions

Individual stock standard solutions (1 mg/mL) were prepared in 0.005 M sulphuric acid. The working standard solutions were a mixture of the three compounds prepared by appropriate dilution of the stock solutions.

### 2.3.4 Extraction and clean-up

After adjustment of the pH to 4.0 with sulphuric acid (1 M) and addition of 186 mg of EDTA di-potassium salt, one litre of the sample was percolated through the Oasis HLB 6 cc (200 mg) cartridge. The cartridge was previously conditioned with 5 mL of methanol and 4 mL of Milli-Q water. The washing step was performed with Milli-Q water at pH 4.0. Then the cartridge was eluted with 4 mL of methanol. This eluate was evaporated to dryness under a gentle stream of nitrogen and the residue was redissolved in 0.5 mL of mobile phase, and filtered through a 0.45-µm membrane filter. The volume of eluate injected was 50 µL.

## 3 Results and discussion

### 3.1 Optimization of HPLC conditions

In recent years, the use of monolithic columns has gained considerable significance, due to its main advantages, namely low pressure drop and short retention time.

The effect of mobile phase composition on the retention of the FQs was investigated. The optimisation focused on proposing an appropriate ratio of methanol, TBA and phosphoric acid for the mobile phase composition and pH.

The pH of the mobile phase is a major factor influencing the chromatographic behaviour of FQs, given that they contain ionogenic functions such as carboxylic acid and amino groups [15]. The carboxyl group is normally a stronger acid than the ammonium group. In the mobile phase proposed, a water rich solution at acidic pH, these FQs are ionized.

Our experiments show that with the Chromolith column the best result in terms of resolution and selectivity was achieved at pH = 3.0 in the presence of TBA, and are in accordance with the results obtained by Forlay-Frick and Fekete [16]. As a consequence of the presence of acidic and basic functional groups, the FQs are prone to chemical tailing due to interactions with the free silanols of the stationary phase. Forlay-Frick and Fekete [16] evaluated different stationary phases for the determination of CIPRO, including a Chromolith Speed ROD column, and stated that silanol activity could be detected in all stationary phases.

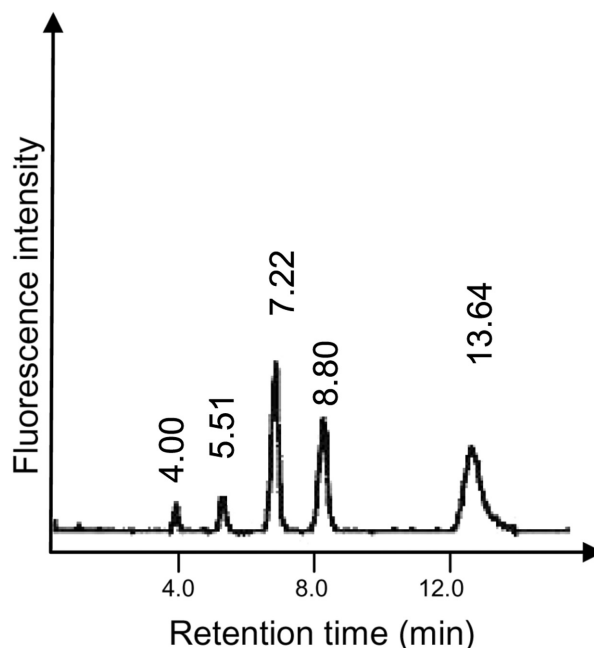
NOR, CIPRO, and ENRO are zwitterionic analytes, and ion pair formation in the mobile phase between the carboxylate anion and the TBA counter ion causes the disruption of the zwitterionic structure, leading to the deliberation of the positive charge and thereupon to elution of FQs [17].

The chromatographic separation was accomplished in 14 min by isocratic elution with a mixture consisting of phosphoric acid adjusted to pH 3.0 with tetrabutylammonium (TBA) and methanol as mobile phase through a monolithic Chromolith Performance RP-18e column (100 × 4.6 mm) with a 10-mm precolumn at room temperature and at a flow rate of 2.5 mL/min. The chromatogram in Fig. 1 shows the separation of the analytes obtained by the proposed method.

On the basis of three parallel determinations, performed on three days, the mean retention times and standard deviations (SD) for NOR, CIPRO, and ENRO were 7.58, 8.83, and 13.86 min, and 0.12, 0.06, and 0.21, respectively.

Excitation-emission scans were performed to establish optimum excitation and emission wavelengths. The maximum wavelengths obtained for the different fluoroquinolones were: NOR  $\lambda_{\text{ex}}$  280 nm,  $\lambda_{\text{em}}$  447 nm, for CIPRO  $\lambda_{\text{ex}}$  279 nm,  $\lambda_{\text{em}}$  445 nm and for ENRO  $\lambda_{\text{ex}}$  277 nm,  $\lambda_{\text{em}}$  446 nm, and thus, chromatographic detection was carried out at wavelengths of excitation and emission of 278 nm and 450 nm, respectively.

Piperazinylquinolones have two  $\text{pK}_{\text{a}}$  values,  $\text{pK}_{\text{a}1} \approx 6$ , and  $\text{pK}_{\text{a}2} \approx 9$  for the carboxylic function and the piperazinyl moiety, respectively [18]. The mobile phase pH con-



**Figure 1.** Liquid chromatogram of a standard solution of NOR, CIPRO and ENRO at 0.05 µg/mL.

tributes for a highest fluorescence, since they exist mostly in cationic form at acidic pH.

Since the goal of these studies was to develop a HPLC method for sensitive determination of these antibiotics, we have proceeded to an interference study. The effect of the solvent of standard solutions of NOR, CIPRO, and ENRO on fluorescence intensity was evaluated. The standard solutions were prepared in mobile phase with and without TBA and in  $\text{H}_2\text{SO}_4$  0.005 M, and no significant interference was observed on native fluorescence intensity, probably due to the high water content of the mobile phase [19].

### 3.2 Optimization of extraction and clean-up

The clean up efficiencies for Oasis HLB 6 cc/200 mg cartridges were studied by adjusting the following parameters: the solvents used in the washing steps, the eluent solvents, and the volumes for eluting FQs from the cartridge. This polymeric reversed-phase sorbent showed no effect of sorbent drying, no silanol interaction, and no breakthrough of polar analyte.

First the cartridges were washed with 4 mL of Milli-Q water pH 4, but this was shown to be unsatisfactory, since many interferences appear in the blank assay chromatogram. Finally, washing was performed with three cartridge volumes of Milli-Q water at pH 4 to obtain clean blank chromatograms without interferences.

After elution of FQs with 4 mL of methanol, a second elution with 2 mL of methanol was assayed. FQs were not

found in this second eluate. Thus our results show that 4 mL of methanol is necessary and sufficient to elute all FQs from the cartridge. This method was therefore applied to analyze FQs in surface water samples.

### 3.3 Method validation

In the validation procedure of the analytical method, the following criteria were considered: sensitivity; linearity; recovery; precision; and evaluation of the matrix effect.

#### 3.3.1 Linearity

The calibration curves were prepared using linear regression analysis and gave good fits over the range 0.05 to 1 µg/mL. The mean regression coefficients ( $R^2$ ) were 0.9945 for NOR, 0.9974 for CIPRO, and 0.9982 for ENRO.

#### 3.3.2 Stability studies

The stability of standard solutions and of sample extracts was evaluated. The stock standard solutions were stored at  $-20^\circ\text{C}$  and analysed during a one-month period, and the working standard solutions were stored at  $4^\circ\text{C}$  and analysed during a one-week period. For the period of study we did not observe any degradation of FQs. The stability of FQs during sample storage at  $-20^\circ\text{C}$  was tested during one week, and no degradation was observed.

#### 3.3.3 Limit of quantification and accuracy

The limit of quantification, calculated according to the lower concentration that provides repeatabilities lower than 20%, was 25 ng/L for NOR, CIPRO, and ENRO.

In order to verify the absence of potential interfering substances around the retention time of FQs, water blank samples ( $n = 6$ ) were analyzed in order to assess the specificity of the method. No interferences were observed in the region of interest where the FQs were eluted (Fig. 2). These results clearly demonstrate that real sample matrices had no effect on the performance of the proposed method, which is therefore suitable for analysis of trace levels of FQs in surface waters.

The accuracy of the method was studied by spiking water samples at three fortification levels (25, 50, and 100 ng/L). Figure 3 represents the chromatogram obtained for fortification assay.

Within-day accuracy and precision data were determined by analyzing, on the same day, three replicates of spiked samples at three levels, and one blank (to check for interferences). The between-day accuracy and precision were also determined by extracting batches of three fortification levels and analysing them on three consecutive days. Accuracy and intra-day and inter-day precision are shown in Table 1. Recoveries for the lower fortification level were generally greater than 76.5, 78.5 and 79.4% for NOR, CIPRO, and ENRO, respectively, and the

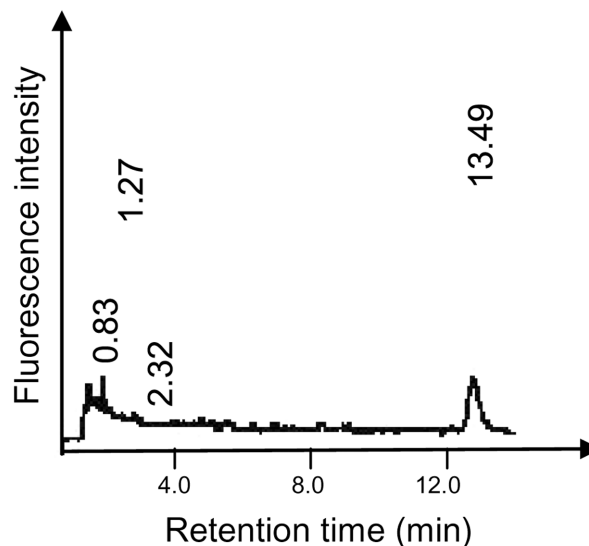


Figure 2. Liquid chromatogram of a blank assay of river water.

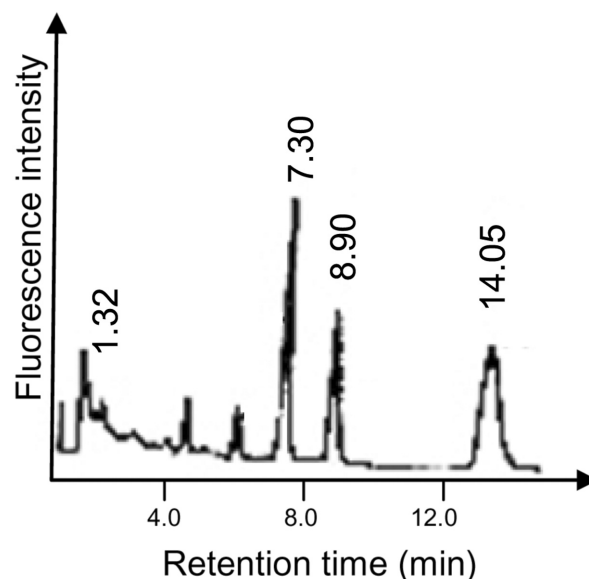


Figure 3. Liquid chromatogram of a spiked sample.

dependence of recovery was negligible, showing good accuracy of the method. For the three fortification levels, the relative standard deviation for all fortification levels on each day for each analyte was less than 12%, demonstrating good method precision.

### 3.4 Application of method

A total of twenty-two samples of water were analyzed under the conditions described, and ENRO and CIPRO were detected in eight samples at levels higher than the LOQ, in concentrations ranging from 67.0 to 102.5 ng/L and from 79.6 and 119.2 ng/L, respectively.

**Table 1.** Inter-day and intra-day assay validation results

Fluoroquinolone antibiotics	Number assays (N)	Fortification level (ng/L)	Recovery mean (%)	RSD Intra-day (%)	RSD Inter-day (%)
NOR	5	25	76.5	4.1	6.2
CIPRO	5		78.5	2.6	8.0
ENRO	5		79.4	4.2	11.8
NOR	5	50	87.5	1.5	5.1
CIPRO	5		88.1	2.1	7.3
ENRO	5		98.0	4.1	6.7
NOR	5	100	91.0	1.6	2.9
CIPRO	5		97.2	1.4	3.6
ENRO	5		93.6	3.4	4.5

Effluence of FQs into the environmental waters occurs mainly as the parent compounds and as a consequence of human and animal use.

FQs are rather resistant to microbial degradation and these compounds may be persistent in environmental waters because of their strong sorption properties. It is known that quinolones and FQs are able to form stable complexes with several divalent and trivalent metal ions [20].

Since ENRO is only permitted in veterinary medicine, the results obtained suggest an input from veterinary and agricultural sources. There is a visible effect from flooded agricultural areas with returning water transporting ENRO residues from topsoils which have been fertilized with liquid manure.

CIPRO is restricted to human medicine but is also the major metabolite of ENRO used in animals, produced by N-deethylation of the ethylpiperazine ring. Unfortunately, it is not straightforward to decide whether the CIPRO presence in river water originates from human or veterinary use.

In the scientific literature FQs have been found in comparatively high concentrations in effluents from sewage treatment plants in European countries, such as France (330–510 ng/L), Italy (290–580 ng/L), Greece (460 ng/L) [21], Switzerland (249–405 ng/L) [22], and also in USA (19–45 ng/L) [21], and Canada (102–506 ng/L) [23].

#### 4 Concluding remarks

The monitoring of antibiotic residues is certainly an important issue in order to avoid possible residues in water systems used for drinking water supplies.

In the present study, a novel HPLC method using a monolithic column has been developed to identify and

quantify trace levels of FQs in real environmental surface water samples from the Mondego River.

The use of a monolithic column combined with fluorescence detection allows the rapid and sensitive analysis required for this type of analysis.

The method was found to fulfil the validation requirements of the analytical methodology for the determination of NOR, CIPRO, and ENRO in environmental surface waters.

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