Chemosphere 84 (2011) 1708–1715

Contents lists available at ScienceDirect

Chemosphere

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

Application of an ELISA to the quantification of carbamazepine in ground, surface and wastewaters and validation with LC–MS/MS

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ARTICLE INFO

Article history:
Received 25 November 2010
Received in revised form 2 February 2011
Accepted 28 April 2011
Available online 17 May 2011

Keywords:
Immunoassay
Pharmaceuticals
Environment
Wastewater treatment plants
Matrix effects
Cetirizine

ABSTRACT

Carbamazepine is a psychiatric pharmaceutical widely detected in aquatic environments. Due to its generalized occurrence and environmental persistence it might be considered as an anthropogenic pollution indicator. In this research, a previously developed enzyme-linked immunosorbent assay (ELISA), based on a commercial monoclonal antibody, was applied to the quantification of carbamazepine in ground, surface and wastewaters and results were validated by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

The performance of the applied ELISA methodology was tested in the presence of high concentrations of sodium chloride and dissolved organic matter. The method was not significantly affected by matrix effects, being adequate for the quantification of carbamazepine in environmental samples, even without sample pre-treatment. This method allows the quantification of carbamazepine in the range of 0.03–10 $\mu g\,L^{-1}$, with a relative error lower than 30%. Due to a pH dependent cross-reactivity with cetirizine, an antihistaminic drug, the assay also enabled the quantification of cetirizine in the samples.

The application of the developed method to the quantification of carbamazepine was performed by using environmental samples with very different matrices, collected in the geographical area of Ria de Aveiro, an estuarine system located in the North of Portugal. Carbamazepine was detected in all analyzed wastewater samples and in one surface water with concentrations between 0.1 and 0.7 $\mu g\,L^{-1}$. Validation with LC–MS/MS revealed that results obtained by ELISA are 2–28% overestimated, which was considered highly satisfactory due to the absence of sample pre-treatments.

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1. Introduction

Nowadays, pharmaceutically active compounds are considered an important group of environmental contaminants (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Calisto and Esteves, 2009). These compounds have received special attention since the 1990s, with a large number of studies reporting their widespread occurrence in environmental matrices (Ternes, 1998; Jørgensen and Halling-Sørensen, 2000; Ternes, 2001; Kolpin et al., 2002; Weigel et al., 2004; Conley et al., 2008). The well-established environmental persistence (Glassmeyer et al., 2008) and interference with non-target organisms at extremely low concentrations (Brooks et al., 2003a,b; Brain et al., 2004) largely contributed to raise a worldwide concern about this issue.

Carbamazepine (CBZ), an antiepileptic drug, is among the most frequently reported pharmaceuticals and has been detected in a large variety of environmental matrices: effluents of waste water treatment plants (WWTP) (Hummel et al., 2006; Bahlmann et al., 2009), ground waters (Focazio et al., 2008), surface waters (Metcalfe et al., 2003; Tixier et al., 2003) and even in treated drinking waters (Heberer et al., 2002). The large number of environmental occurrences can be attributed to the low efficiencies of the removal methods used in WWTPs (typically below 10%) (Zhang et al., 2008), which are considered to be the most relevant source of this aquatic contamination. Moreover, as CBZ was pointed out as being considerably more resistant to bio- and photodegradation than a large number of other pharmaceuticals (Andreozzi et al., 2003; Tixier et al., 2003; Zhang et al., 2008; Calisto et al., 2011), it has been repeatedly proposed as an adequate marker of environmental anthropogenic pollution (Clara et al., 2004; Glassmeyer et al., 2008; Yu et al., 2009). Consequently, the assessment of CBZ contamination levels might constitute a valuable tool for the identification of relevant focal points of pollution.

To monitor pharmaceutically active compounds in the environment, it is mandatory the use of techniques with adequate limits of quantification (between $ng\,L^{-1}$ and $\mu g\,L^{-1}$). In this context, the application of immunoassays to environmental analysis is a

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0045-6535/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2011.04.072

growing field of research (Schneider, 2003; Huo et al., 2007; Shelver et al., 2008; van Emon et al., 2008; Carvalho et al., 2010). When compared with reference techniques such as LC–MS/MS or GC–MS/MS, immunoassay methodologies allow the analysis of a large number of samples in a very limited amount of time, revealing their suitability to perform high-throughput environmental screenings (Buchberger, 2007). These methods are highly sensitive and sample pre-treatments (such as pre-concentration procedures) are usually not required. Taking into account the referred advantages, immunoassay techniques are an inexpensive and time-efficient alternative to chromatographic techniques.

In this research an enzyme-linked immunosorbent assay (ELISA) was applied to the quantification of carbamazepine in ground, surface and wastewaters. The application of the methodology to environmental samples was performed using samples collected in Aveiro (Northern Portugal). In opposition to other countries located in Occidental and Central Europe, very limited data is available about the environmental presence of pharmaceuticals in Portugal. Until now, only a few recent studies were published reporting surface water contamination with endocrine-disruptors (Douro river, and Ria de Aveiro, Mondego and Sado estuarine systems) (Ribeiro et al., 2009a,b,c; Jonkers et al., 2010). In the specific case of CBZ, the available literature data is restricted to the works published by Madureira et al. (2009) and Madureira et al. (2010) which reported CBZ contamination levels around 178 ng L^{-1} in Douro River (Northern Portugal) and also indicated CBZ as a persistent and ubiquitous compound. Taking into consideration the lack of investigation in this field in Portugal, this study also aims at presenting some results regarding the evaluation of CBZ contamination levels in ground, surface and wastewaters.

The methodology applied in this research, based on a commercial monoclonal antibody, was previously developed and validated by LC–MS/MS (Bahlmann et al., 2009). Taking into consideration the nature of the collected samples, the performance of the assay in the presence of high concentrations of organic matter and high salinity has now been evaluated in the study presented here.

2. Experimental

2.1. Reagents

The polyclonal antibody against mouse (IgG F(c) domain, from goat, lot 20 185) and the anti-CBZ monoclonal antibody (mouse IgG1, clone B3212M, lot 5 K32007) were purchased from Acris Antibodies (Germany) and BIODESIGN International (Meridian Life Science Inc., USA), respectively. The tracer was previously produced and characterized as described in Bahlmann et al. (2009). 3,3′,5,5′-Tetramethylbenzidine (TMB, puriss.) and tetrabutylammonium borohydride (TBABH, >97%) were purchased from Fluka. Sodium phosphate dibasic dihydrate (>99%), sodium phosphate monobasic dihydrate (>99%), potassium sorbate (>99%), potassium dihydrogen citrate (>99%), hydrogen peroxide (30%) and Tween™ 20 were also from Fluka. Ethylenediaminetetraacetic acid disodium salt dihydrate (p.a.), and sodium chloride (99.5%) were from Panreac. Dimethylacetamide (DMA), tris(hydroxymethyl) aminomethane (TRIS, p.a.) and bovine serum albumin (for electrophoresis, 98%) were purchased from Sigma. Commercial humic acids (technical) were also obtained from Sigma. Glycine (99.8%) was purchased from VWR Prolabo and sodium hydroxide (>98.0%) was obtained from José Manuel Gomes dos Santos (Portugal). CBZ (99%) and cetirizine dihydrochloride (p.a.) were purchased from Sigma.

Ultra-pure water, used in the preparation of all the solutions, was obtained using a Millipore water purification system (Milli-Q plus 185).

2.2. Materials

Transparent 96 flat-bottom well microtiter plates with high binding capacity (MaxiSorp™) were purchased from Nunc (Thermo Scientific). Membrane filters (pore size 0.22 μm, Millex-GV) and paper filters (pore size 0.45 μm) were from Millipore. A Titramax 100 plate shaker (Heidolph, Germany) and an automatic 8-channel plate washer (Biochrom, ASYS Atlantis) were also used. Optical density was read using a microplate spectrophotometer (Biochrom, ASYS UVM340).

The LC–MS/MS experiments were performed on an Agilent 1100 LC system consisting of a degasser, binary pump, autosampler and column heater. The outlet of the column was coupled to an API 4000 mass spectrometer from Applied Biosystems. A Turbo V™ ion source was used in electrospray positive mode.

2.3. Sample collection and preparation

Water samples (250 mL) were collected in and around Aveiro (NW Portugal).

The geographical distribution of the samples is shown in Fig. 1. Ground water samples were collected from four wells (W1–W4), two mines (M1, M2) and one riverine bank filtration system on Vouga river (BF), mainly located in rural areas, which are currently used for the water supplies of the municipal drinking-water system. Also, 11 surface water samples (S1–S11) were collected from north to south of Ria de Aveiro, including samples from rural areas (S2, S3, S8), urban areas (S4–S7) and touristic coastal areas (S1, S9–S11). Wastewater samples were collected from the two main WWTPs of Aveiro (“North” and “South”) and three collection points were selected: after primary decantation, after secondary biological treatment and after secondary decantation (corresponding to the final treated effluent). Samples were collected during spring (between April and May 2010). Immediately after collection, all the samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore) and stored at −20 °C until analysis. Samples were not subjected to any other cleaning procedure, extraction or enrichment process.

2.4. Immunoassay procedure

The CBZ ELISA procedure applied in this study was previously developed. Detailed information about tracer characterization and assay optimization is presented in Bahlmann et al. (2009).

Briefly, high binding capacity microtiter plates were first coated with polyclonal antibody (anti-mouse IgG, 1 mg L^{-1}, 200 μL per well), covered with Parafilm® to prevent evaporation and incubated overnight (approximately 16–18 h) on a plate shaker at 750 rpm. The antibody dilution was prepared in PBS (10 mM sodium dihydrogen phosphate, 70 mM sodium hydrogen phosphate and 145 mM sodium chloride, pH 7.6). The plates were then washed thrice with PBS 0.05% Tween™ 20 using an 8-channel plate washer. Subsequently, monoclonal antibody against CBZ, also diluted in PBS (12.9 μg L^{-1}, 200 μL per well), was incubated for 1 h. After another three-cycle washing procedure, the tracer solution was added to the plates (177 pmol L^{-1} in sample buffer, 50 μL per well). Sample buffer consisted of 1 M glycine, 3 M sodium chloride and 2% (*w*/*v*) of ethylenediaminetetraacetic acid disodium salt dihydrate, pH 10.5. Variations in the sample buffer composition, introduced to study its influence in performance of the assays, are detailed in Sections 2.6 and 2.7. Then, CBZ standard solutions and samples (150 μL per well) were added; the plates were incubated for 30 min and submitted to a final three-cycle washing step. Afterwards, substrate solution (200 μL per well) was pipetted and incubated for another 30 min. The substrate solution consisted of 540 μL of TMB based-solution (41 mM TMB,

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