Journal of Chromatography A, 1347 (2014) 63–71

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Simultaneous determination of diclofenac, its human metabolites and microbial nitration/nitrosation transformation products in wastewaters by liquid chromatography/quadrupole-linear ion trap mass spectrometry

Victoria Osorio[a], Marta Imbert-Bouchard[a], Bozo Zonja[a], José-Luis Abad[b], Sandra Pérez[a,*], Damià Barceló[a,c]

[a] Water and Soil Quality Research Group, IDAEA-CSIC, c/ Jordi Girona 18-26, 08034 Barcelona, Spain
[b] RUBAM-IQAC-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain
[c] Catalan Institute for Water Research (ICRA), Scientific and Technological Park of the University of Girona, Emili Grahit 101, 17003 Girona, Spain

ARTICLE INFO

Article history:
Received 15 December 2013
Received in revised form 26 March 2014
Accepted 17 April 2014
Available online 24 April 2014

Keywords:
Diclofenac
Transformation products
Nitration
Nitrosation
High resolution mass spectrometry
LC–MS

ABSTRACT

An analytical method was developed and validated for the first determination of five major human metabolites of the non-steroidal anti-inflammatory drug diclofenac as well as two microbial transformation products in wastewater. The method was based on the extraction of diclofenac and the chemically synthetized compounds by solid-phase extraction (SPE), using a hydrophilic–lipophilic balanced polymer followed by liquid chromatography (LC) coupled to hybrid quadrupole-linear ion trap mass spectrometry (QqLIT-MS). Quantitation was performed by the internal standard approach, to correct for matrix effects. The accuracy of the method was generally higher than 40% for raw and treated wastewater with a precision below 12%. In wastewater influent and effluent samples the detection limits for the majority of target compounds were 0.3–2.5 ng L^{-1} and 0.1–3.1 ng L^{-1}, respectively. The method was applied to the analysis of influent and effluent wastewater samples from urban wastewater treatment plants. Moreover, to obtain an extra tool for confirmation and identification of the studied diclofenac-derived compounds, Information-Dependent Acquisition (IDA) experiments were performed, with selected reaction monitoring (SRM) as the survey scan and an enhanced product ion (EPI) scan as the dependent scan. Diclofenac and its major human metabolite, 4′-hydroxydiclofenac were detected in all samples at concentrations of 331–1150 ng L^{-1} and 585–6000 ng L^{-1}, respectively. Neither microbial transformation product of diclofenac was detected in any of the influent samples analyzed, but in effluents, their concentrations ranged from 4 to 105 ng L^{-1}.

1. Introduction

Diclofenac (DCF) is a non-steroidal anti-inflammatory drug (NSAID) widely used for the treatment of inflammatory disorders and painful conditions. In humans the extensive first-pass metabolism reduces the oral bioavailability to about 50% [1,2]. During hepatic metabolism, DCF undergoes hydroxylation to yield predominantly 4′-hydroxydiclofenac (4′-OH-DCF) and to minor extent 5-hydroxydiclofenac (5-OH-DCF), as well as glucuronidation of the carboxylic acid to produce the 1-O-acyl glucuronide (DCF-gluc) (see Table S-1 for structures) [3]. Thus DCF together with its human metabolites enter wastewater treatment plants (WWTPs) through sewers. DCF has been frequently detected in effluents samples collected at European WWTPs at concentrations ranging from 0.1 to over 5 μg L^{-1} [4]. Incomplete removal efficiencies of DCF during conventional activated sludge treatment (7–80%; [5]) translate into its frequent appearance in surface waters. Widespread use of DCF as over-the-counter drug in conjunction with relatively high doses at short dosing intervals and low removals in WWTPs lead to its continuous discharge into the aquatic environment, making it a pseudo-persistent pollutant therein. By definition, removal rates in WWTPs only reflect the disappearance of the compound itself without addressing the processes leading to its removal. If biochemical processes are involved in the removal of organic contaminants, biodegradation proceeds

* Corresponding author at: IDAEA-CSIC, Department of Environmental Chemistry, Jordi Girona 18-26, Barcelona 08034, Spain. Tel.: +34 934006100.
E-mail addresses: spsqam@idaea.csic.es, spsqam@cid.csic.es (S. Pérez).

http://dx.doi.org/10.1016/j.chroma.2014.04.058
0021-9673/

via the formation of a series of intermediates before the compound is – in the ideal case – ultimately mineralized. The treated effluents may thus contain not only remaining parent drug and excreted human metabolites but also microbial transformation products, all of which being discharged into receiving water bodies.

Although the major human metabolites were identified more than three decades ago [6], their presence in wastewater samples has been described only recently: the two hydroxylated metabolites 4′-OH-DCF and 5-OH-DCF were reported to occur in raw wastewater at concentrations ranging from 0.06 to 3.0 $\mu g\,L^{-1}$ and from 0.06 to 0.7 $\mu g\,L^{-1}$, respectively [7–9]. The major metabolite of DCF, 4′-OH-DCF, together with 5-OH-DCF and the lactam of 4′-OH-DCF (4′-OHD-DCF), were detected at levels of 0.71 $\mu g\,L^{-1}$, 0.45 $\mu g\,L^{-1}$, and 0.42 $\mu g\,L^{-1}$, respectively, while DCF concentrations ranged from 1.3 to 3.3 $\mu g\,L^{-1}$ in wastewater samples [10]. However, the human metabolites have been never analyzed in environmental samples. Moreover, to date no quantitative information is available on the occurrence of 4′,5-dihydroxydiclofenac (4′,5-diOH-DCF) and DCF-gluc in WWTPs, but it has been speculated that the conjugate presents hydrolytic instability of its ester bond which may lead to the release of DCF during the biological wastewater treatment and thereby explain the occasional observation of effluent DCF concentrations exceeding those measured in the corresponding influents [11].

Besides the presence of human metabolites of DCF in the WWTPs, formation of microbial transformation products (TPs) is a second aspect to be considered in assessing the overall fate of pharmaceuticals. There is growing interest in the study of the whereabouts of pharmaceuticals in WWTPs and the aquatic environment which is largely facilitated by technological advances in instrumentation suitable for analyzing polar organic compounds in complex matrices. Fate studies were the objective of the work presented by Pérez and Barceló [7] who investigated the transformation of DCF in lab-scale bioreactors loaded with mixed liquor from a municipal WWTP. Through the application of several mass spectrometric approaches, two hitherto unknown TPs of DCF, namely a nitroso derivative (TP323) and nitro derivative (TP339) were described for the first time. However, no detection of the two TPs in WWTP samples was attempted due to the lack of availability of pure standards. One of the main hurdles for measuring TPs in environmental samples is the need for available standards for method development and samples quantification; these are rarely commercially available. An alternative to obtain reference compounds is via “in-house” classical organic synthesis or biochemical synthesis. In order to generate human metabolites not commercially available at the time for further analysis in wastewater samples, Pérez and Barceló [7] biosynthesized 4′-OH-DCF by means of recombinant human cytochrome P450.

Importantly, DCF (along with the pharmaceuticals 17-β-estradiol and 17-α-ethinylestradiol) has been proposed to be included in the EU Commission first watch list of substances in order to gather monitoring data for the purpose of facilitating the determination of appropriate measures to address the risk posed by those substances [12]. In view of the environmental concern about DCF-related metabolites and TPs which are expected to be discharged with wastewater effluent into surface waters, the goal of the present work was to develop and validate a sensitive analytical protocol for the simultaneous determination of DCF, five human metabolites as well as the two chemically synthesized compounds, TP323 and TP339 in order to better understand the overall fate of DCF. The methodology, which relied on solid-phase extraction (SPE) and liquid chromatography/tandem mass spectrometry (LC–MS/MS), was applied to monitor influent and effluent water samples from Spanish WWTPs. To achieve an additional level of confidence in the detection of the target analytes, routinely analyzed in selected reaction monitoring (SRM) mode, the so-called instrument-dependent analysis (IDA) mode was activated on the hybrid triple quadrupole/linear ion trap (QqLIT) mass spectrometer to generate high-sensitivity product ion spectra.

2. Experimental

2.1. Chemicals

While diclofenac was obtained from Sigma–Aldrich (Steinheim, Germany), 4′-OH-DCF, 5-OH-DCF and DCF-gluc were purchased from Toronto Research Chemicals (Toronto, Canada). The human metabolites, 4′,5-diOH-DCF and the lactam form of 5-OH-DCF (5-OHD-DCF), as well as the microbial nitration/nitrosation transformation products TP339 and TP323 were chemically synthesized, purified and characterized according to the information provided in Sections 2.2 and 2.3 (see also supplementary content: Table S-2, Figs. S-1 and S-2). Isotopically labeled compounds, used as internal standards, were mefenamic acid-d_3 (MFA-d_3) purchased from Toronto Research Chemicals (Toronto, Canada) and niflumic acid-d_5 (NFA-d_5) purchased from Santa Cruz Biotechnologies (Santa Cruz, Canada). Sulfadimethoxine-d_6 (SDM-d_6) and lumiracoxib (LMX), used as surrogates, were provided by Sigma–Aldrich and Toronto Research Chemicals, respectively. Individual stock solutions of the analytes and the isotopically labeled internal standards were prepared on a weight basis in methanol (1000 $\mu g\,L^{-1}$) and stored at −20 °C. A mixture of all target analytes were prepared by appropriate dilution of individual stock solutions in methanol/water (5:95, v/v). Working standard solutions were prepared freshly in the same solvent mixture before each analytical run. A separate mixture of isotopically labeled internal standards, used for internal standard calibration, was prepared in methanol (1000 $\mu g\,L^{-1}$) and further dilutions were prepared in methanol/water (5:95, v/v). They were generated using linear regression analysis and afforded good fits over the established concentration range of 0.1–100 ng mL^{-1} ($r^2 > 0.999$). For quantification purposes, the internal standard calibration approach was used, performing eight-point calibration standards daily, and the possible fluctuation in signal intensity was checked by injecting a standard solution at two concentration levels after each 8–10 injections.

The cartridges used for SPE were Oasis HLB (200 mg, 6 mL) and Oasis MAX (150 mg, 6 mL) (Waters, Milford, MA, USA). Glass fiber filters Whatman (Maidstone, Kent, UK) (0.7 μm) and nylon membrane filters (0.45 μm) were purchased from Teknokroma (Barcelona, Spain). HPLC-grade methanol, acetonitrile, water (Lichrosolv), hydrochloric acid (37%) and formic acid (98%) were supplied by Merck (Darmstadt, Germany). Ascorbic acid, ammonium hydroxide and ammonium acetate (99%) were from Sigma–Aldrich.

2.2. UPLC/ESI-high resolution MS analysis of synthetized standards

Accurate mass measurements of the chemically synthetized metabolites and TPs were carried out in full-scan and product ion scan mode using an LTQ Orbitrap Velos interfaced with an Accela 1250 UPLC system (Thermo Scientific, San Jose, CA, USA). Samples were separated on a Waters Acquity BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm particle size) equipped with a precolumn (50 mm × 2.1 mm) of the same packing material. The LC–MS analysis was carried out using an ESI interface working in positive and negative ion modes. For the positive ion mode the mobile phases were (A) formic acid (0.1%) in water, and (B) acetonitrile. After 1 min of isocratic conditions at 85% A, the portion of A was linearly decreased to 3% within 8 min. This condition was held for 2 min and then the initial mobile phase composition was