Journal of Chromatography B, 1029–1030 (2016) 174–183

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Integrated liquid chromatography method in enantioselective studies: Biodegradation of ofloxacin by an activated sludge consortium

Alexandra S. Maia[a,b], Paula M.L. Castro[b], Maria Elizabeth Tiritan[a,c,d,*]

[a] CESPU, Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Rua Central de Gandra, 1317, 4585-116 Gandra PRD, Portugal
[b] Universidade Católica Portuguesa, CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal
[c] Laboratório de Química Orgânica e Farmacêutica, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[d] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR/CIMAR), Universidade do Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal

ARTICLE INFO

Article history:
Received 9 April 2016
Received in revised form 12 June 2016
Accepted 15 June 2016
Available online 16 June 2016

Keywords:
Ofloxacin
Levofloxacin
Macrocyclic antibiotic-based stationary phases
Enantioselectivity
Biodegradation
Activated sludge

ABSTRACT

Ofloxacin is a chiral fluoroquinolone commercialized as racemate and as its enantiomerically pure form levofloxacin. This work presents an integrated liquid chromatography (LC) method with fluorescence detection (FD) and exact mass spectrometry (EMS) developed to assess the enantiomeric biodegradation of ofloxacin and levofloxacin in laboratory-scale microcosms. The optimized enantioseparation conditions were achieved using a macrocyclic antibiotic ristocetin A-bonded CSP (150 × 2.1 mm i.d.; particle size 5 μm) under reversed-phase elution mode. The method was validated using a mineral salts medium as matrix and presented selectivity and linearity over a concentration range from 5 μg L^{-1} (quantification limit) to 350 μg L^{-1} for each enantiomer. The method was successfully applied to evaluate biodegradation of ofloxacin enantiomers at 250 μg L^{-1} by an activated sludge inoculum. Ofloxacin (racemic mixture) and (*S*)-enantiomer (levofloxacin) were degraded up to 58 and 52%, respectively. An additional degradable carbon source, acetate, enhanced biodegradation up to 23%. (*S*)-enantiomer presented the highest extent of degradation (66.8%) when ofloxacin was supplied along with acetate. Results indicated slightly higher biodegradation extents for the (*S*)-enantiomer when supplementation was done with ofloxacin. Degradation occurred faster in the first 3 days and proceeded slowly until the end of the assays. The chromatographic results from LC-FD suggested the formation of the (*R*)-enantiomer during levofloxacin biodegradation which was confirmed by LC–MS with a LTQ Orbitrap XL.

1. Introduction

Quinolone antibiotics, discovered in the 1960s when nalidixic acid was developed, have been gaining great pharmaceutical interest [1,2]. Between 1985 and 1990 ofloxacin (Fig. 1), which belongs to the second generation of fluoroquinolones, became commercially available in some European countries. Ofloxacin is nowadays largely used in human antimicrobial therapies and in veterinary medicine, the latter with both therapeutic and prophylactic value.

Ofloxacin is a racemic mixture and the (*S*)-enantiomer corresponds to levofloxacin. The enantiomerically pure form is up to fourfold more active against numerous bacterial strains than the racemic mixture and with up to 128 times more antibacterial activity when compared to the (*R*)-enantiomer [3,4]. Levofloxacin prevailed in time-kill studies against methicillin-susceptible *Staphylococcus aureus*, when compared to ofloxacin and ciprofloxacin [5]. Because of their fluorinated nature, fluoroquinolones exhibit higher recalcitrance and persistence in the environment [6] and have been labeled as emerging environmental micropollutants [7]. These characteristics make them potential targets for environmental risk assessment studies regarding occurrence of pharmaceutical compounds. In recent years, the biodegradability of fluoroquinolones in different conditions has been assessed in several works [8–10]. Although it is known that biotransformation processes can be enantioselective [11,12] only

* Corresponding author at: CESPU, Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Rua Central de Gandra, 1317, 4585-116 Gandra PRD, Portugal.
E-mail addresses: elizabeth.tiritan@iscsn.cespu.pt, tiritan@yahoo.com.br (M.E. Tiritan).

http://dx.doi.org/10.1016/j.jchromb.2016.06.026
1570-0232/

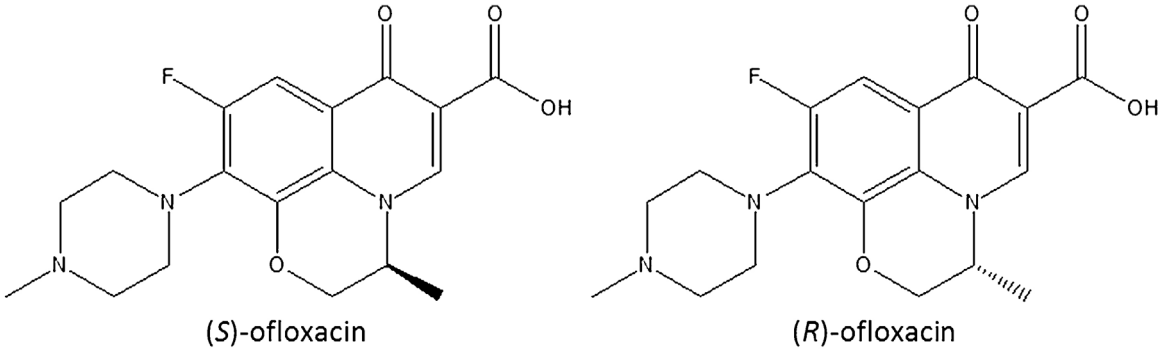

Fig. 1. Chemical structures of ofloxacin enantiomers.

a few classes of pharmaceuticals have been included in enantioselective environmental studies [12–14].

Monitoring the enantiomeric fraction (EF) of chiral pollutants by liquid chromatography in complex natural matrices is a powerful tool to study the enantioselective behavior of such molecules in the environment [11]. However, the difficulty to establish suitable conditions for liquid chromatography with mass spectrometry (LC–MS) analysis is a drawback in enantioselective studies.

The classic approach to select the right chiral stationary phase (CSPs) is based on trial and error studies and usually starts with CSPs with broad applicability such as polysaccharide and macrocyclic antibiotic-based CSPs [15]. In recent years works regarding environmental analysis of chiral pharmaceuticals report the use of protein-based, polysaccharide-based and macrocyclic antibiotic-based CSPs [12] and other derivatization methods [16–18]. Methods for enantioseparation of chiral fluoroquinolones using other CSPs than crown ethers-based and protein-based are still scarce [19,20] and none are compatible with LC–MS.

This work describes the development and optimization of a LC method for the resolution of ofloxacin enantiomers. The optimized analytical method was validated in accordance to international criteria and applied to the quantification of the EF of ofloxacin and its (*S*)-enantiomer levofloxacin during biodegradation assays by an activated sludge inoculum from a municipal wastewater treatment plant. LC–MS with a LTQ Orbitrap XL exact mass spectrometer confirmed the identity of the enantiomers. To the best of our knowledge this is the first report on the enantioseparation of ofloxacin by Chirobiotic based CSPs and validation of the enantioselective method to follow the biodegradation of its enantiomers.

2. Material and methods

2.1. Chemicals and materials

Standards of the fluoroquinolones ofloxacin and levofloxacin were purchased from Sigma-Aldrich. The standards presented a purity degree >98%. Chromatographic gradient grade solvents ethanol, methanol, isopropanol, and acetonitrile, were obtained from Fisher Scientific UK (Leicestershire, UK); hexane was obtained from Merck (Darmstadt, Germany). Triethylamine and diethylamine, both with ≥99% purity, were acquired from Sigma-Aldrich (St. Louis, USA). Acetic acid, formic acid, and trifluoroacetic acid were purchased from VWR International (Fontenay-sous-Bois, France), Merck (Darmstadt, Germany) and Acros Organics (New Jersey, USA), respectively. Ammonium acetate and ammonium formate were both obtained from Sigma-Aldrich (St. Louis, USA). Ultrapure water was supplied by a Milli-Q water system. All chromatographic solvents were filtered prior to use with 0.45 μm glass microfiber filters from Whatman™.

Ofloxacin 1000 mg L^{-1} and levofloxacin ((*S*)-ofloxacin) 500 mg L^{-1} stock solutions were prepared by dissolving the standards in a mixture of water:acetic acid 10% (50/50, v/v). These solutions were stored in amber bottles at −20 °C. Working standard solutions were obtained by dilution of stock solutions in ultrapure water or in ethanol (depending on the elution mode in use) to 1 mg L^{-1} and prepared weekly.

2.2. Instrumentation

Chiral analysis was accomplished in a Shimadzu UFLC Prominence equipment, using two pumps LC-20AD, an autosampler SIL-20AC, a column oven CTO-20AC, a degasser DGU-20A5, a fluorescence detector RF-10AXL, and a system controller CBM-20A. The detection wavelengths were set at 290 and 460 nm for excitation and emission, respectively. Data acquisition was performed using LC Solution, Version 1.24 SP1 from Shimadzu. CSPs included: (*S,S*)-Whelk-O1 and L-Phenylglycine (250 × 4.6 mm i.d., particle size 5 μm, pore size 100 Å) columns, both from Regis Technologies, Inc. (Morton Grove, IL, USA); and macrocyclic antibiotic-based CSPs. The latter were used to perform a sequential optimization of ofloxacin enantiomers separation, namely Chirobiotic V (vancomycin-bonded CSP), Chirobiotic T (teicoplanin-bonded CSP), Chirobiotic TAG (teicoplanin aglycone-bonded CSP), and Chirobiotic R (ristocetin A-bonded CSP). Vancomycin and teicoplanin CSPs with 150 × 4.6 mm i.d. and particle size 5 μm, and teicoplanin aglycone and ristocetin A CSPs with 150 × 2.1 mm i.d. and particle size 5 μm. All the macrocyclic antibiotic-based CSPs were provided from SUPELCO analytical (Sigma-Aldrich, Steinheim, Germany). HPLC Accela with Accela PDA detector, Accela Autosampler and Accela 600 Pump (Thermo Fischer Scientific, Bremen, Germany) coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) was used to analyze biodegradation samples. Data acquisition software was LTQ Tune Plus 2.5.5 and Xcalibur 2.1.0. MS detection settings were as follows: capillary voltage of the electrospray ionization (ESI) 3100 V; capillary temperature 275 °C, sheath gas and auxiliary gas flow rates (nitrogen) set to 40 and 10, respectively (arbitrary unit as provided by the software seetings), capillary voltage 36 V, and tube lens voltage 110 V. The identification was performed in a positive ionization full scan mode ranging from 100 to 1000 *m/z*.

2.3. Optimization of chromatographic conditions

Optimization experiments were performed in isocratic mode. Flow rate variations were between 0.2 and 1.0 mL min^{-1}. Sample injection volume was 10 μL. Mobile phase compositions were: hexane and an additional modifier, ethanol, or isopropanol in normal elution mode approach; and methanol, ethanol, isopropanol, and acetonitrile solo or in combinations, with different propor-