Environmental Pollution 225 (2017) 354–360

Contents lists available at ScienceDirect

Environmental Pollution

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

Mussels as bioindicators of diclofenac contamination in coastal environments☆

S.C. Cunha [a, *], A. Pena [b], J.O. Fernandes [a]

[a] LAQV-REQUIMTE, Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, University of Porto, Rua Jorge de Viterbo Ferreira 228, 4050-313 Porto, Portugal

[b] LAQV-REQUIMTE, Group of Bromatology, Pharmacognosy and Analytical Sciences, Faculty of Pharmacy, University of Coimbra, Polo III, Azinhaga de St[a] Comba, 3000-548 Coimbra, Portugal

ARTICLE INFO

Article history:
Received 7 January 2017
Received in revised form
27 February 2017
Accepted 27 February 2017
Available online 9 March 2017

Keywords:
Diclofenac
Macroalgae
Mussels
Contaminants
LC-MS/MS

ABSTRACT

Diclofenac a nonsteroidal anti-inflammatory drug (NSAID) has been confirmed as an emerging contaminant in the aquatic environment. Toxicology studies have revealed that harmful effects may emerge from diclofenac presence not only for human health, but also for marine organisms, which implies its monitoring. To overcome the demanding challenges of diclofenac quantification in biotic aquatic species, a novel method for the determination of diclofenac in mussels (*Mytilus galloprovincialis* and *Mytilus edulis*) and macroalgae (*Laminaria digitata*) using high performance liquid chromatography coupled to tandem mass spectrometry was developed and validated according to the EC Decision 2002/657/EC. Additionally, a study was done about diclofenac contamination in mussels collected from 8 sites along the 1115 miles of coastline in Portugal in 2015. The results suggested that levels in mussels are closely related to the environmental contamination. Therefore, mussels can be a potential bioindicator of diclofenac contamination in the coastal environment.

1. Introduction

In the last years, the focus of environmental research has been gradually changing from the conventional “priority pollutants”, such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons, and pesticides, to the so-called “emerging pollutants” (EPs) such as flame retardants, disinfection by-products, pharmaceuticals and personal care products (Sousa, 2013; Geissen et al., 2015). These are chemicals that are not commonly monitored in the environment, but which have the potential to enter into the diverse environmental compartments and cause adverse ecological and human health effects (Geissen et al., 2015). Some EPs as most of the pharmaceutical compounds are not new chemicals but substances that have been present for a long time in the environment and whose presence and significance are only now being elucidated (Norman, 2017).

Pharmaceuticals used for the treatment of human or animal illness are commonly excreted via urine and/or faeces, and are thereafter subject to inadequate removal during conventional wastewater treatment. As a result, pharmaceuticals are ubiquitous compounds, often persistent and bioaccumulative in the environment, particular in the aquatic ecosystem (Kot-Wasik et al., 2007).

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID), used in the treatment of post-operative pain, rheumatoid arthritis, and the chronic pain regularly associated with cancer, widespread used worldwide. For example, during the year of 2014 about 1 054 952 packages were provided in the National Health Service in Portugal (Infarmed, 2014). Due to its widespread and growing use, it is needed to create sound knowledge about its incidence, levels and fate in the environment, as well as to explain its long-term risks, ecotoxicity and human health impact (Sousa, 2013).

Several studies have described physiological and behavioural effects on fish when exposed at environmental or near environmental levels of diclofenac. Cytological changes in rainbow and brown trout tissues (kidney and gills) were found to be induced by exposure to diclofenac in aquatic environments (Schwaiger et al., 2004; Hoeger et al., 2005; Triebskorn et al., 2007). Mehinto et al. (2010) reported that exposure of rainbow trout to diclofenac lead

☆ This paper has been recommended for acceptance by Klaus Kummerer.

* Corresponding author. Laboratório de Bromatologia e Hidrologia, Faculdade de Farmácia, Universidade do Porto, Rua Jorge de Viterbo Ferreira 228, 4050-313 Porto, Portugal.

E-mail address: sara.cunha@ff.up.pt (S.C. Cunha).

http://dx.doi.org/10.1016/j.envpol.2017.02.061
0269-7491/

to tissue damage. Additionally, diclofenac can affect the gene expression in fish (Cuklev et al., 2011). This NSAID has also been associated with the serious reduction of the Gyps vultures in Asia due to renal failure and visceral gout, and ultimately death (Oaks et al., 2004; Taggart et al., 2007).

Due to diclofenac direct and indirect toxicity to vertebrates, it has been recently suggested to be added into the list of priority substances in the EU's Water Framework Directive (2013/39/EU). Legislation on maritime water bodies as the Directive 2008/56/EG (European Commission, 2008) (maritime strategy framework directive-MSFD; European Commission, 2008) and the HELCOM (Baltic Marine Environment Protection Commission) (Helcom, 2015) are considering the insertion of some pharmaceuticals in routine monitoring programs, whereas the OSPAR Commission has already recognized clotrimazole as "substance for priority action" and a wider range of pharmaceuticals as "substances of possible concern" (OSPAR, 2013).

Assessment of environmental exposure to chemicals can be achieved through the use of indicator species such as algae or bivalves. These biomarker species accumulate pollutants in their tissues from the surrounding environment being therefore important biomonitoring devices. Filter feeders, such as bivalves (clams and mussels) tend to concentrate metals in their gills or other tissues. *Mytilus edulis* became a species monitored in the United States of America as well as in other countries for changes in levels of water pollution (Phillips and Rainbow, 1993). Seaweeds as *Ulva lactuca* commonly found at and near effluent discharge points of fish farms have been used as an indicator for the presence of antibiotics (Leston et al., 2015). *Laminaria japonica* was found to bioaccumulate polycyclic aromatic hydrocarbons from the surrounding medium (Wang and Zhao, 2008). Recently, experimental studies reported that mussels were able to bioconcentrate diclofenac from the water where the mussels were exposed to (Ericson et al., 2010; Mezzelani et al., 2016). McEneff et al. (2014) reported that mussel samples collected from two sites on Irish coastline are able to uptake pharmaceutics such as trimethoprim, carbamazepine and mefenamic acid, although no evidences of diclofenac accumulation were observed. To our knowledge there are a lack of reports on diclofenac in wild mussels along an entire coastal line.

The growing interest in diclofenac as an environmental contaminant has catalysed the development of analytical methods able to deal with the trace levels of the compound usually found in the different environmental compartments. Recently, some reviews have been published reporting the state-of-the-art of pharmaceutical environmental analysis (Petrovic et al., 2010; Richardson, 2012). Most of the analytical methods use liquid chromatography coupled to tandem spectrometry (LC-MS/MS) for quantitative analysis of low concentrations of diclofenac. Gas chromatography-mass spectrometry (GC-MS) can be also used, although diclofenac residue usually needs to be derivatized before analysis to enhance its volatility. Most methodologies require an extraction procedure usually based on liquid extraction with moderate polar solvents followed by a clean-up step with solid-phase extraction (SPE) prior to LC-MS/MS or GC-MS analysis (Wang and Zhao, 2008; Ericson et al., 2010; Petrovic et al., 2006). Only a few studies have employed QuEChERS method which stands for "quick, easy, cheap, effective, rugged and safe" in pharmaceutical analysis (Cerqueira et al., 2014; Núñez et al., 2015). The advantage of QuEChERS is that it is a rapid, simple, and accurate method that provides a saving of time and consumables (solvents) over existing methodologies.

The aforementioned analytical methods have been applied for the determination of diclofenac in various matrices such as river waters (Johnson et al., 2013), surface waters (Rabiet et al., 2006), wastewaters (Richardson, 2012; Vieno and Sillanpää, 2014), seawaters (Lolić et al., 2015), fish (Mehinto et al., 2010; Kallio et al., 2010) and mussels (McEneff et al., 2014), but none directly applied to macroalgae. Taking this into consideration, the aim of present work was to develop a simple and reliable analytical method that not only assures the unequivocal identification and quantification of diclofenac at very low levels, but also allows a common pretreatment process (preservation, extraction and clean-up) for two kind of matrices (mussels and algae) traditionally unwieldy to analyze. Additionally, the developed method was validated according the requirements of the Commission Decision 2002/657/EC for determination of diclofenac in mussels and algae. It was further applied in the analysis of wild mussels samples collected from 8 different sites distributed over Portugal costal, along five different seasons of 2015.

2. Experimental

2.1. Chemicals and reagents

Diclofenac sodium salt (99.5% purity) and Internal Standard (IS) diclofenac-acetophenyl ring (13C6, 99.99% purity) sodium salt were acquired from Sigma Aldrich (MO, USA). The solvents acetonitrile (MeCN) and methanol (MeOH), both LC-MS grade, were obtained from VWR (PA, USA). Acetic acid (purity >99%) and formic acid (purity >99%) were both obtained from Merck, (Darmstadt, Germany). The salts ammonium acetate ($NH_4CH_3CO_2$, 97% purity), ammonium chloride (NH_4Cl, 99.8% purity) and ammonium formate (NH_4HCO_2, 99.99% purity) were obtained from AppliChem Panreac ITW companies (Barcelona Spain), Merck, and Sigma Aldrich, respectively. The sorbents sulfate magnesium ($MgSO_4$) and Z-sep were both obtained from Sigma-Aldrich. Ultrapure water was obtained daily from a "Seradest LFM 20" system (Seral, Ransbach-Baumbach, Germany). Nitrogen (nitrogen 90, 99.998% purity) was generated in-house with nitrogen generator from Sysadvance (Maia, Portugal). Ultrahigh purity Argon (99.999%) was purchased from Gasin (Maia, Portugal).

2.2. Standard solutions and validation

Individual stock standard solutions of diclofenac and $^{13}C_6$-diclofenac (IS) were prepared in MeOH (1 mg/mL). Individual working standard solution of diclofenac at 20 μg/mL and $^{13}C_6$-diclofenac at 10 μg/mL were prepared from the stock solutions by appropriate dilution in MeOH, and stored at - 20 °C when not in use. The stability and accuracy of diclofenac in solution were guaranteed using two individual stock solutions, prepared and analyzed on days 0, 30 and 60. The same protocol was used to the standard solutions used in recovery and matrix-matched calibration curves.

The method was validated in agreement with internationally recognized principles, such as linearity, recovery, repeatability, sensitivity (limits of detection and quantification), decision limit (CCα), detection capability (CCβ), selectivity, and robustness. Validation criteria were adopted from guidelines for residues according to Commission Decision 2002/657/EC [30].

Linearity was studied in mussels and *Laminaria digitata* samples (free of analytes) spiked at 6 concentration levels, covering a range between 0.5 and 50 ng/g. The relationship between peak area ratios of analyte/IS and concentrations in the investigated concentration range was assessed by the coefficient of determination (R^2).

Recovery and repeatability were evaluated in simultaneous through the analysis of 6 blank samples spiked, before extraction, at 1, 5 and 20 ng/g with diclofenac only. The internal standard was added to the extracts at the end of the sample preparation with the aim of allowing the estimation of analyte loss during processing.