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Böger, Beatriz; Amaral, Bianca do; Estevão, Priscila Lagner da Silveira; Wagner, Ricardo; Peralta-Zamora, Patricio Guillermo; Gomes, Eliane Carneiro

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ARTICLES

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Determinação de carbamazepina e diazepam por SPE-HPLC-DAD nas águas do rio Belém em Curitiba-PR/ Brasil

Beatriz Böger ^{1 *} beatrizboger@gmail.com
Universidade Federal do Paraná, Brazil

Bianca do Amaral ² biancadoamaral_@hotmail.com
Universidade Federal do Paraná, Brazil

Priscila Lagner da Silveira Estevão ²
priscilalagner@hotmail.com
Universidade Federal do Paraná, Brazil

Ricardo Wagner ¹ sorw2003@yahoo.com.br
Universidade Federal do Paraná, Brazil

Patricio Guillermo Peralta-Zamora ² zamora@ufpr.br
Universidade Federal do Paraná, Brazil

Eliane Carneiro Gomes ¹ ecarneirogomes@gmail.com
Universidade Federal do Paraná, Brazil

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Abstract: This work sought to determine the two psychotropic drugs most commonly released by Psychosocial Care Centers (CAPS) into urban river waters (Belém sub-basin, Curitiba, PR, Brazil). A simple analytical method using SPE followed by a HPLC-DAD was developed and validated. Strata-X® cartridges were used to extract (carbamazepine) CZ and diazepam (DZ) from water and SPE conditions were optimized by 2³ factorial design. The validated method was specific for target compounds; correlation coefficients were above 0.9998, recovery between 85.8 and 98.4% and precision below 6.60% (RSD, n=3). This method was successfully applied to analyze river samples and pollution hotspots were identified. The CZ and DZ concentrations found ranged from 0.670 to 0.856 µg L⁻¹ and from LOQ to 0.763 µg L⁻¹, respectively, and confirmed that drug consumption is directly related to river pollution in the studied region.

Keywords: carbamazepine, diazepam, emerging pollutants.

Resumo: Este trabalho teve como objetivo determinar duas drogas psicotrópicas mais dispensadas pelos Centros de Atenção Psicossocial (CAPS) nas águas de um rio urbano (sub-bacia do rio Belém, Curitiba - PR, Brasil). Foi desenvolvido e validado um método analítico SPE seguido de HPLC-DAD. Os cartuchos Strata-X® foram utilizados para extrair carbamazepina (CZ) e diazepam (DZ) das águas e as condições de SPE foram otimizadas por um planejamento fatorial 2³. O método validado foi específico para compostos alvo. Os coeficientes de correlação foram superiores a 0,9998, a recuperação entre 85,8 e 98,4% e a precisão abaixo de 6,60% (RSD, n = 3). Este método foi aplicado com sucesso para analisar amostras de rios e pontos críticos de poluição foram reconhecidos. A CZ e DZ foram encontradas em concentrações variando de 0,670 a 0,856 µg L⁻¹ e de LOQ a 0,763 µg L⁻¹, respectivamente, confirmando que o consumo de drogas está diretamente relacionado à poluição do rio na região estudada.

Palavras-chave: carbamazepina, diazepam, poluentes emergentes.

1. INTRODUCTION

About 450 billion people worldwide have some mental or behavioral disorder, and this number is expected to grow significantly up until 2020 (Menken et al., 2000). Psychiatric drugs are of particular concern because of their toxicity, persistence and the increased consumption in the last decade (Stuart et al., 2012; Subedi and Kannan, 2015; FENAFAR, 2015). Diazepam (DZ) is a psychotropic medication of the benzodiazepine class that are selective central nervous system modifiers and can be classified as anxiolytic. They are the most commonly used in the world (Sebastião and Pelá, 2004). About 1-3% of the Western population has already consumed diazepam regularly for over a year and in Brazil about 10% of adults have been prescribed these drugs (Huf et al., 2000; CREMESP, 2002). Carbamazepine (CZ) is a controlled drug in Brazil and is considered a mood stabilizer (Rang et al., 2003). In 2008, carbamazepine, with anticonvulsant action, was among the five active ingredients most consumed in Brazil (Mota, 2011). Carbamazepine has been detected in the surface of drinking water in more than 29 countries (Ebert et al., 2014). Diazepam was found in 8 of 20 wastewater treatment plants at relatively low concentrations ($< 0.04 \text{ mg L}^{-1}$) with a high frequency of detection in these treatment plants (Cabeza, 2012).

Although the present knowledge in the occurrence and behavior of these compounds in rivers is well documented in the world (Li, 2014; Pal et al., 2013; Patrolecco et al., 2013; Camacho-Muñoz et al., 2009; Madureira et al., 2010), in Brazil it is still very limited (Montagner and Jardim, 2011; Campanha et al., 2015; Almeida et al., 2013). Only eight quantification studies of emerging pollutants of pharmaceutical origin were performed between 2000-2015 in Brazil. Among these studies, only three psychoactive drugs were investigated in aqueous matrices. Also, the southern region has only one study on hospital sewage and none on rivers (Böger et al., 2015).

Pharmaceuticals are released into the environment through excretions via feces and urine in a conjugated or unmetabolized way, and through disposal of outdated medicines in household sewage and effluents of wastewater treatment plants (WWTPs) (Subedi and Kannan, 2015; Alygizakis et al., 2016). While the toxic effects related to the disposition of these compounds are not fully known, recent studies demonstrate some interference in the metabolism and behavior of aquatic organisms (Fent et al., 2006; Morley, 2009), crossing all biological hierarchy, from cells and organs, and even ecosystems (Jorgensen and Halling-Sorensen, 2000). A recent study showed that exposure of fish and benthic invertebrates to psychoactive drugs altered their behavioral responses (Brodin et al., 2014; Rosi-Marshall et al., 2015).

Due to the matrix complexity and the low concentration of the target analytes, the direct analysis of these drugs is not feasible. Also, sample pretreatment is necessary to eliminate the interferences and to achieve desirable limits of detection and quantification. Solid phase extraction (SPE) has been applied to trace analysis to obtain significant

preconcentration factors, with the use of smaller volumes of solvent (Madureira et al., 2010; Huntscha et al., 2012; Amaral et al., 2014).

Recently, the emerging organic pollutants have been investigated worldwide; nevertheless, there is no legislation that considers pharmaceuticals as micropollutants. The analytical techniques for medicine quantification and identification are well-established in governmental agencies such as ANVISA in Brazil. Despite this, conventional methodologies are used for raw material and commercial product quality (Anvisa, 2003). Hence, the development of rapid, cheap, sensitive and accurate methods for monitoring pharmaceuticals at trace level have represented an analytical challenge in the last years. Further, the simultaneous determination of compounds with different chemical nature, from various therapeutic classes (Gros et al., 2006; Gomez et al., 2007) and also track pharmaceuticals in highly polluted aquatic environments, decreased time, and reduced overall cost is still a challenge. Most of these new methodologies are based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) due to its high sensitivity and ability to confirm the compound's identity; however, the application of this sophisticated and expensive technology is not yet available in all laboratories. Taking into account the previously mentioned concerns, the purpose of this work was to develop an analytical method based on a single and efficient preconcentration procedure based on solid-phase extraction (SPE) followed by high-performance liquid chromatography with diode array detection (HPLC-DAD) analysis, demonstrating that this methodology is very useful for detecting levels of carbamazepine and diazepam in polluted water samples. These analytes were chosen based on survey of the most prescribed drug by Psychosocial Care Center (CAPS) in Curitiba, Paraná- BR performed in this work.

2. MATERIALS AND METHODS

2.1. Chemicals and materials

Carbamazepine (CZ, 5*H*-Dibenz[*b,f*]azepine-5-carboxamide) and Diazepam (DZ, 7-Chloro-1-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2(1*H*)-one) were purchased from USP (Rockville, MD, USA).

All the other solvents used were HPLC grade and supplied by J. T. Baker (Philipsburg, NJ, USA). Ultrapure water was obtained using Milli-Q system coupled with a UV lamp (18.2 M(cm, Bedford, MA, USA). 0.45- μm glass fiber filters were purchased from Millipore (Macherey Nagel). Stock solutions for individual standards (1000 mg L⁻¹) were prepared in methanol and stored in the dark at -6°C. Stock solutions were stable, and no evidence of degradation of the analytes was observed on the chromatograms during the six-month study period. Working solutions were prepared daily by diluting the stock solution with a suitable solvent.

The pH of the solutions was measured by OHAUS Starter 2100 pH-meter.

2.2. Sample collection

The river studied was chosen according to the geographical location of the CAPS evaluated. These two CAPS are located on the watershed of Belém. Therefore, the river selected for the study was the sub-basin of Belém River (tributary of the Iguaçu River, which is one of the basins used for water supply in other cities in Paraná), considered a contaminated hotspot. Several samples were collected at three distinct areas of the Belém River: two considered highly polluted since most waste generated in this region is not suitably treated and is directly discharged into the Belém River (the mouth and about 7 km from the headspring of the river) and the other with low levels of pollution as it was near the headspring. The samples were fortified with $2.56 \mu\text{g L}^{-1}$ of DZ and $2.26 \mu\text{g L}^{-1}$ of CZ to monitor retention time (T_R) of drug. The water samples were collected during May, June, July and October. Two liters of river water samples were collected into 2.5 L pre-rinsed amber glass bottles. Upon collection, all samples were immediately transported at 4°C to the laboratory and vacuum-filtered through a $0.45\text{-}\mu\text{m}$ glass fiber filter with a 47 mm diameter (Millipore). The pH of filtered samples was adjusted to 4.00. Each sample was divided into different volumetric flasks, then stored in darkness at 4°C and extracted within a maximum of 24 h after collection.

2.3. Solid-phase extraction

SPE was performed on a PrepSep 20-port vacuum manifold (Waters, Milford, MA, USA) with Strata-X[®] cartridges (Phenomenex, 200 mg/3 mL). The SPE operational variables such as conditioning and elution solvent, analytes concentration and sample volume, were optimized by a two-level factorial design (2^3 with triplicate of center point) according to Table 1. The assays were carried out in a randomized mode. The response evaluated was the recovery of psychoactive drugs.

In the optimized SPE procedure, cartridges were conditioned with two aliquots of acetonitrile (2.50 mL) and two aliquots of ultrapure water pH 4 (2.50 mL) before each run. Sequentially, water samples at 4.00 pH were percolated through the cartridges at a constant flow rate of 3 mL min^{-1} . Afterward, elution was performed with 5.00 mL of acetonitrile. The extracts were evaporated to dryness in a thermostatic bath at 30°C under a gentle nitrogen stream. The residues were dissolved in 1.00 mL of water, and 15 μL was injected into the HPLC system. The pH and reconstitution volume were evaluated separately.

Table 1.
Factors and levels analyzed in two-level full factorial design.

Factor	Level		
	-1	0	+1
Concentration	1.28 $\mu\text{g L}^{-1}$ DZ	2.56 $\mu\text{g L}^{-1}$ DZ	3.84 $\mu\text{g L}^{-1}$ DZ
	1.13 $\mu\text{g L}^{-1}$ CZ	2.26 $\mu\text{g L}^{-1}$ CZ	3.39 $\mu\text{g L}^{-1}$ CZ
Sample volume	100 mL	200 mL	300 mL
Conditioning/elution solvent	Acetonitrila (ACN)	ACN:MetOH (1:1)	Metanol (MetOH)

2.4. HPLC-DAD analysis

Chromatographic analyses were performed with the Agilent 1260 Infinity Quaternary LC system (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, quaternary gradient pump, and diode array detector (DAD) system. Separation was achieved in a 100 mm \times 2.1 mm, 5 μm particle size Waters XBridge BEH C18 column. The data were collected with OpenLab EZChrom Elite software. The chromatographic conditions were optimized based on previous information in literature (INMETRO, 2010; Madureira et al., 2010). Hence, an isocratic elution mode was selected for simultaneous determination of carbamazepine and diazepam with methanol and ultrapure water (60:40 v/v) as mobile phase at a flow rate of 0.4 mL min^{-1} . Separations were performed at a 30°C. The following wavelengths were monitored: 254 nm (DZ) and 286 nm (CZ), which allowed a selective analysis with a suitable absorption of the studied compounds. The optimized chromatographic condition had 8.0 minutes of total run time.

2.5. Method validation

The method validation for the determination of pharmaceuticals in river water was performed with the following parameters: linearity, detection and quantification limits, precision and accuracy ($n=3$ for all assays) according to guidelines described by the National Agency of Health Surveillance (Agência Nacional de Vigilância Sanitária - ANVISA) (Anvisa, 2003). All procedures were performed with preconcentration step.

3. RESULTS AND DISCUSSION

3.1. Psychotropic drugs studied

This study aims to analyze the most commonly used psychotropic drugs in the Psychosocial Network, the Psychosocial Care Center (CAPS) in Curitiba, Paraná, BR, assisted by Unified Health System (SUS), which

consists of healthcare venues for people suffering from mental disorders and needs arising from the use of crack, alcohol, and other drugs.

A transverse quantitative analysis was performed to search for information in the Specific Registration Books of controlled drugs in CAPS for the years 2011 to 2014. There were 10,897 drugs dispensed for emergency use in mental disorders. The Brazilian Common Denomination (DCB), pharmaceutical forms and concentrations that are exempted from the following drug classes was considered: antidepressants, anxiolytics, antiepileptics, antipsychotics, anticholinergics, and hypnotic/sedative. Based on this survey, carbamazepine and diazepam were among the most prescribed antipsychotics (Diazepam 5 mg pill (11.86%) and Carbamazepine 200 mg (9.08%)). Hence, the both psychotropic drugs were selected for further investigation.

3.2. Solid phase extraction

Pre-concentration methods are essential to environmental analysis since the target analytes are at low concentrations (the trace levels - $\mu\text{g L}^{-1}$) (Amaral et al., 2014). Initially, the selection of pH included different values of 4.00, 6.50 and 8.00 ($n = 3$). Water acidified to pH 4.00 showed better recovery results to both analytes because CZ ensures the prevalence of the neutral form of compounds in the solution (around pK_a) and DZ is undissociated; however the best recoveries were obtained in acidic media compared with neutral and basic pH recoveries.

To determine the influence of conditioning and elution solvent, analytes concentration and sample volume on the preconcentration system, a two-level full factorial design, 2^3 with 8 runs was employed. Based on central point standard deviation (CZ: 0.38; DZ: 0.15), 3-way interactions were statistically significant (95% of confidence level) for both analytes. Hence, the factors can not be evaluated individually. As shown in Figure 1, this higher-order effect means there are several conditions which provide proper psychoactive drug recovery.

The optimized SPE conditions were in the low level for all variables (5.00 mL of ACN followed by 5.00 mL of ultrapure water pH 4.00 as conditioning solvents, 5.00 mL of ACN as an elution solvent, 100 mL of sample volume and $1.13 \mu\text{g L}^{-1}$ to CZ and $1.28 \mu\text{g L}^{-1}$ to DZ) and were employed in further experiments. Similar recoveries were estimated by Patrolecco et al. (2013) employing Strata X cartridge (80 to 93% to CZ) and by Madureira et al. (2010) using Oasis HLB cartridges (81 to 91% to CZ and 86 to 91% to DZ).

Reconstitution volume was also investigated. Recoveries of around 80% with RSD below 3.7% were obtained for CZ and DZ at 1.00 mL of ultrapure water. Meanwhile, volumes of 0.500 and 0.250 mL recovered less than 70% of both analytes. Moreover, an enrichment factor of 100 times was achieved.

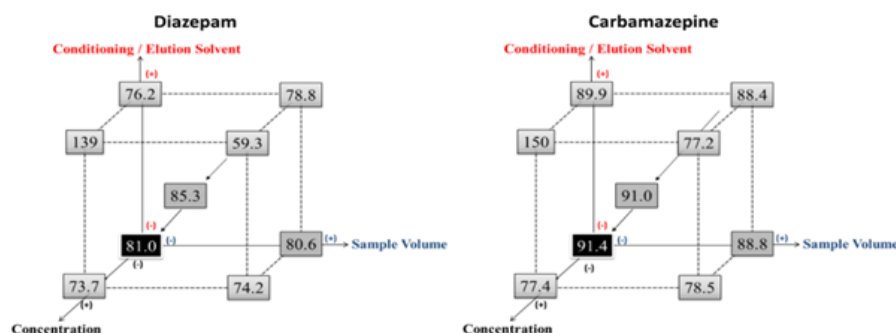


Figure 1.

Graphical representation of SPE optimization by 2^3 factorial design. Recovery observed in each assay appears in the boxes.

3.3. Method validation

Figure 2 shows the chromatograms obtained for the proposed method. Well-resolved peaks were observed, for which the retention times were 1.47 minutes and 3.07 minutes for CZ and DZ, respectively.

The linearity of the method was studied in ten different concentrations of analyte in triplicate in the range between 10.0 e 1500 $\mu\text{g L}^{-1}$. The proposed method showed good linear range between 20 - 1500 $\mu\text{g L}^{-1}$ to CZ and 40 - 1500 $\mu\text{g L}^{-1}$ to DZ, with excellent coefficients of determination ($R > 0.999$) for both analytes, as recommended by ANVISA (2003). Angular coefficients were used to evaluate method sensitivity, which showed slightly more sensitivity to carbamazepine (Figure 2C).

Selectivity was observed by the association of the T_R and DAD spectra, standards in a pure solvent and standards in matrix ensures that the signal measured is not influenced by matrix interferences. This observation guarantees that the method is selective for the pharmaceuticals and can be used for monitoring purposes in river water samples. The RSD values were obtained as a result of a precision estimation of the t_R between the standard in solvent solutions, and spiked matrix were below 2.45% ($n = 18$). The difference in the baseline shift observed at the beginning of chromatograms is entirely related to the absorption of humic substances commonly present in river water samples, which due to its high conjugated system caused this characteristic band (Moffat et al. 2004). However, in our study, this fact did not interfere with the determination of the studied compounds.

The instrumental precision was estimated by repeatability and intermediate precision assays and was expressed as percent relative standard deviation (%RSD) of replicate measurements using the peak areas for calculation. Table 2 summarizes the precision results from replicate measurements at three different concentration levels (50.0 $\mu\text{g L}^{-1}$, 500 $\mu\text{g L}^{-1}$, and 1000 $\mu\text{g L}^{-1}$, $n=3$) for each studied psychoactive compound.

Repeatability of replicate measurements was satisfactory, with RSD values below 6.06% and 3.64% for CZ and DZ, respectively. The intermediate precision showed RSD values below 3.87% for CZ and 6.61% for DZ (Table 2). The Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (INMETRO, 2010) determines a maximum RSD value of up to 20%. ANVISA (2003) recommends that the coefficients of variation should not exceed 5% for the determination of drugs in medicine and more complex matrices, such as serum, blood or plasma, but accepts a value of up to 15%. Hence, the method's precision was satisfactory within the maximum value permitted by applicable Brazilian legislation.

Table 2.
Repeatability, intermediate precision RSD (%) and recovery values (%) at three different concentration levels.

	Concentration levels ($\mu\text{g L}^{-1}$)					
	50.0 (n=3)		500 (n=3)		1000 (n=3)	
	CZ	DZ	CZ	DZ	CZ	DZ
Repeatability (%)	6.06	1.34	0.640	3.64	0.420	2.15
Intermediate precision (%)	3.87	6.61	0.820	0.240	1.56	0.210
Recovery \pm RSD (%)	98.3 \pm 4.46	98.4 \pm 5.32	89.7 \pm 2.25	92.7 \pm 10.7	85.8 \pm 3.86	89.7 \pm 9.07

Accuracy was established based on CZ and DZ recovery, which was performed by extracting and analyzing triplicate ultrapure water samples spiked with analytes ($50.0 \mu\text{g L}^{-1}$, $500 \mu\text{g L}^{-1}$ and $1000 \mu\text{g L}^{-1}$). The results of recovery experiments are reported in Table 2, which shows that the method is accurate within the desired recovery range. Similar recoveries were found in real samples (Patrolecco et al., 2013, Madureira et al., 2010)

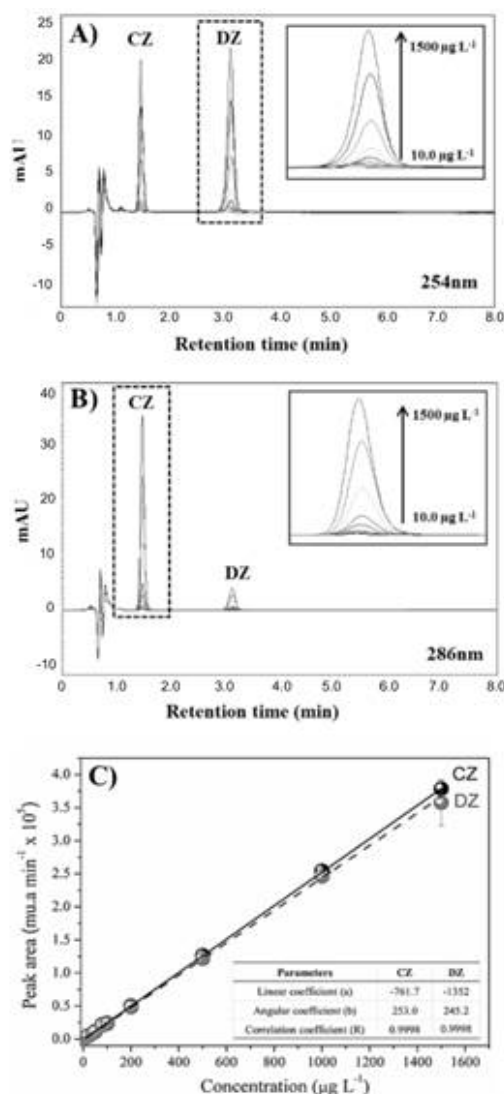


Figure 2.

HPLC-DAD chromatograms of a standard mixture of the psychoactive at 10 to 1500 $\mu\text{g L}^{-1}$ performed under the optimized conditions: A) DZ and B) CZ. C) Analytical curves for CZ at 286 nm and DZ at 254 nm.

Limits of detection and quantification of the developed method were estimated by the relation of the intercept standard deviation (s) and the value of the calibration curve slope (S), according to Equations 1 and 2:

$$(1)$$

$$(2)$$

The LODs and LOQs were 0.0670 $\mu\text{g L}^{-1}$ and 0.209 $\mu\text{g L}^{-1}$ to CZ and 0.130 $\mu\text{g L}^{-1}$ and 0.435 $\mu\text{g L}^{-1}$ to DZ. These results were close to those described in the literature (Madureira et al. 2010; Ebert et al. 2014).

The LOD and LOQ obtained in this work were lower than Madureira et al. (2010) have found with the SPE method (LOD and LOQ to CZ of 3.8 and 15 $\mu\text{g L}^{-1}$, respectively, and to DZ 10.3 and 40 $\mu\text{g L}^{-1}$, respectively). Ebert et al. (2014) related that the global mean concentration in surface water to CZ is between 0.187 and 8.05 $\mu\text{g L}^{-1}$. Based on that, the proposed method would be able to detect CZ in concentrations lower than this minimum limit.

The evaluation of robustness was made by analyses of the influence of variations in column temperature at 20°C, 25°C and 30°C, respectively, and in flow rates of 0.3, 0.4 and 0.5 mL min⁻¹. This data allowed us to correlate each area of peak to the corresponding chromatographic retention time. For robustness evaluation, small changes in flow rate ($\pm 10\%$) and column temperature ($\pm 5^\circ\text{C}$) did not make significant changes to resolution or recovery.

3.4. Psychoactive pharmaceuticals in the Belém River water

To demonstrate the applicability of the developed method, three sampling locations in the Belém River were selected according to the level of pollution in each area (low, medium and high pollution). Water samples were collected in May, June, July and October 2015 (Table 3).

The mouth of the river is the most-polluted site. It is fully inserted in the city of Curitiba; there are many irregular houses on its margins. Many of these houses are not connected to the city's sewage network, thus discarding their sewage into the river.

Table 3.

The average concentration of residues found in different locations in the river in each collection.

Locations	Analytes	Average concentration ($\mu\text{g L}^{-1}$)			
		May	June	July	October
Headspring	CZ	<LOQs	<LOQs	0.371	nd*
	DZ	<LOQs	nd*	nd*	nd*
Middle of river	CZ	<LOQs	<LOQs	<LOQs	nd*
	DZ	nd*	nd*	nd*	nd*
Mouth	CZ	nd*	0.856	<LOQs	nd*
	DZ	nd*	0.763	nd*	nd*

*nd: not detected.

Carbamazepine was found higher than LOQ in headspring (July-CZ = 0.371 $\mu\text{g L}^{-1}$) and the both at the Belém River mouth (June-CZ = 0.856 $\mu\text{g L}^{-1}$, DZ = 0.763 $\mu\text{g L}^{-1}$) in the second and third collections, which are consistent with the results that similar work reported (Ebert et al., 2014, Patrolecco et al., 2013). These collections were performed during a dry period (winter), which increases the analytes concentration and lowers

degradation rates. Further, during the spring (October), the increase of rain volume and warm weather (INMET, 2015) promotes the dilution of analytes and increases degradation rates. Therefore, CZ and DZ were not detected at any of the collection points. The highest frequency of CZ detection is indicative of inadequate sewage disposal along the river localized in the region that this drug is widely distributed by CAPS, as early mentioned.

CZ were found in the Tiber River (Italy) at the concentration level of $0.063 \mu\text{g L}^{-1}$ (Patrolecco et al., 2013). Madureira et al. (2010) found CZ at $0.0327 \mu\text{g L}^{-1}$ and did not detect DZ in the Douro River estuary (Portugal). Campanha et al. (2015) found CZ in 71% of analyzed samples between 0.50 and $0.215 \mu\text{g L}^{-1}$ in the Monjolinho River, São Carlos, SP. According Clara et al. (2004), CZ could be considered a qualified parameter for detecting wastewater in aquatic environments due to its high persistence.

Carbamazepine and diazepam were found to be ubiquitous (present in 58.3% of samples collected) and persistent in river water. Here again, a strong correlation was observed between the amount of pharmaceuticals dispensed in the CAPS and their excretion as an unchanged drug and their concentration levels in the surface water (the amount of CZ that would go to the Belém River would be 19.7 g - estimating that 10% is excreted as unchanged drug, and the amount of DZ would be 0.194 g - calculating that 3% is excreted as unchanged drug) (Moffat et al., 2004). CAPS provide a regionalized service, implying the delineation of specific geographical areas. These two CAPS studied delimited the region on the sub-basin of the Belém River, where houses not connected to the sewage system properly end up discarding their waste into rivers or streams of this subbasin.

Despite diazepam being the most commonly dispensed medication in the community between 2011-2014, carbamazepine was found at much higher concentrations in both the mouth and headspring of the river than diazepam (a maximum concentration of $0.763 \mu\text{g L}^{-1}$ determined in the mouth of the river during dry weather conditions). This can be explained by the fact that carbamazepine is excreted by the human body in approximately 10% unchanged form, while diazepam is excreted approximately 3% as an unchanged compound. Therefore, CZ environmental concentrations are much higher. Other similar observations about carbamazepine are reported (Glassmeyer et al., 2009; Subedi and Kannan, 2015). To date, diazepam has not been extensively reported.

Because of the importance of these substances to public health, according to the ecotoxicological point of view (Alonso et al., 2010; Brodin et al., 2014; Petersen et al., 2014; Rosi-Marshall et al., 2015), it is essential to ascertain their occurrence in surface water systems, even though it is not contemplated in Brazilian environmental legislation. Further studies of this are extremely necessary to support the establishment of legal parameters of drug residues in aqueous matrices.

The proposed HPLC-DAD method showed specificity, accuracy and sensitivity, good linearity for both analytes ($R > 0.9998$) and LOD and LOQ were found to be suitable for environmental analysis with a preconcentration step (micrograms per liter or parts per billion). This paper provided a useful and expressive guidance on HPLC-DAD that is in accordance with validation requirements for pharmaceutical analysis in water samples. To our knowledge, this is the first study reporting the method validation of the psychoactive drugs by SPE-HPLC-DAD for river water, especially in developing countries such as Brazil.

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Author notes

* Corresponding author: Beatriz Böger, e-mail: beatrizboger@gmail.com