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Enantioselective HPLC analysis and biodegradation of atenolol, metoprolol and fluoxetine

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Abstract The accurate quantification of enantiomers is crucial for assessing the biodegradation of chiral pharmaceuticals in the environment. Methods to quantify enantiomers in environmental matrices are scarce. Here, we used an enantioselective method, high-performance liquid chromatography with fluorescence detection (HPLC-FD), to analyze two beta-blockers, metoprolol and atenolol, and the antidepressant fluoxetine in an activated sludge consortium from a wastewater treatment plant. The vancomycin-based chiral stationary phase was used under polar ionic mode to achieve the enantioseparation of target chiral pharmaceuticals in a single chromatographic run. The method was successfully validated over a concentration range of 20–800 ng/mL for each enantiomer of both beta-blockers and of 50–800 ng/mL for fluoxetine enantiomers. The limits of detection were between 5 and 20 ng/mL and the limits of quantification were between 20 and 50 ng/mL, for all enantiomers. The intra- and inter-batch precision was lower than 5.66 and 8.37 %, respectively. Accuracy values were between 103.03 and 117.92 %, and recovery rates were in the range of 88.48–116.62 %. Furthermore, the enantioselective biodegradation of atenolol, metoprolol and fluoxetine was followed during 15 days. The (S)-enantiomeric form of metoprolol was degraded at higher extents, whereas the degradation of atenolol and fluoxetine did not show enantioselectivity under the applied conditions.

Keywords Pharmaceuticals · Chiral ecotoxicity · Enantioselective biodegradation · HPLC-FD · Polar ionic mode · Chirobiotic V

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Introduction

Pharmaceutical ingredients and their metabolites have been frequently detected in the environment at concentrations ranging from μg/L to ng/L and are often resistant to degradation. The main origins of this type of contamination rely on the improper elimination occurring at wastewater treatment plants and on the discharge of untreated domestic sewage (Tong et al. 2011). Biodegradation is the most important step to eliminate polar pharmaceuticals in wastewater treatment plants. Many chiral pharmaceutical ingredients are usually administered as racemates, but can be found in wastewater treatment plant effluents and in aquatic environments with different enantiomeric ratios due to the possible enantioselective pharmacokinetic and pharmacodynamic in humans or enantioselective biodegradation occurring in wastewater treatment plants (Ribeiro et al. 2012).
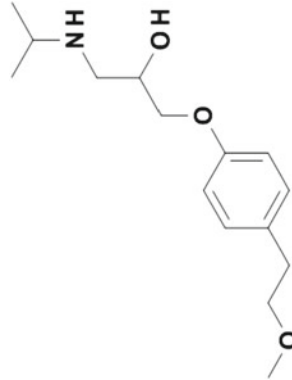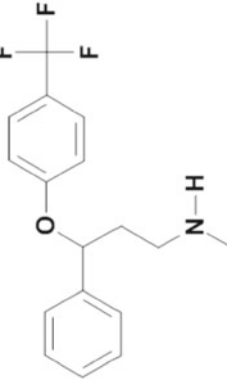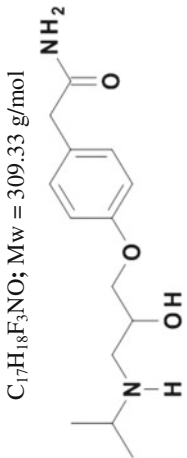
Beta-blockers such as metoprolol and atenolol and the antidepressant fluoxetine (Table 1) are chiral pharmaceuticals frequently prescribed in most countries as racemic mixtures. Metoprolol was classified as toxic to daphnids and algae in a toxicity study comprising the beta-blockers such as propranolol, metoprolol and atenolol (Cleuvers 2005). In other study,

Table 1 Chemical data, chromatographic (k_1 = capacity factor, α = selectivity factor, Rs = resolution) and validation (RSD = relative standard deviation) parameters obtained for target enantiomers, using the chiral high-performance liquid chromatography with fluorescence detection

Enantiomer	Chemical data	Chromatographic parameters			Linearity parameters			Method limits		Validation parameters			
	Chemical structures, formula and molecular weight (Mw)	k_1	α	Rs	Range (ng/mL)	Calibration equation	Correlation levels (R^2)	Limit of quantification (ng/mL)	Limit of detection (ng/mL)	Accuracy (%)	Recovery (%)	Intra-batch precision (%RSD)	Inter-batch precision (%RSD)
(S)-metoprolol		1.53	–	–	20–800	$y = 5835.2x + 14133$	0.9999	20	5	[107.85–111.32]	[100.45–101.70]	[0.63–2.01]	[1.84–4.13]
(R)-metoprolol	$C_{15}H_{25}NO_3$; Mw = 267.36 g/mol	1.80	1.17	1.65	20–800	$y = 5807.3x - 1078.9$	0.9999	20	5	[106.10–108.72]	[99.88–103.56]	[0.71–3.51]	[2.17–4.55]
(S)-fluoxetine		2.63	1.47	4.35	50–800	$y = 4120.9x - 196555$	0.9943	50	20	[110.57–117.92]	[89.15–116.62]	[2.82–4.68]	[2.95–7.36]
(R)-fluoxetine	$C_{17}H_{18}F_3NO$; Mw = 309.33 g/mol	2.93	1.12	1.30	50–800	$y = 3818.6x - 166105$	0.9940	50	20	[110.35–112.10]	[88.48–114.96]	[2.58–5.30]	[5.01–8.37]
(S)-atenolol		3.40	1.16	1.68	20–800	$y = 11136x + 49948$	0.9998	20	5	[103.03–113.81]	[97.70–101.08]	[0.48–5.66]	[2.58–6.51]
(R)-atenolol	$C_{14}H_{22}N_2O_3$; Mw = 266.34 g/mol	3.87	1.14	1.46	20–800	$y = 11176x + 14767$	0.9999	20	5	[104.38–117.09]	[93.69–98.57]	[0.80–2.83]	[4.54–7.69]

The chromatographic and validation parameters provided an accurate quantification of the enantiomers of metoprolol, fluoxetine and atenolol in the biodegradation studies

atenolol was considered not harmful at concentrations normally found in the environment, although *Daphnia magna* and *Pseudokirchneriella subcapitata* revealed to be more sensitive to (S)-atenolol than to (R)-atenolol (De Andrés et al. 2009). Concerning fluoxetine, it was reported as toxic at low concentrations to several aquatic species (Foran et al. 2004; Flaherty and Dodson 2005; Henry and Black 2008; Sánchez-Argüello et al. 2009); both enantiomers were classified as toxic to *Daphnia Magna,* and (R)-fluoxetine was considered harmful to *Pseudokirchneriella subcapitata* (De Andrés et al. 2009). Enantioselective toxicity of fluoxetine was also demonstrated using *Pimephales promelas*, (S)-fluoxetine being more toxic than the (R)-form (Stanley et al. 2007). Furthermore, fluoxetine was classified as an endocrine disrupting chemical (Piersma 2009) and has been considered as a pharmaceutical with high environmental risk (Christen et al. 2010).

In this study, an enantioselective HPLC method was validated using the antibiotic-based chiral stationary phase vancomycin to quantify the enantiomeric fractions of metoprolol, atenolol and fluoxetine during biodegradation assays by an activated sludge inoculum from a wastewater treatment plant. The analytical method was validated in accordance with the International Conference Harmonization (Validation of Analytical Procedures: Text and Methodology Q2(R1) 1996) to quantify the enantiomers of these pharmaceuticals in assays supplemented with the compounds individually and with the mixture of all of them.

Experimental

Activated sludge

Activated sludge inoculum was collected from the aerated tanks of a municipal wastewater treatment plant (Parada, Maia, Portugal) for the biodegradation assays. The inoculum was washed three times prior to inoculate minimal salts medium supplemented with the target compounds. The composition per liter of minimal salts medium was as follows: $Na_2HPO_4 \cdot 2H_2O$, 2.67 g; KH_2PO_4, 1.4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 0.5 g and 10 mL of a trace elements solution with the following composition per liter: NaOH, 2.0 g; $Na_2EDTA_2 \cdot 2H_2O$, 12 g; $FeSO_4 \cdot 7H_2O$, 2 g; $CaCl_2$, 1 g; Na_2SO_4, 10 g; $ZnSO_4$, 0.4 g; $MnSO_4 \cdot 4H_2O$, 0.4 g; $CuSO_4 \cdot 5H_2O$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.1 g; H_2SO_4 98 %, 0.5 mL.

Chemicals

Metoprolol (+)-tartrate, fluoxetine hydrochloride, (+)-(S)-fluoxetine hydrochloride, (−)-(R)-fluoxetine hydrochloride, atenolol, (−)-(S)-atenolol and (+)-(R)-atenolol were purchased from Sigma-Aldrich (Steinhein, Germany). All reference

standards were of >98 % purity. Stock solutions were prepared by dissolution of metoprolol and the enantiomerically pure compounds in ethanol, to obtain a concentration of ca. 1,000 µg/mL of the enantiomeric mixture of metoprolol and 500 µg/mL of the individual enantiomers of atenolol and fluoxetine. Stock solutions were then diluted in ultrapure water supplied by a Milli-Q water system to obtain working solutions of 26 µg/mL of metoprolol, (S)-atenolol, (R)-atenolol, (S)-fluoxetine and (R)-fluoxetine.

Methanol and ethanol LiChrosolv (HPLC grade) were obtained from Merck (Darmstadt, Germany). Acetic acid 100 % Chromanorm (HPLC grade) and triethylamine (≥99 %) were purchased from VWR International (Fontenay-sous-Bois, France) and Sigma-Aldrich (Steinheim, Germany), respectively. HPLC solvents grade were filtered with 0.45-µm glass microfiber filters (Whatman™).

Chromatographic measurements

A Shimadzu UFLC Prominence System equipped with two Pumps LC-20AD, an Autosampler SIL-20AC, an Oven CTO-20AC, a Degasser DGU-20A₅, a System Controller CBM-20A and a LC Solution version 1.24 SP1 (Shimadzu) was used. The Fluorescence Detector coupled to the LC System was a Shimadzu RF-10AXL. A vancomycin-based chiral column, Astec Chirobiotic™ V (150 × 4.6 mm, I.D., 5-µm particle size) supplied by SUPELCO Analytical (Sigma-Aldrich, Steinhein, Germany) was set at 35 °C. The optimized mobile phase was ethanol/methanol (50:50, v/v) with 0.075 % of triethylamine and 0.225 % of acetic acid to adjust the pH to 6.7, at isocratic mode with a flow rate of 0.6 mL/min. The injection volume was 20 µL. The excitation and emission wavelengths of the Fluorescence Detector were set at 230 and 290 nm, respectively. The elution order was established based on the injection of solutions of each enantiomer separately, except for metoprolol for which elution order was assessed by the deviation of the polarized light using a polarimeter detector Jasco OR-2090 Plus coupled to the HPLC.

Method validation parameters

The method was validated according to the International Conference Harmonization Validation of Analytical Procedures: Text and Methodology Q2(R1) (1996), considering the following parameters: selectivity, linearity and range, accuracy, recovery, precision, limits of detection and quantification.

Selectivity was verified by comparing the chromatograms of standards dissolved in ethanol and spiked in the matrix to assess the matrix interferences. The linearity and the range were evaluated using calibration curves performed with three sets of seven different standard concentrations of the

working solution (metoprolol, (S)- and (R)-atenolol and (S)-and (R)-fluoxetine) spiked in the matrix. Briefly, working solutions of metoprolol, (S)- and (R)-atenolol and (S)- and (R)-fluoxetine were diluted in 25 mL of minimal salts medium inoculated with 1 mL of activated sludge to obtain working calibration standards in the concentration range of 20–800 ng/mL for each enantiomer of metoprolol and atenolol and of 50–800 ng/mL for each enantiomer of fluoxetine. After shaking at 50 rpm during 10 min, aliquots of 1 mL were centrifuged at 14,200 rpm for 5 min and the supernatant was analyzed. Centrifugation was performed in a Mikro 200 microcentrifuge (Hettich, Germany). Limits of detection and quantification were calculated from spiked samples through the signal-to-noise ratio of 3 for limits of detection and 10 for limits of quantification.

Accuracy, intra- and inter-batch precision were determined by analyzing three replicates of three quality control standard solutions, with three different concentrations: 75, 300 and 700 ng/mL. Precision was expressed as the relative standard deviation (RSD) of the replicate measurements, and the accuracy of the method was evaluated as the percentage of agreement between the method results and the nominal amount of compound added. Minimal salts medium inoculated with activated sludge (blank matrixes) were fortified at the three quality control concentrations and used for recovery assays. The recovery was calculated by comparing the peak areas of the standards in aqueous solutions with those of similar concentrations from the supernatant of centrifuged aliquots collected from the spiked activated sludge after 2 h of shaking at 50 rpm.

Biodegradation assays

Biodegradation experiments were performed in batch mode in 100-mL flasks filled with 25 mL of minimal salts medium inoculated with 1 mL of activated sludge with an optical density at 600 nm of ca. 0.3 monitored by a spectrophotometer (Helios Gamma, Unicam Instruments, UK) and supplemented with each enantiomeric mixture to obtain concentrations of each enantiomer of 1 and 10 μg/mL for the single supplementation assays. Assays with a mixture of metoprolol, fluoxetine and atenolol, at initial concentrations of 0.5 and 5 μg/mL of each enantiomer were carried out. All these assays were repeated supplementing the medium with an additional carbon source, sodium acetate at a concentration of 100 μg/mL. All experiments were performed in triplicate. The cultures were incubated on a shaker (50 rpm) at 25 °C. Abiotic degradation of the compounds exposed to light and in the dark (flasks covered with aluminum foil) was also assessed by supplementing minimal salts medium with each enantiomer at initial concentrations of 1 and 10 μg/mL. The assays were monitored during 15 days using the HPLC-FD

validated method, by injecting 20 μL of the supernatant obtained after centrifuging 1-mL aliquots at 14,200 rpm for 5 min.

The degradation rate constant (k, day^{-1}) was determined considering the first-order kinetic expression (1):

$$C_t = C_0 \cdot e^{-kt} \tag{1}$$

being C_0 and C_t the concentrations of each enantiomer at the start of the experiment and at time (t, days), respectively.

The half-life ($t_{1/2}$, days) was also calculated by using the expression (2):

$$t_{1/2} = \ln 2/k \tag{2}$$

The enantiomeric fraction (EF) was used to express enantioselectivity, being $C(+)$ and $C(-)$ the concentrations of the (+) and (−) enantiomers of the compounds, respectively.

$$EF = C(+)/C(+) + C(-) \tag{3}$$

The average of the module variation of enantiomeric fraction (ΔEF) compared to the initial enantiomeric fraction was calculated by using the expression (4):

$$\Delta EF = \text{average}\big(|EF_t - EF_0|; |EF_{t+1} - EF_0|\ldots|EF_{tf} - EF_0|\big) \tag{4}$$

The enantiomeric fraction of the metoprolol, fluoxetine and atenolol working solutions was measured as EF = 0.54 ± 0.012 ($n = 8$), EF = 0.49 ± 0.011 ($n = 8$) and EF = 0.50 ± 0.004 ($n = 8$), respectively.

Results and discussion

Chiral chromatographic separation

The chromatographic conditions adequate to determine the enantiomeric fraction were achieved with a vancomycin-based chiral stationary phase Chirobiotic^{TM} V. The mobile phase was ethanol/methanol (50:50, v/v) adding 0.075 % of triethylamine and 0.225 % of acetic acid (pH 6.7). The pH adjustment of the mobile phase plays an important role in the optimization of the enantioselective separation of ionizable compounds since the functional groups of chiral selectors interact with the analytes by ionic interaction (Berthod 2009). The optimal acid/base ratio was explored testing several proportions of acetic acid and triethylamine to adjust the selector ionization state in order to achieve the highest resolution combined with a short run time. The column oven temperature is also important in the chromatographic parameters (Aboul-Enein and Ali 2002; Berthod et al. 2004). Increasing the temperature allowed a good resolution, a shorter run time and a minimal consumption of the mobile phase.

Fluorescence Detector was used due to the high selectivity and sensitivity of the detector. The similar structure of the target compounds allowed working at the same excitation and emission wavelengths. Thus, optimal chromatographic separation of metoprolol, fluoxetine and atenolol enantiomers extracted from the spiked matrix followed the elution order (S)-metoprolol, (R)-metoprolol, (S)-fluoxetine, (R)-fluoxetine, (S)-atenolol and (R)-atenolol (Fig. 1) and was processed in less than 20 min.

Method validation

Selectivity was verified by comparing the chromatogram with standards, blank matrix and spiked blank matrix. Since the ionic interactions of vancomycin-based chiral stationary phases like Chirobiotic™ V are very important concerning the interaction sites of polar and ionizable analytes, matrix components such as ionizable salts composing the minimal salts medium can slightly affect the resolution of the peaks. However, the chromatographic parameters and the constant behavior of the Chirobiotic™ V demonstrated to be suitable to analyze all the enantiomers from the spiked matrix (Table 1).

A linearity assay was performed for all enantiomers ranging from their limits of quantification to 800 ng/mL. The range, calibration equation and correlation levels ($R^2 > 0.99$) of each analytical curve (Table 1) are in agreement with international guidelines Validation of Analytical Procedures: Text and Methodology Q2(R1) (1996). The RSD of each matrix calibration points ($n = 3$) varied from 0.46 to 16.19 % for all calibration standards and limits of detection and
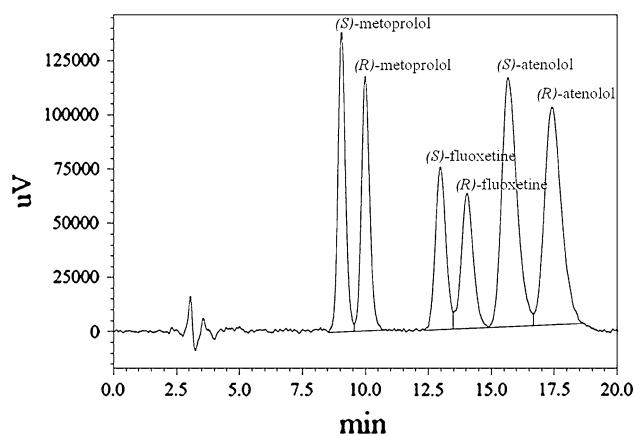
quantification were calculated from spiked samples through the signal-to-noise ratio being the limit of detection the concentration that yields a signal-to-noise ratio of 3 and the limit of quantification the concentration yielding a signal-to-noise ratio of 10 (Validation of Analytical Procedures: Text and Methodology Q2(R1) 1996). The values of limits of detection and quantification for the different compounds (Table 1) are suitable to an adequate monitoring of the target compounds in biodegradation assays.

The accuracy ranged from 103.03 to 117.92 % (Table 1). The recovery rates were calculated through the ratio of the peak area of the standards analyzed in the supernatant of the spiked matrix after 2 h of shaking, and the peak area of the aqueous standard solutions to assess the loss of compound by sorption processes. The recovery rates ranged between 88.48 and 116.62 %. The precision results evaluated by intra- and inter-batch assays demonstrated that this method is precise, with RSD values lower than 5.66 % for intra-batch precision and lower than 8.37 % for inter-batch precision (Table 1). This is in agreement with international criteria, which recommends RSD values lower than 20 % (Validation of Analytical Procedures: Text and Methodology Q2(R1) 1996).

Biodegradation assays

The biodegradation of the beta-blockers (metoprolol and atenolol) and the antidepressant fluoxetine was monitored during 15 days using the validated chiral HPLC-FD method described above. Figure 2 shows the overall degradation of the target enantiomers in biotic and abiotic conditions at initial concentration of 1 μg/mL. The inoculum was able to degrade both enantiomers of metoprolol at an initial concentration of 1 μg/mL, the (S)-form being degraded at higher extents (Fig. 2), which is corroborated by the lower half-life of (S)-metoprolol (Table 2). The addition of acetate led to a slight increase in the biodegradation rate, 7.8 % to the (S)-form and 10.5 % to the (R)-form. Regarding abiotic controls, non-enantioselective degradation was observed, both in the light and in the dark, but higher extents of degradation in the presence of light were observed. Concerning higher initial concentration, the pattern was similar (data not shown).

Activated sludge was able to remove both enantiomers of fluoxetine (Fig. 2). The removal percentage was ca. 80 % at an initial concentration of 1 μg/mL in the presence and absence of the extra carbon source. The half-life of both enantiomers was similar and biodegradation seemed to be non-enantioselective (Table 2). There was degradation of both enantiomers in abiotic controls, with higher extents of degradation in the assays directly exposed to the light. The experience at the higher initial concentration presented a similar pattern (data not shown).
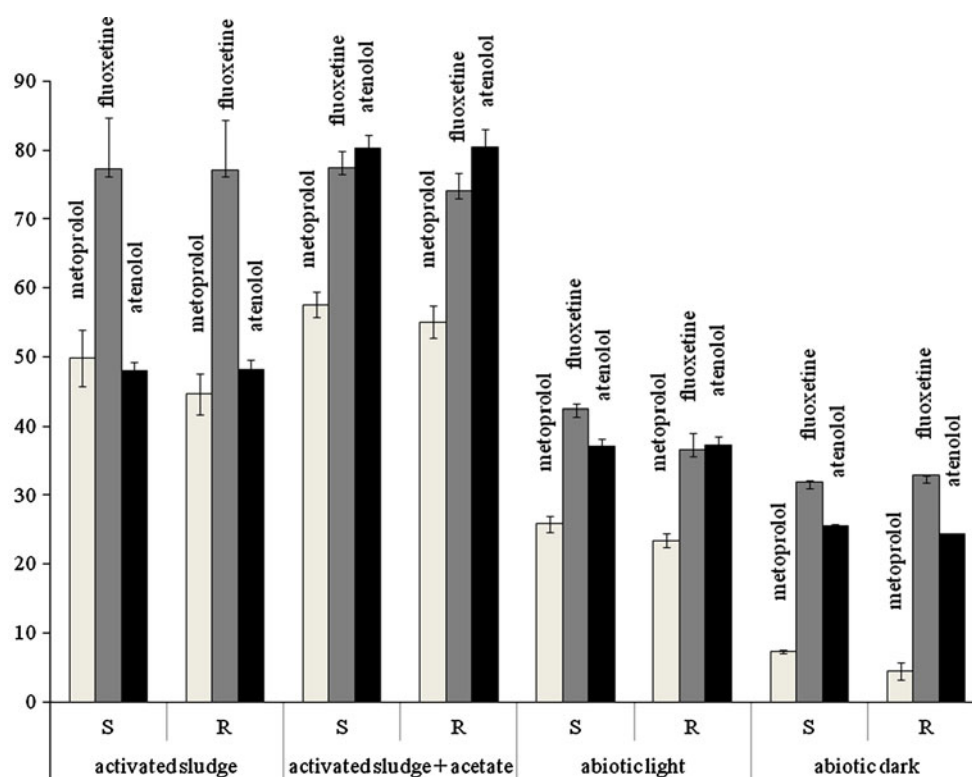

Fig. 1 Chromatogram of the enantiomers extracted from the spiked matrix of metoprolol, fluoxetine and atenolol at 500 ng/mL, showing the short elution time and the following elution order: (S)-metoprolol, (R)-metoprolol, (S)-fluoxetine, (R)-fluoxetine, (S)-atenolol and (R)-atenolol. Conditions: Chirobiotic™ V column (150 × 4.6 mm, I.D., 5-μm particle size); mobile phase: ethanol/methanol (50:50, v/v), 0.075 % triethylamine, 0.225 % acetic acid (pH 6.7); flow 0.6 mL/min; column oven temperature 35 °C; volume injection 20 μL. λ_{Exc} (nm)/λ_{Em} (nm) = 230/290

Fig. 2 Overall degradation (15 days) of (S)- and (R)-enantiomers of metoprolol, fluoxetine and atenolol at single compound supplementation of 1 μg/mL by activated sludge, by activated sludge supplemented with acetate, in the abiotic controls under light and dark conditions. The activated sludge inoculum was able to degrade both enantiomers of metoprolol at an initial concentration of 1 μg/mL, the (S)-form being degraded at higher extents. Activated sludge was also able to remove both enantiomers of fluoxetine and atenolol, but in a non-enantioselective manner. The addition of acetate led to an increase in the biodegradation rate of both beta-blockers, being more pronounced in the case of atenolol. Non-enantioselective degradation was observed to all pharmaceuticals, both in the light and dark conditions, but higher extents of degradation in the presence of light were observed

Table 2 Degradation rate constant (k, day^{-1}) and half-lives ($t_{1/2}$, days) of enantiomers of metoprolol, fluoxetine and atenolol and mean variation of enantiomeric fraction (ΔEF) during the biodegradation assays at single compound supplementation of 1 μg/mL by activated sludge, by activated sludge supplemented with acetate, and in the abiotic controls under light and dark conditions

Enantiomer	Activated sludge			Activated sludge + acetate			Abiotic light			Abiotic dark		
	k (day^{-1})	$t_{1/2}$ (day)	ΔEF	k (day^{-1})	$t_{1/2}$ (day)	ΔEF	k (day^{-1})	$t_{1/2}$ (day)	ΔEF	k (day^{-1})	$t_{1/2}$ (day)	ΔEF
(S)-metoprolol	0.0452	15.34	0.023	0.0542	12.79	0.016	0.0196	35.36	0.013	0.0045	154.03	0.011
(R)-metoprolol	0.0388	17.86		0.0513	13.51		0.0179	38.72		0.0031	223.60	
(S)-fluoxetine	0.0859	8.07	0.013	0.0839	8.26	0.005	0.0321	21.59	0.003	0.0300	27.29	0.001
(R)-fluoxetine	0.0854	8.12		0.0772	8.98		0.0277	22.65		0.0253	27.40	
(S)-atenolol	0.0390	17.77	0.008	0.1127	6.15	0.006	0.0303	22.88	0.001	0.0188	36.87	0.001
(R)-atenolol	0.0385	18.00		0.1021	6.79		0.0303	22.88		0.0186	37.27	

The lower half-life of (S)-metoprolol shows the higher degradability of the (S)-form of this beta-blocker by the activated sludge inoculum at an initial concentration of 1 μg/mL. In the case of fluoxetine and atenolol, there are no differences in the half-lives of their enantiomers in the biotic experiments. The presence of the extra carbon source accelerated the degradation rate of both enantiomers of metoprolol and atenolol by the activated sludge inoculum. All enantiomers were degraded in the abiotic controls, with higher extents of degradation in the assays directly exposed to light

Concerning atenolol, biodegradation was non-enantioselective and the presence of the extra carbon source accelerated the degradation rate of atenolol by activated sludge at concentrations normally found in the environment. The inoculum was able to degrade both enantiomers at the same extent, attaining ca. 80 % of removal in the presence of the extra carbon source and ca. 50 % in its absence, at the single supplementation of 1 μg/mL. This effect was also observed

at the higher concentration of atenolol. Abiotic controls showed a similar behavior and no enantioselectivity, with higher extents of degradation in the presence of light at both 1 and 10 µg/mL (data not shown).

Metoprolol exhibited a slightly enantioselective biodegradation profile, the (S)-enantiomer being degraded faster, whereas biodegradation of atenolol and fluoxetine seemed to be non-enantioselective. Reports on enantioselective biodegradation of chiral pharmaceuticals are scarce, although there are some studies related to xenobiotic compounds, including the pharmaceutical warfarin (Lao and Gan 2012; Ma et al. 2009; Patrick et al. 2011; Wang et al. 2012). Concerning the target pharmaceuticals, there are only some reports on the variation of enantiomeric fraction in wastewater treatment plants. Fono et al. (2006) found in microcosms experiments that the enantiomeric fraction of metoprolol decreased from the effluent to downstream, also suggesting enantioselective biodegradation of enantiomers (Fono et al. 2006). In three different wastewater treatment plant's effluents in Canada, the enantiomeric fraction of atenolol was different for the different effluents, also indicating that the different microbial consortia would affect the enantioselectivity of the biodegradation (MacLeod and Wong 2010). In a recent study developed in our laboratory, fluoxetine was enantioselectively degraded by a bacterial strain with a preferential degradation of the (R)-fluoxetine observed (unpublished work).

Biodegradation profile of the cocktail was similar to that of the single supplementation, with the degradation rate diminished for all enantiomers in the mixture (data not shown). This effect was more pronounced in the case of fluoxetine and atenolol, which showed up to a 14 % decrease in degradation rate. It is important to assess the mixture effect, since pharmaceuticals can affect the metabolism of each other (Kümmerer 2009; Kasprzyk-Hordern 2010). These phenomena may be explained by competition mechanisms for the same enzyme and can affect biodegradation in the real environment.

Conclusion

The chiral chromatographic method using Chirobiotic™ V chiral stationary phase demonstrated to be selective, accurate and precise to monitor the enantioselective biodegradation of two beta-blockers (metoprolol and atenolol) and the antidepressant fluoxetine, allowing their quantification at ng/mL level. A simple sample preparation method was established using only a centrifugation step and avoiding extractions with organic solvent. The short chromatographic run and the low flow rate added to the fact that a cheap and conventional detector can be used are advantages of the method. The method was applied to a biodegradation study using activated sludge. It was demonstrated that biodegradation of metoprolol occurred in a stereoselective manner, with (S)-form being slightly faster degraded. Regarding fluoxetine and atenolol, the biodegradation by the activated sludge used was non-enantioselective, demonstrated by the same half-life for both enantiomers.

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