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Fast and easy extraction combined with high resolution-mass spectrometry for residue analysis of two anticonvulsants and their transformation products in marine mussels

M.J. Martínez Bueno*, C. Boillot, H. Fenet, S. Chiron, C. Casellas, E. Gómez

Hydrosciences Montpellier UMR 5569, Department of Environmental Sciences and Public Health, University of Montpellier I, 34000 Montpellier, France

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ABSTRACT

Environmental field studies have shown that carbamazepine (Cbz) is one of the most frequently detected human pharmaceuticals in different aquatic compartments. However, little data is available on the detection of this substance and its transformation products in aquatic organisms. This study was thus mainly carried out to optimize and validate a simple and sensitive analytical methodology for the detection, characterization and quantification of Cbz and oxcarbazepine (Ox), two anticonvulsants, and six of their main transformation products in marine mussels (*Mytilus galloprovincialis*). A modified QuEChERS extraction method followed by analysis with liquid chromatography coupled to high resolution mass spectrometry (HRMS) was used. The analyses were performed using two-stage fragmentation to reveal the different fragmentation pathways that are highly useful for the identification of isomeric compounds, a common problem when several transformation products are analyzed. The developed analytical method allowed determination of the target analytes in the lower ng/g concentration levels. The mean recovery ranged from 67 to 110%. The relative standard deviation was under 11% in the intra-day and 18% in the inter-day analyses, respectively. Finally, the method was applied to marine mussel samples collected from Mediterranean Sea cultures in southeastern France. Residues of the psychiatric drug Cbz were occasionally found at levels up to 3.5 ng/g dw. Lastly, in this study, other non-target compounds, such as caffeine, metoprolol, cotinine and ketoprofen, were identified in the real samples analyzed.

1. Introduction

The presence of different pharmaceutical compounds in surface and marine water has been largely attributed to the low efficiencies of municipal wastewater treatment plants (WWTPs) for removing many of these compounds [1–3]. The coastline is becoming increasingly urbanized and faces a double pollution threat: by oil slicks or chemical spills, as well as, by pollution generated by inland activities which discharge their wastes into marine coastal waters via streams, rivers and wastewater. In order to improve and maintain the quality of surface water along the European coasts, the European Parliament and the Council approved Recommendation 2002/413/CE concerning the implementation of Integrated Coastal Zone Management in Europe [4].

The carbamazepine (Cbz), is a prescribed drug widely sold for the treatment of epilepsy and other psychotherapy applications. It has been regarded as a potential tracer in surface water due to its poor elimination during wastewater treatment and its persistency, which makes Cbz a pharmaceutical of high environmental relevance [3,5]. It has frequently been detected in WWTP effluent (up to 2.1 μg/L) [2,3,6], river water (up to 1.1 μg/L) [3,7], drinking water (30 ng/L) [8] and even seawater (up to 1.1 μg/L) [7,9]. Cbz is predominantly metabolized in the liver to carbamazepine-10,11-epoxide (Epoxy), a pharmacologically active compound which is further metabolized into 10,11-dihydro-10,11-trans-dihydroxycarbamazepine (TRANS). Oxcarbazepine (Ox) is a keto analogue of Cbz which generates transformation products common of those of Cbz, such as TRANS and 10-hydroxy-10,11-dihydrocarbamazepine (10OH), its therapeutically active metabolite. In 2003, Miao and Metcalfe reported, for the first time, on the presence of Cbz transformation products in Canadian WWTP effluents and surface water [10]. Their study showed that TRANS transformation products exhibited threefold higher concentrations than Cbz itself (up to 1.3 μg/L in effluents and 2.2 ng/L in surface waters). Similar results on these by-products were recently published in French WWTPs by Leclercq et al. [6]. However, little information on the detection of Cbz and Ox in marine organisms and no data on its transformation products are available in literature. This issue could be partly explained by the high complexity of the matrix and the lack of suitable protocols, which include

* Corresponding author. Tel.: +33 04 11 75 94 18; fax: +33 04 11 75 94 61.
E-mail address: mjbueno@ual.es (M.J. Martínez Bueno).

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effective extraction methods and sensitive and specific analytical methods to detect these analytes at trace levels (ng/g or lower).

Most methods for the analysis of organic micropollutants from aquatic organisms are based on lipid isolation, which often involves complicated extraction and clean up procedures to generate extracts ready for analytical determination. SPME (solid-phase micro-extraction) [11], MASE (microwave-assisted solvent extraction) [12], MSPD (matrix solid phase dispersion) [13], SFE (supercritical-fluid extraction) [14], and especially PLE (pressurized liquid extraction) [15–18], have been reported. Nevertheless, most of these methods are long, tedious, time-consuming and require large volumes of organic solvents. In 2003, Anastassiades et al. [19] developed the approach called QuEChES (quick, easy, cheap, effective, rugged and safe). This procedure has frequently been used for the extraction of pesticides in food matrices (milk, olive oil, several fruits and vegetables) [20,21]. However, there are few reported studies related to pharmaceutical and personal care products (PPCPs) determination in fish [22] and it has not been applied for the extraction of target anticonvulsants from mussel samples.

Mussel *Mytilus galloprovincialis* is a common filter feeder that occurs along the European sea coasts. This species is an excellent sentinel for monitoring of organic micro-contaminants from environmental waters, because the mussels can bioaccumulate substances through their gills (dissolved substances) and/or digestive tract (substances sorbed on particles). In a recently publication, Gomez et al. [23] reported the bioconcentration of two pharmaceuticals (benzodiazepines) and two personal care products (UV filters) in such marine organisms.

Accordingly, our aim was to develop and validate a simple, rapid and sensitive analytical strategy for detection, characterization and quantification of two anticonvulsants (Cbz and Ox) and six of their main transformation products in mussels by accurate mass measurements in MS and MS/MS modes. For that, an easy QuEChERS extraction method followed by analysis with a liquid chromatography coupled to full scan high resolution-mass spectrometry (LC–Orbitrap-MS) system was developed and the procedure is described in this study. The effects of several parameters were investigated and reported. The more demanding requirements regarding mass spectrometric confirmation currently set by EU regulations (Commission Decision 2002/657/EC and SANCO/10684/2009 Guideline) were taken into account when confirming and quantifying the target compounds [24,25]. We reported results obtained during the optimization of the QueChERS extraction method, evaluating the influence of several sorbents in the clean-up step while comparing with other conventional procedures used for the analysis of mussel samples. Moreover, retrospective analysis has been applied to identify other non-target compounds (not initially included in the method) by manual processing of previously recorded and stored spectral data. Finally, another innovative aspect of the present study concerns the use of marine organisms as a tool for exposition assessment of micropollutants in aquatic environments.

2. Experimental

2.1. Chemicals and reagents

A comprehensive overview of the reagents used in this study has been included as supplementary data.

2.2. Sample preparation

2.2.1. QuEChERS extraction and d-SPE clean-up

Freeze-dried mussels (2 g ± 0.01) were weighed in a 50 mL polypropylene centrifuge tube and then 100 μL of a 2 mg/L methanolic surrogate standard solution was added (Cbz-d8 and Epoxy-d10). Next, the mussels were rehydrated by adding 10 mL of ultrapure water, and the mixture was vortexed for 30 s. The tubes were then manually and vigorously shaken for 1 min, after the addition 10 mL of AcN. Then 4 g Na_2SO_4 (anh), 1 g NaCl, 1 g $Na_3Cit{:}2H_2O$ and 0.5 g $Na_2HCit{:}3H_2O$ were added directly into each tube and the mixture was immediately vigorously shaken (manually) to avoid salt agglomeration for 1 min more. A centrifugation (3500 rpm, 5 min) was performed, and the mixture was then allowed to stand for 5 min, 2.5 mL of the upper AcN layer was transferred into a 15 mL polypropylene tube, containing clean-up sorbent (750 mg Na_2SO_4, 125 mg PSA, 125 mg C18), and 50 μL of formic acid was added and shaken for 1 min. After a second centrifugation step (5000 rpm, 5 min), 1 mL of mixture was evaporated to dryness at 35 °C under a nitrogen stream. The residue was reconstituted in 1 mL of AcN/water (1:9, v/v). Finally, the sample was centrifuged at 10,000 rpm for 10 min to separate the residual lipids and the extract was filtered directly into an analysis vial using a 0.45 μm PTEF syringe filter.

2.2.2. PLE extraction and clean-up

This was performed on a Dionex ASE 350 (Dionex, Sunnyvale, CA) system. Several experimental extraction variables were optimized (data not included). Finally, the best results were obtained when two GlassFiber filters (Dionex), 7 g of Florisil (60–100 mesh, Sigma–Aldrich), another GlassFiber filter and 50 g of glass beads (1 mm diameter, Assistent) were placed in the stainless-steel extraction cells (66 mL). 3 g ± 0.1 of homogenized mussel enriched with standard was next placed in the cells, mixed with glass beads and finally covered with 15 g of glass beads. Extraction was carried out with water/acetone (3/2, v/v). The extraction conditions were as follows: cell heating time (5 min), static time (10 min), pressure (1500 psi), temperature (80 °C), purging time (200 s), flushing volume (60%) and cycles (2). After extraction, the acetone content was evaporated using a rotary evaporator at 35 °C. The remaining aqueous matrix was diluted with 100 mL of water and filtered with a throw Glass Fibre filter (Whatman). This matrix was cleaned by SPE using Oasis HLB extraction cartridges preconditioned with 2 mL of MeOH and 2 mL of H_2O. The extracts were passed through cartridges and then dried. Elution was carried out with 6 mL of MeOH (method 1) or 2 mL MeOH + 4 mL EtAc (method 2). The eluate was then evaporated to dryness at 40 °C, under a nitrogen stream (Turbovap). The residue was dissolved in 1 mL of AcN/water (1:9). After homogenization, centrifugation was carried out at 12,000 rpm for 8 min. The final extract was transferred into vial after filtration using a 0.45 μm PTEF filter (Note: a dilution 1:1 was necessary to apply the extracts obtained using method 1 before injection into the LC–MS).

2.3. LC–MS analysis

A detailed discussion of analytical method and validation study developed in this work has been included as supplementary data. In summary, the analyses were run on an Exactive mass spectrometer (Thermo Fisher Scientific, USA) equipped with a heated electrospray ionization probe (HESI) source in positive ion mode. Data were acquired by continuously alternating scan events: one without and one with fragmentation. Several instrumental settings were tested to maximize the analyte signals: mass range, resolution, automatic gain control (AGC) target, tube lens, heated capillary temperature, capillary voltage and gas flow rate. The optimal conditions were as follows: HCD (10 eV), resolving power (50,000), AGC (5×105) and scan range (100–400 m/z) in both scan events. The identification criteria applied to the target analytes were: (i) retention times and (ii) 2 diagnostic ions (the protonated molecular ion and one product ion) together a mass accuracy < 5 ppm. Thus, an