



Degradation of fluoroquinolone antibiotics and identification of metabolites/transformation products by liquid chromatography–tandem mass spectrometry[☆]



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ABSTRACT

Antibiotics are a therapeutic class widely found in environmental matrices and extensively studied due to its persistence and implications for multi-resistant bacteria development. This work presents an integrated approach of analytical multi-techniques on assessing biodegradation of fluorinated antibiotics at a laboratory-scale microcosmos to follow removal and formation of intermediate compounds. Degradation of four fluoroquinolone antibiotics, namely Ofloxacin (OFL), Norfloxacin (NOR), Ciprofloxacin (CPF) and Moxifloxacin (MOX), at 10 mg L⁻¹ using a mixed bacterial culture, was assessed for 60 days. The assays were followed by a developed and validated analytical method of LC with fluorescence detection (LC–FD) using a Luna Pentafluorophenyl (2) 3 μm column. The validated method demonstrated good selectivity, linearity ($r^2 > 0.999$), intra-day and inter-day precisions (RSD < 2.74%) and accuracy. The quantification limits were 5 μg L⁻¹ for OFL, NOR and CPF and 20 μg L⁻¹ for MOX. The optimized conditions allowed picturing metabolites/transformation products formation and accumulation during the process, stating an incomplete mineralization, also shown by fluoride release. OFL and MOX presented the highest (98.3%) and the lowest (80.5%) extent of degradation after 19 days of assay, respectively. A representative number of samples was selected and analyzed by LC–MS/MS with triple quadrupole and the molecular formulas were confirmed by a quadrupole time of flight analyzer (QqTOF). Most of the intermediates were already described as biodegradation and/or photodegradation products in different conditions; however unknown metabolites were also identified. The microbial consortium, even when exposed to high levels of FQ, presented high percentages of degradation, never reported before for these compounds.

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1. Introduction

In the last couple of decades the presence of active pharmaceutical ingredients (API) in different environmental compartments has been an evolving subject in the environmental science field [1,2]. They are named as “pseudo-persistent” pollutants [3] as

their transformation vs. elimination rate is balanced by their uninterrupted input into the environment [4]. Wastewater treatment plants (WWTP) represent a critical spot of contribution to the increase of pharmaceuticals in the environment since they are not designed with the ability to completely eliminate all organic compounds at low levels of concentration [5,6]. A raising number of reports spotted trace levels of API in WWTP effluents [7–9], surface and ground waters [10–12] and even in drinking water supplies [13].

Antibiotics are an extensively studied class of API due to the development of multi-resistant bacteria and their high frequency in aquatic environmental compartments [14–16]. The levels reported are usually within the ng L⁻¹ to μg L⁻¹ range, however reports of effluents from pharmaceutical manufacturing facilities located in India and China presented higher levels, reaching concentrations up

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to mg L^{-1} [17,18]. Pharmaceutical industries do not represent the main source of pharmaceuticals in the environment, as indicated in the environmental risk assessment study for wastewater and river water [19], however in some cases a direct association has been reported [17,18,20].

According to the 2010 European Centre for Disease Prevention and Control report on antimicrobial consumption in Europe, the consumption proportion of quinolone antibacterials regarding systemic use ranged from 2.5% in the United Kingdom to 13.3% in Portugal. Ciprofloxacin (CPF) accounted for most of the consumption of quinolone antibacterials [21]. Considering the 2010 European Medicines Agency report on veterinary antimicrobial agents' sales in 19 countries, the major antimicrobial classes sold, as oral solutions, were tetracyclines (24%) and fluoroquinolones (FQ) (20%). FQ accounted for 2.2% of the total sales of antimicrobial veterinary medicine products [22]. The presence of FQ in many environmental matrices has been described, including marine aquaculture samples in Pearl River Delta, South China, in concentrations between 1.88 and 11.20 ng g^{-1} (dry weight) [23]; sea water in Hong Kong, China [24] and also in drinking water ranging from 1.0 to 679.7 ng L^{-1} , in a study performed in Guangzhou, China [13]. Recently, a study described the occurrence of several FQ and the presence of FQ-resistant genes in bacterial species in surface waters [25]. In general, FQ are described to be resistant to hydrolysis [26,27] and readily adsorbed to particulate matter [28], exhibiting WWTP removal efficiencies over 80% [29] or around and above 50% [15,28,30,31]. Along with adsorption to particulate matter, photodegradation and biodegradation are reported as the most important removal processes for elimination of API in WWTP [32]. However, photodegradation and biodegradation tend to produce metabolites that are rarely quantified in monitoring studies of WWTP effluents.

This work describes the biodegradation of four important FQ antibiotics: ofloxacin (OFL), norfloxacin (NOR), CPF and moxifloxacin (MOX), by a microbial consortium able to degrade fluorinated aromatic compounds. An integrated approach was used to identify the metabolites/transformation products originated in the biodegradation studies. FQ primary degradation was followed by the validated LC–FD method and the metabolites/transformation products were identified by LC–MS/MS with a triple quadrupole analyzer (TQD) and confirmed by a time of flight (TOF) couple to a quadrupole analyzer (QqTOF). Most of the intermediates were already described as biodegradation and/or photodegradation products in different conditions. QqTOF analysis provided relevant data with new molecular formulas, which allowed the proposal of structures of two new metabolites, not described before.

2. Material and methods

2.1. Chemicals and standards preparations

Antibiotics OFL, NOR and CPF standards were purchased from Sigma Aldrich. MOX standard was donated by Bayer. All the standards presented a purity degree above 98%. The ethanol HPLC grade was purchased from Merck. Triethylamine with $\geq 99\%$ purity was obtained from Sigma–Aldrich. Acetic acid and trifluoroacetic acid were purchased by Panreac and Acros Organics, respectively. Ultra-pure water was supplied by a Milli-Q water system.

OFL, NOR and CPF stock solutions were prepared at 1000 mg L^{-1} in water:acetic acid 10% (50:50, v/v). MOX stock solution was prepared at 1000 mg L^{-1} in water. These solutions were stored at -20°C in amber bottles. The working solution of the four FQ was obtained by a dilution of the stock solutions in ultra-pure water to a concentration of 50 mg L^{-1} and prepared weekly.

2.2. Chromatographic conditions

A Shimadzu UFLC Prominence System equipped with two Pumps LC-20AD, an Autosampler SIL-20AC, a column oven CTO-20AC, a Degasser DGU-20A5, a System Controller CBM-20A and a LC Solution, Version 1.24 SP1 (Shimadzu) was used to follow the primary degradation. The fluorescence detector coupled to the LC System was a Shimadzu RF-10AXL. The column was a Luna Pentafluorophenyl (2), pore size 100 Å, particle size 3 μm , 150 mm $\times$ 4.6 mm, a modified reverse phase column from Phenomenex, with pentafluorophenyl groups bound to silica surface that offer high aromatic selectivity due to highly electronegative fluorine atoms on the periphery of each phenyl ring. The optimized mobile phase consisted in an isocratic mixture of 0.1% triethylamine solution acidified to pH 2.2 adjusted with trifluoroacetic acid (eluent A) and ethanol (eluent B) 64:36 (v/v) at a flow rate of 0.6 mL min^{-1} . The injection volume was 10 μL and the column oven temperature was maintained at 38 $^\circ\text{C}$. The fluorescence detector was set to an excitation wavelength of 290 nm and an emission wavelength of 460 nm.

A Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole) was used to analyze selected samples. Data acquisition software was MassLynx V 4.1 with MetaboLynx XS option (Waters). MS detection settings of Waters TQD mass spectrometer were as follows: source temperature 140 $^\circ\text{C}$, desolvation temperature 350 $^\circ\text{C}$, desolvation gas flow rate 900 L h^{-1} , cone gas flow 50 L h^{-1} , capillary voltage 3.00 kV, and cone voltage 30 V. The data were obtained in a positive ionization scan mode ranging from 50 to 1000 m/z in time 0.5 s intervals for the metabolite detection using MetaboLynx XS. After the metabolite detection, MS/MS spectra (daughter scans) were obtained using 15 eV and 25 eV collision energy. For each metabolite/transformation products two MRM transitions were optimized using the most intense fragments of the MS/MS spectra. The chromatographic separations were held on a Water Acquity HSS T3 column, particle size 1.8 μm , 150 mm $\times$ 2.1 mm. The injection volume was 5 μL with a flow rate of 0.4 mL min^{-1} . Mobile phase consisted in water 0.1% formic acid/acetonitrile 0.1% formic acid. Elution was performed with gradient 99/1 to 0/100 in 14 min (v/v).

The identity of the metabolites/transformation products was confirmed by high resolution mass spectrometry with a Dionex RSLC LC-system (Bruker Daltonics, Germany) equipped with an ESI source, coupled to a Bruker QqTOF maXis impact mass spectrometer. Identification was performed by 80–1300 m/z full scan and auto MS/MS and bbCID at 5 Hz. The chromatographic condition was the same used for TQD analysis except for the injection volume (2–5 μL). Data acquisition software was DataAnalysis with MetaboliteTools (Bruker).

2.3. Fluoride release

The measurement of fluoride released into the culture supernatant was performed using an ion-selective electrode (CH-8902 Mettler-Toledo GmbH). A calibration curve was carried out by using newly prepared standard solutions of sodium fluoride in minimal mineral medium (MM) in six concentration levels: 0.01 mM, 0.05 mM, 0.10 mM, 0.50 mM, 1.00 mM, and 5.00 mM. This method was adapted from previous studies of the work group [33].

2.4. LC–FD method validation parameters

The method was validated according to International Conference on Harmonization Guidelines [34], considering the following parameters: selectivity, linearity and range of application,

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