Development and Optimization of a HPLC–DAD Method for the Determination of Diverse Pharmaceuticals in Estuarine Surface Waters

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Abstract

This paper describes the development and validation of a simple analytical method using solid-phase extraction followed by a high-performance liquid chromatography with diode array detection (HPLC–DAD) analysis. Target compounds included six pharmaceuticals (carbamazepine, diazepam, fluoxetine, propranolol, sulfamethoxazole, and trimethoprim) and the active metabolite of fenofibrate (fenofibric acid). Briefly, this method consisted of the preconcentration of water samples (2 L) on 500 mg Oasis HLB cartridges and HPLC analysis using a RP-$_{18}$ analytical column in a gradient mode with a flow rate of 1 mL/min. The validation parameters revealed that this method was highly specific for all assayed compounds (> 99%), and the linearity of the calibration curves always showed a correlation higher than 0.99. The detection limits were in the ng/L level, and recovery rates were > 70% for most of the target compounds. Analysis of samples from polluted areas of the Douro River estuary indicated that propranolol and carbamazepine are present in concentrations ranging from 22.0 to 54.0 ng/L and 21.3 to 32.7 ng/L, respectively. Thus, it is concluded that this method can be successfully applied for screening pharmaceuticals in polluted estuarine areas.

Introduction

The occurrence of pharmaceutical residues in aquatic environments has been considered one of the most relevant topics in environmental research (1). Pollution caused by pharmaceuticals is becoming such a major concern in many countries that a new area designated as "Pharmaceuticals in the Environment" (PIE) has emerged recently (2). Human and veterinary pharmaceuticals can reach the environment by excretion via faeces and urine and also by improper disposal of unused medications (3). The wastewater treatment plants (WWTP) are normally unable to completely remove this type of residue. They are permanently introduced into the environment and are considered as pseudo-persistent pollutants (4). Consequently, many studies have demonstrated that human and veterinary pharmaceuticals have been detected in WWTP effluents (5–9), lakes (10), river surface waters (11–14), groundwater (15), and even in drinking water (16,17). Usually, the concentrations found are between the low ng/L to µg/L levels (18). Albeit at low concentrations, the continuous inputs of these non-regulated emerging contaminants (19) may give rise, over a long exposition period, to potential ecotoxicological effects that can affect aquatic organisms and human health (20). Some of the effects of PIE exposure are already established for aquatic organisms (21). Among them, the most cited is the endocrine disruption phenomenon caused by estrogens, which are active even at ng/L concentration levels (21). The increase of bacterium resistance in aquatic environments caused by the presence of antibiotics is also reported and is actually a cause of great concern (22).

Currently, the main interest of many research groups is the development of accurate and sensitive chromatographic analytical methods, which allow the quantification of pharmaceuticals and/or their metabolic and degradation products in aquatic environments at ng/L levels (23). The new challenge is to develop methods that focus on the simultaneous determination of acidic, neutral, and basic compounds belonging to different therapeutic classes (24,25) and which have the ability to screen pharmaceuticals in highly polluted aquatic environments as routine analysis with shortened time and overall cost reduction (26). 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 compounds identity (27); however, the application of this sophisticated and expensive technology is not yet available in all laboratories. Taking into account the previously mentioned concerns, the main purpose of this work was to develop an analytical method based on a single and efficient preconcentration step by solid-phase extraction (SPE) followed by a high-performance liquid chromatography with diode array detection (HPLC–DAD) analysis, demonstrating that this methodology is very useful for pharmaceutical screening in polluted water samples. The current analytical method was validated following the parameters established by the International Conference on Harmonization (ICH) (28). The target com-

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pounds selected for this study belong to six different therapeutic groups: antibacterials [sulfamethoxazole (SUM) and trimethoprim (TMP)], antidepressants [fluoxetine (FX)], β-blockers [propranolol (PHO)], lipid regulators [fenofibric acid (FA), metabolite of fenofibrate (F)], anticonvulsants [carbamazepine (CBZ)], and tranquilizers [diazepam (DZ)]. The selection of these seven pharmaceutical compounds was not only based upon their recent worldwide occurrence and ubiquity in aquatic environments (4,20,29–31) but also on the differences in their chemical structures (Figure 1), physico-chemical properties (Table I), therapeutic effects, and on the consumption rates of these pharmaceuticals in Portugal between 2001–2005 (32). The main purpose of selecting this series of compounds was to obtain a representative group with distinct physico-chemical properties that can serve as a model for a variety of interactions and different chemical behaviours. Therefore, the method has the appropriate conditions to be applied to other pharmaceuticals belonging to those groups and also for the correct chemical evaluation of aquatic environments. Because recent studies at the Douro River estuary demonstrated the occurrence of several endocrine disrupting chemicals (33), the developed method was applied to investigate the presence of other anthropogenic compounds, such as PIE, in this study area. The method proved to be valuable for the quantification of the selected pharmaceuticals in concentrations of µg/L to ng/L levels in surface water samples from the Douro River estuary. To our knowledge, this is the first study to report the simultaneous analysis in estuarine water of these pharmaceuticals by HPLC–DAD.

Experimental

Chemicals and materials

Standards of the pharmaceuticals used in this study: carbamazepine (CBZ), diazepam (DZ), fenofibrate (F), fluoxetine hydrochloride (FX), propranolol hydrochloride (PHO), sulfamethoxazole (SUM), and trimethoprim (TMT) were purchased from Sigma Aldrich (Steinheim, Germany). The fenofibric acid (FA) was obtained as the hydrolysis product of fenofibrate (F) as described elsewhere (34,35). Triethylamine (TEA) with ≥ 99% purity and 85% ortho-phosphoric acid were obtained from Sigma Aldrich and Riedel-de Haën (Seelze, Germany), respectively. All the other solvents used were HPLC grade and supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained using Milli-Q system (Millipore, Molsheim, France). The cartridges used for SPE were Oasis HLB (Hydrophilic-Lipophilic Balance) (500 mg, 12 cc) and Oasis MCX (Mixed-mode Cation-exchange) (500 mg, 6 cc) from Waters (Milford, MA). 0.45-µm glass fiber filters were purchased from Millipore (Carrigtwohill, Ireland).

Stock solutions of individual standards (1000 µg/mL) were prepared in ethanol, transferred to amber bottles, and stored in the dark at –20°C to minimize their potential degradation. Stock solutions were stable, and no evidence of degradation of the analytes was observed on the chromatograms during the study period (four months). Working solutions were prepared daily by diluting the stock solution with ethanol. From the stock solutions, seven nominal calibration standards were prepared in the following range of concentrations according to each pharmaceutical compound: 0.80–3.20 µg/mL (FA); 0.15–0.60 µg/mL (CBZ); 0.40–1.60 µg/mL (DZ); 8.00–32.00 µg/mL (FX); 0.20–0.80 µg/mL (PHO); 0.35–1.40 µg/mL (SUM), and 2.00–8.00 µg/mL (TMT). These solutions were used to prepare the solvent and matrix-matched calibration curves. For precision, accuracy, and recovery assays, three concentration levels (low, medium, and high) of each pharmaceutical were used to spike 2 L of water sample according to the compounds linearity range: 1.20, 2.00, 3.20 µg/mL for FA; 0.22, 0.38, 0.60 µg/mL for CBZ; 0.60, 1.00, 1.60 µg/mL for DZ; 12.00, 20.00, 32.00 µg/mL for FX; 0.30, 0.50, 0.80 µg/mL for PHO; 0.52, 0.88, 1.40 µg/mL for SUM; and 3.00, 5.00, 8.00 µg/mL for TMT.

Sample collection and preparation

Several samples were collected at two distinct areas of the Douro River estuary: one considered highly polluted (about 1–2 km from the Atlantic Ocean) and the other with low levels of pollution (about 20 km from the Atlantic Ocean). Water samples from the area with low pollution were used to prepare the fortified matrix calibration standards. To demonstrate the applicability of the method, samples were collected at several points in areas of both high and low pollution. The water samples were collected during October 2007 and January 2008.

Two liters of surface water samples were collected into 2.5 L pre-rinsed amber glass bottles with Teflon-lined caps from a

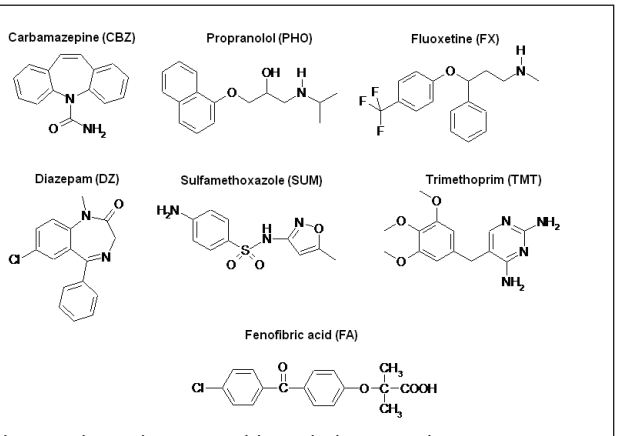

Figure 1. Chemical structures of the studied compounds.

Table I. Physico-chemical Properties of the Target Analytes

Compounds	Molecular formula	Molecular weight (g/mol)	log K_{ow}	pK_a
FA	$C_{17}H_{15}ClO_4$	318.76	4*	
CBZ	$C_{15}H_{12}N_2O$	236.27	2.45†	7‡
DZ	$C_{16}H_{13}ClN_2O$	284.76	2.82*	3.3§
FX	$C_{17}H_{18}F_3NO$	309.33	3.82**	8.7**
PHO	$C_{16}H_{21}NO_2$	259.35	1.2–3.48††	9.5††
SUM	$C_{10}H_{11}N_3O_3S$	253.28	0.89†	2.0 or 5.5††
TMT	$C_{14}H_{18}N_4O_3$	290.32	0.91†	1.32 or 7.12‡‡

*54. †11. ‡30. §56. **25. ††50. ‡‡55.

depth of approximately 1 m using a WS 300 peristaltic sampler pump (Global Water, Gold River, California). Upon collection, all samples were immediately transported at 4°C to the laboratory and vacuum filtered through a 0.45-µm glass fiber filter with a 47 mm diameter (Millipore). The filter was washed with 5 mL of methanol, which was added to the filtrate. Filtered samples were then stored in darkness at 4°C and extracted within a maximum of 72 h after collection.

Solid-phase extraction

The establishment of the sample volume was based on a previous work done with other micropollutants and the same type of matrix, in which 2 L of sample was considered the ideal volume for preconcentration, allowing the quantification by HPLC–DAD (33).

The extraction efficiency of Oasis HLB 500 mg and Oasis MCX 500 mg cartridges was carried out for 2 L of ultrapure water spiked with a standard solution prior to the extraction with all seven pharmaceutical compounds assayed in this study according to general procedures illustrated in the diagram in Figure 2. The potential recovery of Oasis HLB sorbent was assayed with water at different pH values (2, 4, 7, and 8).

The optimized SPE procedure was carried out with Oasis HLB cartridges, conditioned sequentially with 32 mL of dichloromethane, 32 mL of methanol, and 32 mL of ultrapure water, at a flow rate of 1 mL/min. Sequentially, water samples at neutral pH were percolated through the cartridges at a constant flow rate of 10 mL/min using a vacuum manifold system connected to a vacuum pump. Afterwards, the cartridges were washed with 32 mL of water and then dried under vacuum for 30 min to dry out residual water. Elution was performed with 32 mL methanol–dichloromethane (70:30, v/v) at 1 mL/min. The extracts were evaporated to dryness in a thermostatic bath at 40°C under a gentle nitrogen stream. The residues were dissolved in 200 µL of ethanol, and 20 µL was injected into the HPLC system (Figure 2).

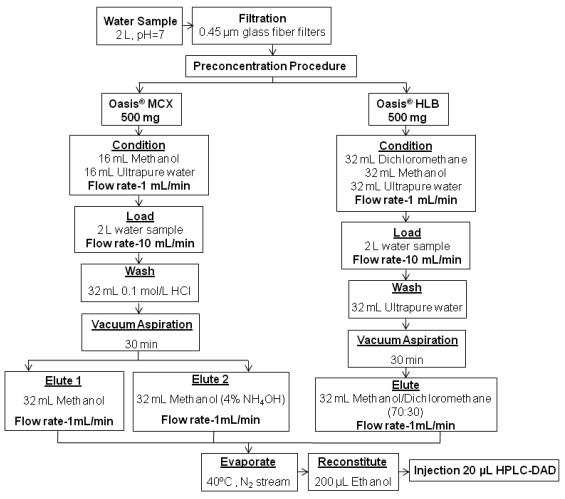

Figure 2. Diagram of the optimized preconcentration method with Oasis HLB and Oasis MCX.

HPLC–DAD analysis

The analyses were carried out on a Merck-Hitachi LaChrom HPLC instrument (Whitehouse Station, NJ) equipped with a quaternary L-7100 pump, an interface D-7000, a L-7455 DAD, and an autosampler L-7200 with the injection volume set to 20 µL. Chromatograms were processed by a HPLC System Manager HSM D-700 (Merck-Hitachi).

Chromatographic separation was achieved with a Merck LiChroCART C_{18} reversed-phase analytical column (250 mm × 4 mm i.d., 5 µm, from Darmstadt, Germany) with mobile phase consisting of a binary mixture of solvents: (A) water (containing 1.5 mL/L of TEA, adjusted to pH 4.5 with 85% ortho-phosphoric acid) and (B) acetonitrile. The solvents of the mobile phase were filtered through 0.45-µm glass fiber filters. The gradient increased from 17 to 70% of B over 23 min followed by 10 min of equilibration time. Separations were performed at room temperature, and the flow rate was maintained at 1 mL/min. The compounds were monitored at the following wavelengths: 254 nm (DZ and TMT), 265 nm (FX), 270 nm (SUM), 286 nm (CBZ), 290 nm (PHO), and 295 nm (FA). In some cases, the maximum wavelengths were not selected for the analysis because they correspond to low wavelengths, which lead to the absorption of matrix interferences. Thus, the selected wavelengths allowed a selective analysis with a suitable absorption of the studied compounds.

Method validation parameters

The method was validated according to internationally accepted criteria (28,36) considering the following parameters: selectivity, linearity and range of application, precision, accuracy, recovery, limit of detection (LOD), and limit of quantification (LOQ).

Selectivity

The interference of matrix compounds was assessed by retention times (t_R), UV spectra, and peak purity tests for all pharmaceutical compounds in both standard solutions and in the fortified matrix. The parallelism between the calibration curves obtained in both solvent and spiked matrices was also considered.

Linearity and range

The linearity of the assay was checked in triplicate with a set of seven different mixtures of pharmaceutical standards in the range of 0.15–32 µg/mL, depending on the compound. Calibration curves for standard

Table II. Chromatographic Retention Times (t_R) and Calibration Results

Compounds	t_R (min)	Range (µg/mL)	Solvent calibration			Matrix Calibration		
			Intercept	Slope	r	Intercept	Slope	r
FA	16.34	0.80–3.20	469.67	22685	0.996	2565.4	21696	0.995
CBZ	14.75	0.15–0.60	516.39	47992	0.996	1801.8	52211	0.996
DZ	21.09	0.40–1.60	2184.20	30263	0.996	1318.8	31493	0.996
FX	19.90	8.00–32.00	933.71	1964.6	0.995	2042.4	2003.1	0.994
PHO	13.90	0.20–0.80	822.18	30250	0.996	1341.2	32199	0.993
SUM	10.91	0.35–1.40	2097.10	84249	0.995	3555.5	80299	0.994
TMT	6.25	2.00–8.00	782.49	10536	0.996	3519.3	10392	0.994

stock solutions and fortified matrices were obtained for each target compound by plotting the analyte concentration versus the peak area at the selected absorption wavelengths.

Precision and accuracy

Method precision was evaluated by repeated intra-day and inter-day analyses at three concentration levels (low, medium, and high) using three replicates per concentration in one day and for three different days, respectively, expressing it as the relative standard deviation (RSD).

The accuracy values were back-calculated considering the recoveries obtained for each compound at each concentration level and were expressed as the percentage of agreement between the method results and the nominal amount of compound added.

Recovery

For the determination of recovery percentages, three replicates were done for each of the three concentration levels (low, medium, and high) in ultrapure and surface estuarine waters. Extraction recoveries of target compounds were evaluated by the peak area rate of extracted samples to those non-extracted standard solutions with similar concentration.

LOD and LOQ

The LOD of the whole method were determined based on the standard deviation of the response and the slope of the matrix calibration curves with the following equation:

$$LOD = 3.3 \times s/S$$

here, s is the standard deviation of y-intercepts, and S is the slope of the calibration curves.

The LOD and LOQ were verified in an estuarine water sample spiked with standards. The LOQ was considered the lowest concentration in the calibration curve.

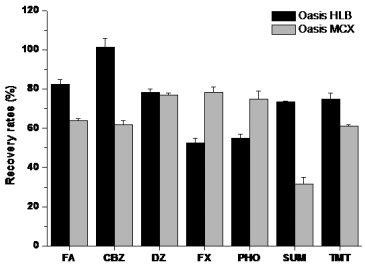

Figure 3. Recoveries obtained for the target pharmaceuticals in ultrapure water (spiked at a medium linearity level for each compound) using Oasis HLB and Oasis MCX cartridges.

Results and Discussion

Solid-phase extraction

SPE efficiency is linked to a large number of parameters such as the selection of the SPE sorbent, sample pH adjustment, flow rates, and the composition/volume of washing and elution solvents used in each step of the procedure (37,38). The common problem encountered is the selection of the experimental conditions, which represent the best performance for all compounds in a series characterised by different physico-chemical properties (39).

At first, preliminary studies were carried out with two different SPE materials, including a polymeric sorbent Oasis HLB (Hydrophilic-Lipophilic Balance) and Oasis MCX (Mixed-mode Cation-exchange). Oasis HLB has been the sorbent most widely employed for the simultaneous extraction of acidic, neutral, and basic compounds (37). The MCX cartridges can also extract compounds with a wide range of polarities at low pH values because the cation-exchange moiety binds the basic compounds, which are in the ionized form, and the polar and non-polar moieties can retain both acidic and neutral compounds (40). Therefore, the basic compounds (PHO and FX) achieved higher recoveries when Oasis MCX were employed, but the best overall SPE recoveries were achieved using Oasis HLB (Figure 3).

Several papers reported a sample pH adjustment prior to extraction with values ranging from acid to alkaline pH levels (37). In this study, several pH values were assayed and best results (higher recovery rates and reduced matrix interference) were obtained at pH 7. These data are consistent with previous studies which confirm that the co-extraction of matrix components are significantly reduced at pH 7 (41). The presence of matrix

Table III. Performance Data for Pharmaceuticals Using 2 L of Surface Water Spiked with 200 µL at the Three Nominal Concentrations

		1st day		2nd day		3rd day	
Compounds	Nominal conc. (µg/mL)	RSD (%) (n = 3)	Accuracy (%)	RSD (%) (n = 3)	Accuracy (%)	RSD (%) (n = 3)	Accuracy (%)
FA	1.20	2.53	94.2	3.05	87.2	0.49	82.6
	2.00	1.18	105	0.32	83.1	1.19	95.0
	3.20	0.56	113	1.48	94.6	0.21	91.6
CBZ	0.22	0.80	84.4	1.12	80.4	1.73	86.4
	0.38	0.99	94.6	1.70	99.8	0.39	85.5
	0.60	1.82	94.8	1.40	99.2	0.85	97.2
DZ	0.60	1.15	69.3	1.88	70.9	1.35	93.5
	1.00	0.59	88.9	2.82	75.8	1.50	90.1
	1.60	1.21	93.7	0.28	80.9	0.17	88.2
FX	12.00	0.64	70.5	0.34	74.3	1.63	70.3
	20.00	1.34	67.3	3.32	71.4	2.39	72.4
	32.00	0.71	89.3	0.93	81.7	3.87	81.9
PHO	0.30	2.49	101	1.06	112	1.70	104
	0.50	0.71	116	1.54	122	0.76	106
	0.80	0.35	102	0.74	97.4	0.79	102
SUM	0.52	2.79	84.9	0.97	86.3	1.75	102
	0.88	3.05	109	0.42	87.5	2.36	86.4
	1.40	1.71	116	2.33	87.1	0.84	86.3
TMT	3.00	2.74	74.8	1.47	76.7	1.72	76.8
	5.00	2.74	80.8	1.82	72.9	1.30	79.2
	8.00	1.14	89.7	0.31	80.6	1.09	83.2

interferences such as humic and fulvic acids is common in groundwater, surface, and estuarine waters and usually represents the majority of the dissolved organic matter in water samples (42). The co-elution of these acidic substances is intensified when the samples are preconcentrated at low pH values because their adsorption in the HLB cartridge is higher at non-ionized form. Humic substances are UV absorptive, and so they are responsible for the initial broad band at the beginning of chromatograms or a hump in the middle, depending on the mobile phase gradient applied in the separation. This situation may hamper the determination of the early-eluting peaks from the most polar analytes (42,43).

Different washing and elution solvents were also tested such as 100% of water and a mixture of methanol–water (05:95, v/v) for washing while the elution was assayed with 100% of methanol, 100% of ethyl acetate, and a mixture of methanol–dichloromethane (70:30, v/v).

Water (100%) and methanol–dichloromethane (70:30, v/v) were the solvents selected for the washing and elution steps, respectively, because they provide the best extraction recoveries for the majority of the target compounds in the series.

Chromatographic separation

The challenge to optimizing the chromatographic separation was to achieve the mobile phase conditions for this series of compounds with a wide range of retention factors within an acceptable analysis time. A large number of methods are reported in the literature for multi-residue HPLC analysis of compounds belonging to the same therapeutic group (44,45); however, very few provide conditions for a multi-class analysis (46).

The gradient elution condition developed provided excellent chromatographic parameters such as $R_s > 2$ and $N > 2000$ for all compounds within a short elution time (23 min) and 10 min to equilibrate the system before a new injection (Figure 4). In Table II, the t_R of all assayed pharmaceuticals are shown.

When ionizable compounds are analyzed, the pH adjustment of the mobile phase plays an important role in the optimization of chromatographic separation. Thus, in this work, it was explored in order to achieve high resolution with good retention times. The best chromatogram was obtained with ultrapure water at pH adjusted to 4.5 as aqueous mobile phase (Figure 4). The TEA as silanol suppressor is well-established (47,48), and it was used in the mobile phase to block the secondary interactions of dissociated silanol groups of the stationary phases and the charged basic analytes in order to improve the peak symmetry of PHO and FX.

Table IV. Recoveries Obtained for Target Analytes Using 2 L of Ultrapure and Surface Water Spiked with 200 µL at the Three Nominal Concentrations

Compounds	Nominal conc. (µg/mL)	Recoveries (%) (RSD)	
		Ultrapure water (n = 9)	Surface water (n = 9)
FA	1.20	84.7 (6.00)	57.4 (5.58)
	2.00	86.8 (4.06)	51.4 (9.69)
	3.20	84.3 (3.73)	69.7 (9.68)
CBZ	0.22	81.9 (3.00)	101 (9.69)
	0.38	85.9 (1.86)	94.4 (1.05)
	0.60	91.0 (7.24)	91.8. (6.79)
DZ	0.60	91.9 (5.95)	88.0 (15.4)
	1.00	88.1 (7.27)	79.1 (7.92)
	1.60	86.6 (3.42)	81.3 (6.14)
FX	12.00	59.4 (0.66)	54.4 (13.8)
	20.00	63.2 (5.77)	48.7 (4.05)
	32.00	56.1 (9.61)	59.7 (4.74)
PHO	0.30	74.9 (3.53)	91.7 (11.5)
	0.50	79.1 (3.20)	83.1 (2.86)
	0.80	78.1 (7.89)	91.1 (10.7)
SUM	0.52	64.7 (6.20)	54.2 (8.80)
	0.88	61.8 (9.97)	46.2 (11.4)
	1.40	65.0 (4.00)	55.3 (14.9)
TMT	3.00	78.1 (5.19)	59.8 (5.98)
	5.00	81.8 (5.66)	48.4 (4.35)
	8.00	73.9 (4.70)	65.4 (4.64)

Method validation parameters

The selectivity was observed by association of the t_R, peak purity tests, UV spectra, and the parallelism of the calibration curves. The RSD values obtained as a result of precision estimation of the t_R between the standard in solvent solutions and in spiked matrix were lower than 0.34% (n = 20). The peak purity tests performed by the HPLC–DAD software revealed that all peaks had their purity levels higher than 99%, independent of the matrix (Figure 5). The difference in the baseline shift observed at the beginning of chromatograms represented in Figures 4 and 5 is entirely related to the absorption of humic substances commonly present in estuarine water samples, which due to its high conjugated system caused this characteristic band. However, in our study this fact didn't interfere with the determination of the early elution compounds.

Moreover, the parallelism between the slopes of both calibration curves, standards in 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 seven pharmaceuticals and can be used for monitoring purposes in estuarine water samples.

The linearity and range of application were established by the calibration curves in the ranges given at Table II with coefficients of correlation (r^2) values between 0.993–0.996. The RSD of each calibration standard (n = 3) varied from 0.02 to 2.65% with accuracy values ranging from 92.9 to 106% for solvent calibration curves and from 0.14 to 4.09% with accuracy data ranging from 93.6 to 109% for matrix calibration curves.

Accuracy, intra-day and inter-day precision were also evaluated. Data obtained for accuracy (67.3–122%), intra-day and inter-day precision experiments (RSD < 4%) are summarized in Table III, which are in accordance with the ICH parameters (28).

The recovery percentages were established by three replicates for each of the three concentration levels (low, medium, and high) in ultrapure and estuarine surface waters. The extraction efficiencies were acceptable for all compounds analyzed at the three concentration levels. Recoveries, obtained for all target compounds ranged from 56.1 to 91.9% in ultrapure water and from 46.2 to 101% in surface water. The RSD values were between 0.66 and 15.4% (Table IV). It was observed that the recovery values obtained for ultrapure and surface water were in some cases significantly distinct, but there was no direct correlation between matrix influence or even concentration depen-

dence. The lowest recoveries obtained were not an obstacle for the reliable quantification of pharmaceuticals because the RSD values obtained for this method are in conformity with the ICH validation requisites (28).

The LOD and LOQ obtained for the whole method are shown in Table V. The LOD and LOQ obtained for spiked surface waters of the Douro River estuary were in the range of 3.8–59.7 ng/L (with the exception of FX, 233.7 ng/L) and 15.0–200.0 ng/L (with the exception of FX, 800.0 ng/L), respectively. The LOQs are in the range of some LC–MS and gas chromatography (GC)–MS methods (6, 41). According to data previously reported, it can be assumed that FA (29), CBZ (11,12,49,50), DZ (12), PHO (51), SUM (52,53), and TMT (52) can be quantified by the HPLC–DAD method established in this work.

Table V. Detection and Quantification Limits of the Method

Compounds	LOD (ng/mL) (n = 3)*	LOQ (ng/mL) (n = 3)*
FA	21.7	80.0
CBZ	3.8	15.0
DZ	10.3	40.0
FX	233.7	800.0
PHO	6.6	20.0
SUM	10.8	35.00
TMT	59.7	200.0

* The LOQ and LOD were obtained using the method preconcentration factor of 10.000.

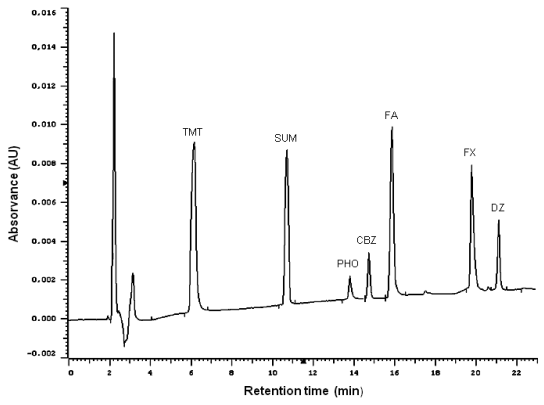

Figure 4. Chromatogram of a standard mixture of all target compounds. Chromatographic conditions: LiChroCART C_{18} RP (250 mm × 4 mm i.d., 5 µm); mobile phase, 0–23 min, 17–70% of eluent B; flow rate 1.0 mL/min; λ = 270 nm.

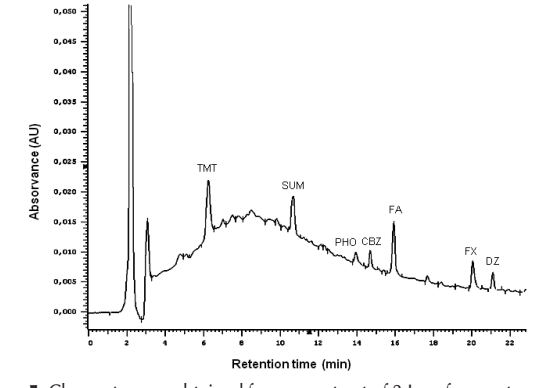

Figure 5. Chromatogram obtained from an extract of 2 L surface water sample spiked with all analytes in the study. Chromatographic conditions: LiChroCART C_{18} RP (250 mm × 4 mm i.d., 5 µm); mobile phase, 0–23 min, 17–70% of eluent B; flow rate 1.0 mL/min; λ = 275 nm.

Application of the method to water samples collected from the Douro River estuary (Portugal)

To demonstrate the applicability of the developed method, two sampling locations in the Douro River estuary were selected for the level of pollution in each area (low and high pollution). Water samples were collected in October 2007 and January 2008. CBZ was detected in this study at the lowest polluted site in winter (21.3 ng/L) and in higher levels (up to 32.7 ng/L) at the more polluted area in autumn. PHO was measured at the most polluted area showing concentrations that ranged from 22.0 ng/L in winter and 54.0 ng/L in autumn. As the most polluted site is located downstream from a wastewater treatment plant, the results obtained show an increase of these two pharmaceutical compounds at this location. CBZ is considered a qualified parameter for detecting wastewater in aquatic environments due to its high persistence, which is in accordance with the data obtained (15).

To our knowledge, this is the first study reporting the method validation of the target compounds by HPLC–DAD for estuarine surface water. It is also important to stress that this is the first study that reports the presence of pharmaceutical compounds in the Douro River in Portugal.

Conclusion

An optimized analytical method based on SPE-HPLC–DAD has been developed and validated for determining six classes of pharmaceuticals with distinct physico-chemical properties including carbamazepine, diazepam, fenofibric acid, fluoxetine, propranolol, sulfamethoxazole, and trimethoprim in estuarine water samples. Recoveries obtained for all target compounds using Oasis HLB cartridges were in the range of 46.2–101%, which is consistent with the analytical methods published that deal with low levels of contaminants in environmental samples. The detection limits achieved with the developed method were in the ng/L range for surface waters, thus providing a reliable tool for a rapid and less costly analysis.

Preliminary results demonstrated the applicability of the method in surface water samples of the Douro River estuary. Carbamazepine and propranolol were quantified in the range of 21.3–32.7 ng/L and 22.0–54.0 ng/L, respectively. This low cost and simple methodology, based on SPE-HPLC–DAD, can be used in all laboratories for screening a wide range of different classes of pharmaceutical compounds in aquatic environments.

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