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Development and Validation of an Analytical Method for the Detection and Quantification of Bromazepam, Clonazepam and Diazepam by UPLC-MS/MS in Surface Water

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Abstract
The development of analytical methods capable of determining micropollutants is essential for quality control of drinking water. Benzodiazepines, a class of pharmaceuticals with anxiolytic properties, have received increasing attention as micropollutants. The purpose of this study was to develop an analytical method for determination of three benzodiazepine drugs (bromazepam, clonazepam and diazepam) in surface water. For the extraction of the matrix analytes, SPE cartridges (C18, 500 mg/3 mL) were used. The method was validated according to the quality criteria of the USEPA 8000D Validation Guide. The developed and validated method showed recovery values between 57 and 100%, RSD < 20% and $R^2 > 0.9949$. LD ranged between 2.70 and 5.00 ng L^{-1} for bromazepam and clonazepam respectively whereas LQ was 0.01 µg L^{-1} for all analytes. The matrix affected the signal intensity of clonazepam thus evidencing the matrix effect by analysis statistic (F test).

Keywords PPCPs · Micropollutants · Surface waters · Benzodiazepines · UPLC-MS/MS

Large number of organic and inorganic contaminants are continuously discharged into receiving water bodies along with municipal and industrial effluents. Among these, pharmaceuticals and personal care products (PPCP) end up in water bodies, posing risks to aquatic ecosystems (Luo et al. 2014; Arbeláez et al. 2015; de Almeida et al. 2015; Stipaničev et al. 2017). Given the growing concern about the presence, fate and potential effects on ecosystems and human health, and the knowledge that such contaminants are inefficiently removed in water treatment and sewage treatment systems (Matsuo et al. 2011; Silveira et al. 2013; Tran et al. 2013; Dai et al. 2014; Caldas et al. 2016; Mann et al. 2016; Valls-Cantenys et al. 2016), it is necessary to develop analytical methods capable of detecting and quantifying these contaminants.

According to the International Narcotics Control Board (INCB 2015), the most representative benzodiazepine drugs consumed worldwide were those with anxiolytic and antiepileptic effects. Diazepam (DZP), bromazepam (BZP), and clonazepam (CZP) are found among these drugs and also among the less investigated ones in surface water (Cunha et al. 2017), regardless the increase in their production (INCB 2015) and their persistence in conventional biological treatment systems (Cunha et al. 2017).

In order to achieve an analytical method with low limit of detection (LD) and limit of quantification (LQ) for psychoactive drugs – particularly benzodiazepines – in surface water, different methods have been applied, as reported in an extensive literature survey (Cunha et al. 2017). The solid phase extraction followed by liquid chromatography coupled to mass spectrometry detection is the most common applied strategy. However, aspects concering matrix effects and possible interferents in polluted waters, to the best of our knowledge, has not been addressed.

The Guandu river basin is an important water supplier for the metropolitan region of Rio de Janeiro and Rio de

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Janeiro city in Brazil, including approx. 9 million inhabitants and several economic sectors (Cetesb 2011). Due to the high demographic density, the presence of an important industrial park in the basin and failure in pollution control, the river is exposed to the discharge of untreated or partially treated urban sewage and industrial effluents, besides the runoff from agricultural areas. Additionally, the presence of benzodiazepines in this river has been previously reported (Ferreira 2014).

The objective of this investigation was to develop and validate a robust analytical method using liquid chromatography coupled to mass spectrometry to detect three widely used psychoactive drugs – BZP, CZP and DZP – the most frequently consumed in Brazil. Investigations on possible interferents and matrix effects caused by pollution was included in our goals and, for this reason, the Guandu river – RJ, subject to intensive discharge of insufficiently treated or untreated domestic and industrial wastewater was taken as a challenging case study.

Materials and Methods

The reference substances BZP, CZP and DZP, with purity (> 99%) were purchased from Brazilian Pharmacopeia (Brazil). The internal standard (IS) DZP-d^5 was purchased from Sigma-Aldrich (Brazil). Methanol, acetonitrile (LC–MS grade) and ammonium hydroxide (28.0%–30.0%) were purchased from J.T. Baker® (Brazil), Milli-Q water (ultra-pure) was obtained from a Milli-Q Direct 8 (Millipore®).

BZP, CZP and DZP (1000 µg mL^{-1}) and DZP-d^5 (10.0 µg mL^{-1}) stock solutions were prepared in methanol and stored at − 4°C. The individual stock solutions were used to prepare mix stock solution of 10.0 µg mL^{-1} and the working mix solution of 100.0 µg L^{-1}, by dilution of the stock solutions in methanol. The working mix solutions for spiking were stored at 4°C, for a period of 1 month.

Solid phase extraction (SPE) cartridges were used to promote the clean-up and pre-concentration of the analytes in the preparation of real samples. Extraction parameters were optimized for the best recovery of the analytes using OASIS HLB and Bond Elut C18 cartridges, elution with 4 and 5 mL of methanol, in one or two steps: 1 × 5 mL and 2 × 2.5 mL. The final protocol included the use of Bond Elut C18 (500 mg/3 mL), SPE cartridges, previously conditioned with 5 mL of methanol, followed by 5 mL of water, at a flow rate of 1 mL min^{-1}. After the conditioning step, 500 mL of the sample was percolated along the cartridge at a flow rate of 3 mL min^{-1}. After sample application, the analytes were eluted with two steps of 2.5 mL methanol at a flow rate of 1 mL min^{-1}.

Analysis were performed by ultraperformance liquid chromatography (Waters Acquity UPLC) couple to triple quadrupole mass spectrometry (MS/MS) Waters Xevo TQD. Liquid chromatographic separations were performed using a BEH C18 column (50 × 2.1 mm^2, 1.7 µm) set at 50°C. The mobile phase consisted of eluent A (water containing 0.01% ammonium hydroxide) and eluent B (methanol containing 0.01% ammonium hydroxide), with gradient mode: the content of eluent B increased from 2% to 99% of in 5 min, returning to the initial composition in 3 min. The injection volume was 5.0 µL. Nitrogen is used as the nebulizer gas and argon as the collision gas. Table 1 shows ionization forms, cone voltage and collision energy for the compounds. The mass spectrometer was operated in the MRM mode, selecting two transitions for each compound, one for quantitation and the other to confirmation.

All validation experiments were performed according to United States Environmental Protection Agency (USEPA) 8000D guidelines (USEPA 2014). The selectivity of the developed method was determined by analyzing a non-spiked matrix (surface water) and spike matrix (surface water containing analytes and IS). The precision and accuracy of the analytical method were determined by analyzing sets of 5 replicates of each of the three concentrations of spiked samples using matrix (surface water) as solvent. Surface water samples were collected in four different sites along the Guandu river: Paracambi (− 22.663144; − 43.742502), Seropédica (− 22.806417; − 43.626079), Nova Iguaçu (− 22.817486; − 43.624333) and Santa Cruz (− 22.897108; − 43.734804) and a composite sample was prepared by mixing. The composite sample was adopted in order to gather all possible interferents and matrix effect that we should find in real samples collected at these different locations. The site in Paracambi is described as a rural area, with low population density; Santa Cruz is an industrial area; Nova Iguaçu and Seropédica have high population density exposed mostly to discharge of untreated domestic wastewater.

Table 1 Characteristic of the mass spectrometer

Analyte	Ionization	m/z	Cone (V)	Product ion	Collision energy (V)
BZP	Positive	316	50	261[a]	26
				209[b]	25
CZP	Positive	316	55	270[a]	35
				214[b]	32
DZP	Positive	285	55	193[a]	32
				154[b]	26
DZP-d^5	Positive	290	60	198[a]	30
				159[b]	30

[a]Quantification ion

[b]Confirmation ion

Prior to fortification, the composite sample was filtered on glass fiber filter, with pore size of 1.0 µm. Spiked matrix were prepared by fortification of the filtered sample at concentration levels of 0.01 µg L^{-1} (low), 0.25 µg L^{-1} (medium) and 0.50 µg L^{-1} (high) in two days (intra-day 1 and 2). In all solutions, IS was added to final concentration of 0.25 µg $L^{-1.}$

The precision of the method was evaluated in terms of the relative standard deviation (RSD). Accuracy was calculated by comparing the measured concentration with the nominal concentration as the mean recovery percent (%). The limit of quantification (LQ) was defined as the lowest intensity point of the analytes that can be accurately quantified (RSD < 20%) and accuracy (50%–120%). Limit of detection (LD) were determined by multiplying the Student's *t* value appropriate for 99% confidence level and a standard deviation estimate, with (n − 1) degrees of freedom, of the analyte concentrations quantified at the lowest concentration level. Five-point calibration curves were obtained by passing the entire extraction and clean-up method (SPE) on spiked matrix at the following concentration levels: 0.01; 0.05; 0.10; 0.25 and 0.50 µg L^{-1}. Five different standard solutions at each concentration level were injected and used for the validation protocol. For comparison, five-point calibration curves prepared in ultra-pure water, in the same concentration range, were also analyzed. In all solutions, IS was added to final concentration of 0.25 µg L^{-1}.

The matrix effect (ME) is related to the degree of suppression/increase of the ion intensities. Such behavior may vary from sample to sample, from compound to compound, and may also depend on the concentration of the analyte, as well as the concentration ratio between the matrix and the analyte. Standard deviations of the calibration factors (mean ratio between the intensity of the signal of the analyte relative to the intensity of the signal of the IS and the concentration of the analyte) were calculated for the analytical curves prepared in ultra-pure water and in the spiked matrix. F test was then applied to evaluate the ME, assuming a confidence limit of 95% (24 degrees of freedom).

Results and Discussion

The selectivity of the method was established by the analysis of non-spiked and spiked matrix at 10.0 µg L^{-1}. The chromatograms of the injections (Fig. 1) were evaluated and the absence of signal in the chromatogram of the blank injection was verified, at the same retention time of the analytes observed in the injection of the spiked matrix.

It is worth noting, in Fig. 1, that the test intensities range from 1.13×10^3 to 2.88×10^3 (for the IS and DZP, respectively, in the non-spiked matrix) and from 1.16×10^6 to 5.37×10^6 (for the IS and DZP, respectively, in the spiked matrix). Thus, the matrix does not show any signal at the retention times of each analyte. The developed method is therefore capable of detecting and/or quantifying each analyte.

Recoveries and relative standard deviations were determined for each analyte, meeting the criteria of validation: RSD < 20% and accuracy between 50% and 20% (USEPA 2014). Inter-day recoveries for BZP were found between 90.25% (0.5 µg L^{-1}, RSD = 5.50%) and 96.75% (0.25 µg L^{-1}, RSD = 8.30%). For CZP, recoveries ranged from 55.14% (0.25 µg L^{-1}, RSD = 6.80%) to 57.68% (0.50 µg L^{-1}, RSD = 5.30%). DZP showed recoveries ranging from 89.13 (0.50 µg L^{-1}, RSD = 7.10%) to 93.23 (0.01 µg L^{-1}, RSD = 11.60%).

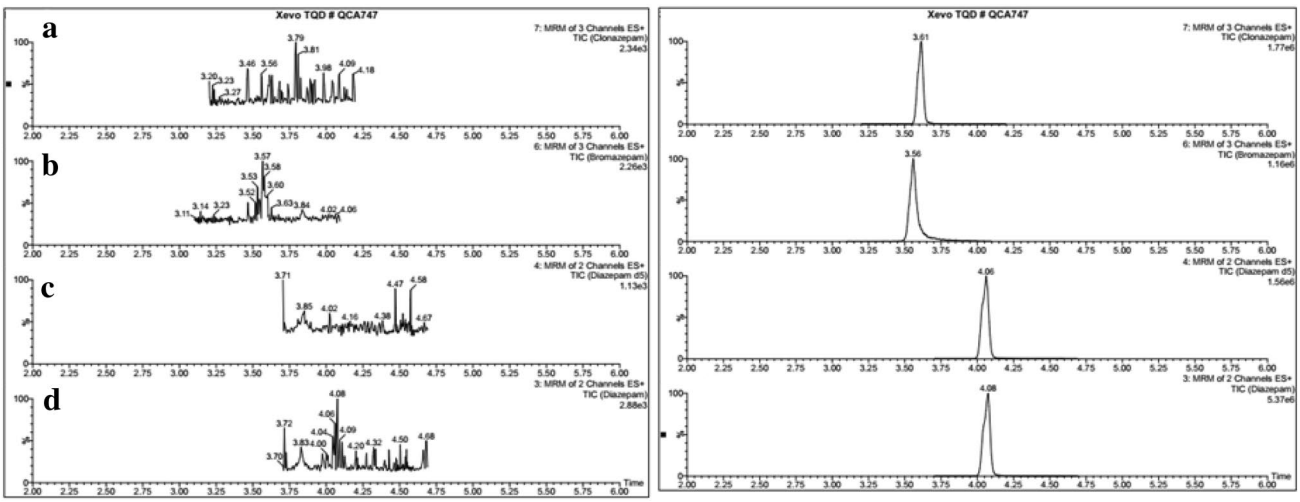

Fig. 1 Left: non-spiked matrix (only surface water). Right: spiked matrix (surface water + Analytes + IS). (*a*) CZP; (*b*) BZP; (*c*) DZP-d^5; (*d*) DZP

The LD is defined as the minimum concentration of a substance that can be detected with 99% confidence when the analyte concentration is greater than zero (USEPA, 2014). In this work, the LD of the analytes varied between 2.70 and 5.00 ng L^{-1} for BZP and CZP respectively. The LQ attained by the procedure was 0.01 µg L^{-1} according to the criteria of validation (RSD < 20% and accuracy between 50 and 120%). Since they are emerging contaminants, maximum contaminant levels (MCL) for benzodiazepines have not been established yet. Therefore, low LQ values are needed. LQ and LD values reported in the present study are similar to previously reported values, considering the SPE-LC–MS/MS applications (Andreu et al. 2016; Aymerich et al. 2016; Brieudes et al. 2016; García-Galán et al. 2016).

Analytical curves were prepared in surface water and ultra-pure water, at concentrations levels ranging from 0.01 to 0.50 µg L^{-1}. Results are shown in Table 2. Determination coefficients (R^2) range from 0.9949 to 0.9991, indicating excellent linearity for all compounds.

Matrix effects (ME) may severely influence sensitivity, linearity, accuracy and precision of quantitative LC–MS/MS determinations (Postigo et al. 2008; Gros and Petrovic 2009; Gros et al. 2012). A comparison between the intensities of the signals of the analytes obtained from analysis of the 0.01 µg L^{-1} standard solutions prepared in ultra-pure water and in the spiked matrix reveals that the ion intensities increase for CZP and DZP, but decrease, for BZP. Moreover, F tests reveal that, for all analytes, the variances of the ion intensities obtained for the standards in ultra-pure water and in the spiked matrix are not significantly equal, assuming a confidence interval of 95%. Therefore, ME is observed for all analytes. Benzodiazepines such as DZP, BZP and CZP are subject to ME, since the pH of the sample (and other components of the matrix) can change their protonation degree (Bonfiglio et al. 1999) and their interactions in the SPE.

Similar statistical analysis was performed for the calibration factors, for all analytes, considering the analytical curves obtained from the standards prepared in ultra-pure water and in the spiked matrix. Calculated F values (Fcalc) are shown in Table 2. For CZP, the calculated F value is higher than the critical F (24 degrees of freedom and confidence interval of 95%). The analytical curves are not equal and the matrix significatively influences the determination of this analyte. Therefore, it is imperative to adopt an analytical curve prepared in spiked matrix, for the determination of CZP, or (by suggestion) the standard addition method should be adopted. For BZP and DZP, the calculated F values are lower than the critical F, suggesting that the analytical curves in ultra-pure water and in spiked matrix are significantly equivalent. Nevertheless, due to the observed ME, quantification of these analytes from matrix spiked analytical curves is also suggested.

In order to test this proposed method, four samples were collected at Paracambi (− 22.663144; − 43.742502) in april, may, june and july of 2018. The determination of DZP, CZP and BZP followed the procedure described above and mean concentrations (conc) are given as follows. DZP was found in all samples (conc = 0.15 µg L^{-1}), whereas BZP was quantified in 50% of the samples (conc = 0.54 µg L^{-1}) and CZP was quantified in 25% of the samples (conc = 0.25 µg L^{-1}). These results reveal a more worrying scenario, with higher concentration levels of these benzodiazepines than some previously reported for samples collected at a site near our collection point (Ferreira 2014).

A method for the determination of three benzodiazepines – bromazepam, clonazepam and diazepam – was developed and validated based on SPE extraction followed by liquid chromatography coupled to mass spectrometry, providing a simple and rapid procedure for the determination of these compounds in surface water samples. The SPE component allows the extraction and cleaning of surface water samples in a single step, generating little residue. The proposed SPE-LC–MS/MS method presents low chromatographic run time (8 min), selective, LQ = 0.01 µg L^{-1}, RSD < 20%, accuracy > 57%, $R^2 > 0.99$, and showed high matrix effect for all analytes, specially clonazepam, which the adoption of the standard addition method highly recommended. Based on the results for the preliminary monitoring campaign and some previously reported determinations, it can be concluded that our proposed methodology is successful and suitable for the determination of reliable concentration levels of these benzodiazepines in surface waters.

Table 2 LQ, LD, slope, intercept, determination coefficients (R^2) and F values (Fcalc) for the analytical curves in ultra-pure water and in spiked matrix

Analyte	LQ[a]	LD[b]	Ultra-pure water			Matrix			Fcalc[c]
			Slope	Intercept	R^2	Slope	Intercept	R^2	
BZP	0.01	2.70	0.0162	− 0.0088	0.9949	0.0072	0.0009	0.9987	1.05
CZP	0.01	5.00	0.0311	− 0.0073	0.9981	0.0348	0.0501	0.9991	15.89
DZP	0.01	4.60	0.0410	− 0.0009	0.9985	0.0426	0.0031	0.9991	1.42

[a]Concentration in µg L^{-1}

[b]Concentration in ng L^{-1}

[c]Fcritical = 1.98 (confidence interval: 95%)

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References

Andreu V, Gimeno-García E, Pascual JA et al (2016) Presence of pharmaceuticals and heavy metals in the waters of a Mediterranean coastal wetland: potential interactions and the influence of the environment. Sci Total Environ 540:278–286. https://doi.org/10.1016/j.scitotenv.2015.08.007

Arbeláez P, Borrull F, Pocurull E, Marcé RM (2015) Determination of high-intensity sweeteners in river water and wastewater by solid-phase extraction and liquid chromatography-tandem mass spectrometry. J Chromatogr A 1393:106–114. https://doi.org/10.1016/j.chroma.2015.03.035

Aymerich I, Acuña V, Barceló D et al (2016) Attenuation of pharmaceuticals and their transformation products in a wastewater treatment plant and its receiving river ecosystem. Water Res 100:126–136. https://doi.org/10.1016/j.watres.2016.04.022

Bonfiglio R, King RC, Olah TV, Merkle V (1999) The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. Rapid Commun Mass Spectrom 13(12):1175-1185. https://doi.org/10.1002/(SICI)1097-0231(19990630)13:12<1175::AID-RCM639>3.0.CO;2-0

Brieudes V, Lardy-Fontan S, Lalere B et al (2016) Validation and uncertainties evaluation of an isotope dilution-SPE-LC-MS/MS for the quantification of drug residues in surface waters. Talanta 146:138–147. https://doi.org/10.1016/j.talanta.2015.06.073

Caldas SS, Rombaldi C, de Oliveira Arias JL et al (2016) Multi-residue method for determination of 58 pesticides, pharmaceuticals and personal care products in water using solvent demulsification dispersive liquid–liquid microextraction combined with liquid chromatography-tandem mass spectrometry. Talanta 146:676–688. https://doi.org/10.1016/j.talanta.2015.06.047

Cetesb (2011) Guia Nacional de Coleta e Preservação de Amostras - Água, Sedimento. Comunidades Aquáticas e Efluentes Líquidos, Cia Ambient do Estado São Paulo

Cunha DL, de Araujo FG, Marques M (2017) Psychoactive drugs: occurrence in aquatic environment, analytical methods, and ecotoxicity—a review. Environ Sci Pollut Res 24:24076–24091. https://doi.org/10.1007/s11356-017-0170-4

Dai G, Huang J, Chen W et al (2014) Major pharmaceuticals and personal care products (PPCPs) in wastewater treatment plant and receiving water in Beijing, China, and associated ecological risks. Bull Environ Contam Toxicol 92:655–661. https://doi.org/10.1007/s00128-014-1247-0

de Almeida CAA, Oliveira MS, Mallmann CA, Martins AF (2015) Determination of the psychoactive drugs carbamazepine and diazepam in hospital effluent and identification of their metabolites. Environ Sci Pollut Res 22:17192–17201. https://doi.org/10.1007/s11356-015-4948-y

Ferreira AP (2014) Environmental investigation of psychiatric pharmaceuticals: Guandu River, Rio De Janeiro State, Southeast Brazil. J Chem Heal Risks 4:25–32

García-Galán MJ, Petrovic M, Rodríguez-Mozaz S, Barceló D (2016) Multiresidue trace analysis of pharmaceuticals, their human metabolites and transformation products by fully automated on-line solid-phase extraction-liquid chromatography-tandem mass spectrometry. Talanta 158:330–341. https://doi.org/10.1016/j.talanta.2016.05.061

Gros M, Petrovic M (2009) Tracing pharmaceutical residues of different therapeutic classes in environmental waters by using liquid chromatography/quadrupole-linear ion trap mass spectrometry and automated library searching. Anal Chem 81:898–912

Gros M, Rodríguez-mozaz S, Barceló D (2012) Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem. J Chromatogr A 1248:104–121. https://doi.org/10.1016/j.chroma.2012.05.084

INCB (2015) International Narcotics Contol Board Psychotropic Substances—Statistics for 2013. INCB, New York

Luo Y, Guo W, Ngo HH et al (2014) A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci Total Environ 473–474:619–641. https://doi.org/10.1016/j.scitotenv.2013.12.065

Mann O, Pock E, Wruss K et al (2016) Development and validation of a fully automated online-SPE–ESI–LC–MS/MS multi-residue method for the determination of different classes of pesticides in drinking, ground and surface water. Int J Environ Anal Chem 96:353–372. https://doi.org/10.1080/03067319.2016.1160381

Matsuo H, Sakamoto H, Arizono K, Shinohara R (2011) Behavior of pharmaceuticals in waste water treatment plant in Japan. Bull Environ Contam Toxicol 87:31–35. https://doi.org/10.1007/s00128-011-0299-7

Postigo C, De Alda MJL, Barceló D (2008) Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on-line chromatography—electrospray-tandem mass spectrometry. Anal Chem 80:3123–3134. https://doi.org/10.1021/ac702060j

Silveira MAK, Caldas SS, Guilherme JR et al (2013) Quantification of pharmaceuticals and personal care product residues in surface and drinking water samples by SPE and LC-ESI-MS/MS. J Braz Chem Soc 24:1385–1395. https://doi.org/10.5935/0103-5053.20130176

Stipaničev D, Dragun Z, Repec S et al (2017) Broad spectrum screening of 463 organic contaminants in rivers in Macedonia. Ecotoxicol Environ Saf 135:48–59. https://doi.org/10.1016/j.ecoenv.2016.09.004

Tran NH, Hu J, Ong SL (2013) Talanta Simultaneous determination of PPCPs, EDCs, and arti fi cial sweeteners in environmental water samples using a single-step SPE coupled with HPLC–MS/MS and isotope dilution. Talanta 113:82–92. https://doi.org/10.1016/j.talanta.2013.03.072

USEPA (2014) SW 846 test method 8000D: determinative chromatographic separations.

Valls-Cantenys C, Scheurer M, Iglesias M et al (2016) A sensitive multi-residue method for the determination of 35 micropollutants including pharmaceuticals, iodinated contrast media and pesticides in water. Anal Bioanal Chem 408:6189–6200. https://doi.org/10.1007/s00216-016-9731-5