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Short communication

Liquid chromatography–tandem mass spectrometry detection of diclofenac and related compounds in water samples

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ABSTRACT

A frequently studied environmental contaminant is the active substance diclofenac, which is removed insufficiently in sewage treatment plants. Since its inclusion in the watch list of the EU Water Framework Directive, the concentrations in surface waters will be determined throughout Europe. For this, still, more precise analytical methods are needed. As a reference, HPLC-MS is frequently employed. One of the major metabolites is 4'-hydroxydiclofenac (4'-OH-DCF). Also, diclofenac lactam is important for assessing degradation and transformation. Aceclofenac (ACF), the glycolic acid ester of diclofenac is used as a drug, too, and could potentially be cleaved to yield diclofenac again. In various sewage treatment plant influent samples, diclofenac, 4'-OH-DCF, DCF lactam and ACF could be determined with detection limits of 3 μg/L, 0.2 μg/L, 0.17 μg/L and 10 ng/L, respectively.

1. Introduction

Diclofenac (DCF) is a common environmental contaminant found in surface waters and sometimes in drinking water. It is used in both human and veterinary medicine. Diclofenac administered orally is excreted to 65-70% with the urine and to 20-30% via faeces mainly in the form of its metabolites [1–4]. It is also heavily used as a topical treatment for any kind of muscular pain. Only 6% of DCF are taken up from the ointment over the skin, the remainder is washed off. Excessive use and incomplete removal in wastewater treatment plants (21-40% [5]) result in up to 75% of the DCF consumed entering the water cycle. An influence on the food chain by enrichment (e.g., fish [6–10], vulture [11,12]) has already been demonstrated. Even at environmental concentrations, DCF is harmful to many species [6–9,13]. Microgram per Litre concentrations already trigger toxic effects in liver and kidney [14]. Liver damage was first attributed to the covalent binding of reactive DCF metabolites to proteins [15,16], but this did not ultimately prove to be the main cause. European wastewater concentrations show an average of 0.7 μg/L with a maximum of 11 μg/L [17]. In ground or drinking water, the concentrations reach 1-7 ng/L [18]. Determination was performed by LC-MS, GC-MS [19] or by immunoassays [12,19–21]. However, DCF is not the only compound in the water with impact on the ecosystem. In addition to the parent compound, both metabolites and degradation products are present in wastewater which may have a higher toxicity than DCF itself [22]. Apart from biotic metabolism, DCF can undergo photodegradation [22–24]. Due to the continuous input from the treatment plants, a pseudo-constant concentration exists in surface waters. This is one of the reasons, why diclofenac was included in the watch list of the EU Water Framework Directive [25]. In Spanish wastewater treatment plants, the main metabolite 4′-hydroxydiclofenac (4'-OH-DCF), which is formed by the enzyme CYP2C9 [2,26], was present in much higher concentrations than DCF [27]. A known secondary reaction is cyclization, which DCF can undergo under various conditions. The resulting DCF-lactam has already been detected in sewage samples [28]. As to the best of our knowledge, no concentrations in water samples have been determined of this degradation product. Another active ingredient is aceclofenac (ACF), which is the glycolic acid ester of diclofenac. ACF was never quantified, too. In addition to quantification, the aim of this work was to investigate possible adulterations of the orig-

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inal contamination levels during sample preparation. A common sample preparation is pre-concentration by means of solid-phase extraction (SPE). However, it is known that organic acids present in the samples are also concentrated [29]. Cyclization takes place under acidic conditions. Therefore, cyclization on the cartridges cannot be ruled out. If this was the case, previous publications could have underestimated the real concentrations. It has already been reported that DCF metabolites cyclize in a sample pre-treatment step for gas chromatography [3]. In addition to the quantification of DCF-lactam and ACF, and review of a possible cyclization of DCF during pre-concentration, it was also our objective to study whether ACF may be subject to an ester cleavage under the conditions of pre-concentration.

2. Experimental

2.1. Reagents and Materials

All standard chemicals and reagents were purchased from Sigma-Aldrich (Steinheim, Germany). All solvents were chromatography grade. Diclofenac sodium salt, aceclofenac, $^{13}C_6$-labeled diclofenac sodium salt, 4′-hydroxydiclofenac and 1-(2,6-dichlorophenyl)-2-indolinone were from Sigma Aldrich. Fmoc Ahx Wang resin was supplied by Iris Biotech GmbH (Marktredwitz, Germany). For HPLC the following solvent compounds were used: ammonium acetate (NH_4Ac, analytical grade, 99.3%, Fischer Chemicals AG, Zurich, Switzerland), glacial acetic acid (AcOH, analytical reagent grade, Fischer Chemicals), methanol (MeOH, HPLC gradient grade, J.T. Baker, Griesheim, Germany). Folded filters (qual., grade 1288) were from Sartorius stedim (Göttingen, Germany) and Strata^{TM}-X 33 μm polymeric Reversed Phase SPE cartridges (500 mg, 6 mL) from Phenomenex (Aschaffenburg, Germany). Ultrapure water was taken from a Milli-Q water purification system (Milli-Q Synthesis A10, Millipore, Schwalbach, Germany).

2.2. LC-MS/MS

An Agilent 1260 Infinity LC system with a binary pump, degasser, autosampler, column heater and UV detector was used. Chromatographic separation was achieved on a Kinetex XB-C18, 2.6 μm, 150 mm x 3 mm analytical LC column with a UHPLC C18, 3 mm column guard (both Phenomenex). The mobile phases were ultrapure water with 0.1% (v/v) acetic acid (A) and MeOH with 0.1% (v/v) acetic acid (B). The flow rate was 350 μL/min and the column heater temperature was 55 °C. An elution gradient was applied, starting with 70% A, held for 3 min. Afterwards A was decreased to 5% within 11 min and held constant for the next 4 min, increased back to 70% A within 0.5 min and held for the next 7.5 min to re-equilibrate the column. The injection volume was 10 μL. The quantification was performed by an AB SCIEX 6500 triple quad^{TM} mass spectrometer (SCIEX, Darmstadt, Germany). Parameters used to produce fragment ions in selected reaction monitoring mode (SRM) and collision energies (CE) are given in Table 1. The electrospray ionization source (ESI) was operated in positive ionization mode.

The parameters used for ionization were a temperature of 400 °C, 4500 V ion spray voltage, an entrance potential (EP) of 10 V, a declustering potential (DP) of 90 V, a collision cell exit potential (CXP) of 15 V, a curtain gas with 35 psi (1 psi = 0.0689 bar = 6,890 kg/(m*s^2) (SI unit)), a nebulizer gas (GS1) with 62 psi, a turbo gas (GS2) with 62 psi and a collision gas with 8 psi. Analyst® version 1.6.2 software (SCIEX) was used to control the instrument, acquire data and evaluate the results.

Table 1
Selected reaction monitoring (SRM) transitions for diclofenac and metabolites including the ^{13}C labeled internal standards, and collision energies (CE).

Compound	SRM transition	CE (V)
DCF	296 → 250 quantifier	22
	296 → 214 qualifier	30
$^{13}C_6$-DCF	302 → 256 quantifier	22
	302 → 220 qualifier	30
4′-OH-DCF	312 → 266 quantifier	22
	312 → 230 qualifier	30
DCF-lactam	278 → 151 quantifier	70
	278 → 214 qualifier	40
	278 → 179 qualifier	60
$^{13}C_6$-DCF-lactam	284 → 157 quantifier	70
	284 → 220 quantifier	40
	284 → 185 quantifier	60
ACF	354 → 250 quantifier	22
	354 → 214 qualifier	30

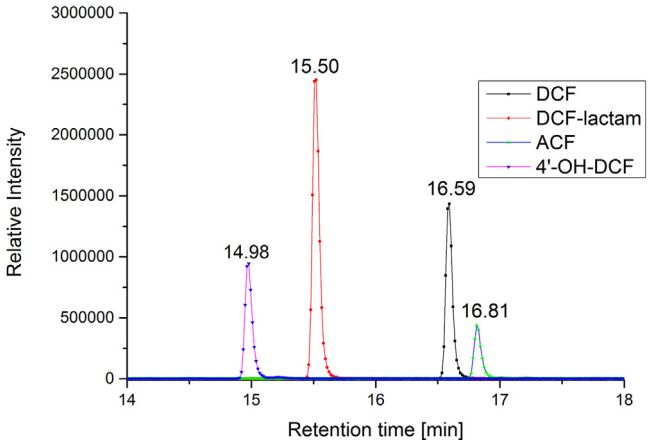

Fig. 1. HPLC-MS/MS chromatogram of DCF (mass transition 296 → 250), 4′-OH-DCF (312 → 266), ACF (354 → 250), and DCF-lactam (278 → 214).

2.3. Solid-phase extraction

SPE was performed with an AutoTrace^{TM} SPE workstation (Dionex, Idstein, Germany). Firstly, Strata^{TM}-X cartridges were washed with 10 mL MeOH, equilibrated with 10 mL ultrapure water, followed by loading the respective sample volume of 1000 mL. The flow rate for each step was 10 mL/min. Afterwards, the cartridges were dried by flushing N_2 (20 psi) for 15 min and the adsorbates were eluted with 10 mL MeOH. The eluate was concentrated in a flow of N_2 to less than 1 mL. The concentrate was filled up with ultrapure water to 1.00 mL and filtrated through a syringe glass fibre filter (No. 7-8808, neoLab, Heidelberg, Germany). After filtration, the samples were stored at 4 °C.

3. Results and Discussion

3.1. HPLC-MS/MS method

Fig. 1 shows exemplarily chromatograms obtained of DCF and related substances,

DCF having a retention time of 16.59 min. The molecular weight of protonated DCF is 296 Da. As described in 2. Experimental, two mass transitions can be used for detection and quantification. The fragmentation is shown in Fig. 2. It leads to protonated fragments with molecular weights of 250 Da and 214 Da.

For measurements of isotopically labelled DCF, 4′-OH-DCF or ACF, analogous fragmentations were determined. 4′-OH-DCF has a retention time of 14.98 min, ACF of 16.81 min. The detection and quantification of DCF-lactam requires two fragments. The fragment