Postprint of: Dogan A., Kempińska-Kupczyk D., Kubica P., Kot-Wasik A., Analysis of chiral pharmaceutical residues in influent and effluent samples at racemic and enantiomeric level using liquid chromatography-tandem mass spectrometry, Microchemical Journal, Vol. 159 (2020), 105517, DOI: 10.1016/j.microc.2020.105517

Analysis of chiral pharmaceutical residues in influent and effluent samples at racemic and enantiomeric level using liquid chromatography-tandem mass spectrometry

Aysegul Dogan[1,2*], Dagmara Kempińska-Kupczyk[2], Paweł Kubica[2], Agata Kot-Wasik[2]

[1]Department of Analytical Chemistry, Faculty of Pharmacy, Hacettepe University, 06100, Sihhiye, Ankara, Turkey.
[2]Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, 11/12 G. Narutowicza Street, 80-233 Gdańsk, Poland

Author for correspondence e-mail: *ayseguld@hacettepe.edu.tr*

Highlights:

- Development of method for chiral pharmaceuticals determination.
- Greenness assessment of developed and validated method.
- d-SPE extraction of chiral compounds from wastewater samples.
- Seasonal monitoring of selected pollutants in wastewater at enantiomeric level.

Abstract:

In this work, two different chromatographic methods for seasonal monitoring of pharmaceutical residue in wastewater samples were developed. In the case of enantiomeric separation of selected compounds, LC-MS technique combining with vancomycin based chiral stationary phase was used. The performance of chiral analysis enabled to monitor the pharmaceutical contamination at the enantiomeric level. The d-SPE procedure was developed as sample preparation step and compared with SPE protocol in terms of recoveries and environmental friendliness. Due to satisfactory recoveries (around 60%) and greener character assessed using

GAPI and AGREE tools, d-SPE-LC-MS/MS method was applied in further analysis. The concentration of detected enantiomers in wastewater collected in different seasons did not exceed 10 μg L^{-1}, whereas the evaluated EF values were generally in the range of 0.4-0.7. Moreover, no significant changes in EF values after wastewater treatment were observed.

Keywords: Chiral analysis; GAPI; AGREE; wastewater; dispersive solid phase extraction; chiral pharmaceuticals

1. Introduction

The monitoring of pharmaceutical residues in effluent can be considered as an indicator of the total pollution of the aquatic ecosystem. The sources of human pharmaceuticals in wastewater are mainly based on hospitals, health-care facilities and households, while the animal origin pharmaceutical sources are mostly from husbandry including those for non-therapeutic purposes. The amount of drugs observed can range from ng L^{-1} to μg L^{-1} levels [1–3] with regard to season, geographical location and local administration practices. Due to lipophilic character of some of these pollutants, they may enter the food-chain and accumulate in the fat tissues of aquatic organisms. The effects of presence of pharmaceuticals in the environment are different, but they may cause zooplankton and phytoplankton extinction, feminization of male individuals, bacterial resistance to antibiotics, and in fish, even kidney and bronchial damage [3,4]. Due to the water cycle and migration, many of them can be quantified in tap water and groundwater [5,6].

The National Association of Clean Water Agencies report [7] states that even if the observed quantities of pharmaceuticals in various water sources are 1000 smaller than the toxic levels, the accumulative effect on the exposed group (children, the elderly) draws attention. Even in advanced wastewater treatment plant (WWTP), where ozone and UV-assisted treatment methods are used, the pharmaceutical residues in the effluent are still at detectable levels [8–10]. Currently, there is no ecotoxicity prevention in this area, but it may be concluded that increasing use of pharmaceuticals will be the foremost environmental and human pollutants in the coming future. So far, there is no legal obligation applied for pharmaceutical residues elimination in wastewater treatment processes, but the contamination of drinking water, vegetables, meat, fishes and seafood or dairy products is a fact with growing attention while the effect of long-term exposure of pharmaceuticals to humans are not clarified by the researchers [3].

The impact of pharmaceuticals on urban pollutant load must be considered on enantiomeric levels, due to the fact that approximately 80% of the drugs are sold as a racemate mixture. The enantiomers of these racemic mixtures exhibit different physiological and toxicological effects [11]. Therefore, enantiomeric determination of pharmaceuticals in wastewater will provide important information on both the level of environmental toxicity and the concentration of pharmaceuticals that may reach human beings again [12]. However, due to the latest trends, the procedure for enantiomeric determination should be developed in accordance with

64 the principles of Green Analytical Chemistry (GAC). The aim of GAC is to reduce the impact of
65 analytical procedures on the environment. One of the most common mode of GAC application is
66 the reduction of the extraction steps in the sample preparation. Moreover, it is proposed to skip
67 some sample preparation steps, apply direct analysis, and to use eco-friendly mobile phases. In
68 case of chiral analysis, the environmental friendly approaches should be given more attention,
69 as the chromatographic run is mainly performed with high amounts of toxic solvents. Besides,
70 the elimination of the derivatization step of the analytes can be qualified as green approach as well
71 [13].

72 The aim of this study is to develop an analytical approach to the determination of chiral
73 pharmaceuticals in wastewater samples and assess its greenness. Firstly, a reversed- phase liquid
74 chromatography coupled with tandem mass spectrometry (RP-LC-MS/MS) was used to develop a
75 method for the monitoring of presence of selected pharmaceuticals in influent and effluent samples.
76 Secondly, a chiral-LC-MS/MS method was developed and validated to determine the enantiomers
77 of the selected pharmaceutical residues together with enantiomeric factor calculation. Moreover,
78 sample preparation step was carried out using two different types of extraction; solid phase
79 extraction (SPE) and dispersive solid phase extraction (d-SPE). The development of d-SPE
80 protocol enabled to reduce the solvent consumption, time needed for extraction and the labor.

2. Experimental

2.1. Analytes Selection

The selection of pharmaceuticals for analysis varied depending on the types, uses, seasons, and demographic structure in which samples were collected. The amount of drugs unchanged excreted should be at very low levels in large volume of wastewater, therefore, it was decided that frequently used pharmaceuticals would be subjected to this analysis. The six pharmaceuticals selected for these studies; atenolol (ATE), fluoxetine (FLX), ibuprofen (IBU), ketoprofen (KET), omeprazole (OME) and ofloxacin (OFL), are characterized in the **Supplementary Material, Table S1**. These pharmaceuticals belong to different groups of drugs, such as β-blockers, serotonin reuptake inhibitors, non-steroidal anti-inflammatory drugs (NSAID), proton pumps and antibiotics, which were believed to have a high frequency of occurrence in wastewater samples.

2.2. Chemicals and Materials

All standards were of the analytical purity and commercially available. The standards of pharmaceuticals, all in racemic form, were purchased from Sigma-Aldrich (St. Louis, USA). Ammonium formate, formic acid and methanol were all HPLC and bought from Merck (Darmstadt, Germany). The ultrapure water was prepared using HPL5 system from Hydrolab (Wiślina, Poland). The cartridges used for SPE were Strata-X Polymeric RP (200 mg, 3 mL) purchased from Phenomenex (Torrance, USA), Oasis HLB (200 mg, 6 mL) obtained from Waters Corporation (Milford, USA) and Lichrolut NH2 (200 mg/3 mL) purchased from Merck (Darmstadt, Germany). The d-SPE sorbents used were made of reverse-phased polymeric sorbent, silica gel modified with octadecyl group and an amino-modified silica gel, obtained from Phenomenex (Torrance, USA), Macherey-Nagel (Dueren, Germany) and Merck (Darmstadt, Germany) respectively.

The buffer solution was prepared by dissolving the required amount of ammonium formate in water and pH was maintained to 3.6 with formic acid. Mobile phase for Chiral-LC-MS/MS was prepared by dissolving required amount of ammonium formate in methanol with 0.005% formic acid.

Stock solutions of pharmaceuticals were prepared in methanol. All solutions were kept in 4°C while prepared samples were stored in -20°C until the analysis. Sodium N-methylcyclohexyl sulfate was used as the internal standard (IS).

2.2. Instruments and Analytical Conditions

All analyses were performed using a liquid chromatograph (Nexera X2, Shimadzu, Japan) coupled with triple quadrupole mass spectrometer (LCMS 8060, Shimadzu, Japan) equipped with an electrospray ionization (ESI) source operating in positive mode for ATE, FLX, OME, OFL and IBU and negative mode for KET and IS. The multiple reaction monitoring mode (MRM) was chosen for qualitative and quantitative analysis. The optimization of MRM conditions were performed using 100 ng mL^{-1} solutions of each analyte and the LC-MS system was set to work in the flow injection analysis mode (FIA). The direct injection of individual standard solutions of each analyte allowed to choose the compound precursor ion. Then, each precursor ion was fragmented in the collision cell to obtain specific product ions. Two the most intense ions were chosen as the MRM transitions for analytes. The optimized parameters of MS/MS mode are presented in Table 1.

Table 1. Parameters of the monitored ion transitions

Analyte	Precursor ion [m/z]	Product ions	Collision Energy [V]	Q1 Prerod [V]	Q3 Prerod [V]
ATE	267.00	145.20 190.20	-25 -18	-13 -13	-30 -12
FLX	310.10	44.16 148.30	-11 -8	-10 -14	-18 -15
IBU	207.10	45.20 89.10	-20 -12	-10 -16	-18 -20
KET	253.10	209.10	9	17	21
OME	346.00	198.15 151.25	-12 -20	-20 -12	-20 -14
OFL	362.10	318.20 261.15	-20 -27	-12 -10	-21 -12
IS	192.20	79.90	29	14	29

The parameters for capillary voltage (4 kV), drying gas flow (10 L min^{-1}); nebulizing gas flow (3 L min^{-1}), interface temperature (300°C), desolvation line temperature (250°C) and heat block temperature (450°C) were optimized by injecting a mixture of the analytes. The LabSolutions Software was used for data acquisition.

ACE Ultracore 2.5 SuperC18 (100 x 2.1 mm, 2.5 µm) was chosen for the analysis in RP mode. The column temperature was kept at 45°C. The flow rate was 0.7 mL min^{-1} and the injection volume was 2 µL all through the analysis. The mobile phase used for the separation consisted of 25 mM ammonium formate (pH 3.6. using formic acid) (Component A) and methanol (Component B). The gradient elution used for the chromatographic separation was as follows: 10% B in 0 min, 10% B in 1 min, 95% B in 8 min, 95% B in 10 min. After each analysis the initial conditions were restored in 5 min.

Chiral-LC-MS/MS analysis were performed using Astec Chirobiotic V column (150 x 4.6 mm). The column temperature was kept at 25 °C, the flow rate was 0.5 mL min^{-1} and the injection volume was 10 µL all through the analysis. The mobile phase used for the separation was consist of 4 mM ammonium acetate and 0.005% formic acid in methanol.

2.3. Calibration Solutions and Validation Formulas

Six-point calibration curves were prepared and analyzed several times (n = 3). Calibration solutions were prepared in MeOH in range of 0.5-25 ng mL^{-1}. For each solution, the same amount of IS was added (10 µL). Calibration curves were constructed using the internal standard method, where the ratio of analyte peak area to IS peak area was taken under the consideration. The values of limit of detection (LOD) and limit of quantification (LOQ) were calculated from the following equations: $LOD = 3.3 \times S_b/a$ and $LOQ = 3 \times LOD$, where S_b is standard deviation of the intercept of the calibration curve, and a is a slope of the calibration curve. For method validation, standard solutions were prepared at three levels (1, 5 and 10 ng mL^{-1}). These samples were used for evaluation of the accuracy and precision of the developed procedure. One series of standard sample (5 ng mL^{-1}, n= 5) was analysed for the next three days to determine the repeatability.

2.4. Sample Collection and Preparation

Average daily influent (INF) and effluent (EFF) samples were collected in winter, spring, summer and autumn from urban WWTP located in Northern Poland (Pomeranian Voivodeship). This WWTP is using mechanical, chemical, and activated biological treatment, and purify about 55 000 m^3 sewage per day. This place is surrounded by tourist cities and villages located nearby

the Baltic Sea and received mainly domestic and industrial discharges, especially from food industry (e.g., fish processing).

Collected wastewater samples were stored in amber glass bottles in a refrigerator at 4°C until extraction (no longer that 48 hours). In case of SPE, 50 mL of influent or effluent samples were passed through the conditioned cartridges, after which the cartridges were dried for 20 minutes under vacuum. Next, the analytes were eluted by methanol, mixture of methanol, acetone and ethyl acetate and finally by ammonia solution (2:1:1 v/v). The excess of solvent was removed to dryness under a gentle stream of nitrogen at 45^{o}C. Finally, the residues were dissolved in 1 mL of methanol. In case of d-SPE, 200 mg of sorbent was added to centrifuge tubes with 45 mL of filtered influent or effluent sample. The samples were shaken for 45 minutes and then centrifuged for 10 minutes. The supernatants were removed and the extraction solvents were added. Then, the extracts were filtrated in order to remove the sorbent from the samples and later evaporated to dryness. The dried extracts were dissolved in 1mL of methanol.

3. Results and Discussion

3.1. Separation of Analytes Using RP-LC-MS/MS Mode and Polar Organic Chiral Mode

In case of this study, a series of experiments was performed in order to obtain separation of six analytes, short analysis time (less than 10 minutes), as well sensitivity and reproducibility needed to determine analytes in the samples. The column with narrow diameter (2.1 mm) was chosen to reduce the amount of mobile phase used. All analyses were performed in the gradient elution mode, which was optimized together with the temperature of separation. A buffer with pH 3.6 was chosen due to improved peak shape and resolution, whereas methanol was chosen as an organic component of mobile phase. Suspecting that the test analytes in the samples are in form of racemates, the chiral separation was performed. The Chirobiotic V column bed is based on bonding vancomycin, so only basic molecules such as FLX and ATE were selected for further optimization. Due to highly polar character of FLX and ATE, long analysis time was expected. As it is performed in the literature [2,14,15], buffers with ammonium salts (formate, acetate) are effectively used in enantiomeric separation. Hence, two different mobile phase compositions were tested. The first one consisted of methanol and ammonium acetate buffer (9:10 v/v), whereas the second one

consisted of 99.95% methanol with 4 mM ammonium acetate with addition of 0.005% of formic acid. The addition of formic acid caused enantiomer peaks to be well resolved from baseline (R_s>1.5). Higher flow rates were also tested in order to minimize the analysis time but the resolution between ATE enantiomers decreased (R_s<1.0).

3.2. Chiral-LC-MS/MS Method Validation

The method dedicated to chiral analytes was validated according to the guidelines for analytical method validation [16]. The parameters such as linearity, LOD, LOQ, recoveries and repeatability were studied. The results from validation are presented in Table 2 and 3. All constructed calibration curves were linear in the analysed concentration range (0.5-25 ng mL^{-1}), with R^2 above 0.997 and LOD below 0.1 ng mL^{-1}. Due to the strict connection between obtained results and the developed method, it is recommended to calculate LOD values based on the calibration curve. The recoveries obtained for *S*-ATE, *R*-ATE, *S*-FLX and *R*-FLX were around 100%. Hence, the obtained results are satisfactory in terms of accuracy, precision and repeatability. Therefore, the developed method is suitable for determining the enantiomers of ATE and FLX in the INF and EFF samples.

Table 2. Data gathered from equations of calibration curves.

Analyte	Calibration Curve Equation	S_a	S_b	LOD [ng mL^{-1}]	LOQ [ng mL^{-1}]	R^2
S-ATE	y = 0.8641x - 0.018	0.0067	0.014	0.051	0.15	0.9991
R-ATE	y = 0.9013x - 0.020	0.0063	0.012	0.045	0.13	0.9989
S-FLX	y = 0.1335x + 0.0030	0.0015	0.0028	0.070	0.21	0.9982
R-FLX	y = 0.1335x + 0.0026	0.0019	0.0036	0.088	0.27	0.9976

Table 3. Accuracy, precision and recovery values obtained for the studied compounds.

Analyte	Spiking level [ng mL^{-1}]	Mean recovery [ng mL^{-1}] (%); n=5	SD [ng mL^{-1}]	CV [%]	Repeatability, n=5			
					Day	Mean recovery [ng mL^{-1}] (%)	SD [ng mL^{-1}]	CV [%]
S-ATE	1	1.003 (100)	0.025	2.5	1	4.81 (96)	0.12	4.2
	5	4.81 (96)	0.12	4.2	2	5.00 (100)	0.11	2.2
	10	9.89 (99)	0.39	3.9	3	4.95 (99)	0.13	2.7
R-ATE	1	1.040 (104)	0.022	2.1	1	5.02 (100)	0.20	4.0
	5	5.02 (100)	0.20	4.0	2	5.25 (104)	0.10	1.9
	10	10.04 (100)	0.27	2.7	3	5.20 (104)	0.16	3.2
S-FLX	1	1.044 (104)	0.021	2.0	1	5.08 (102)	0.22	4.3
	5	5.08 (102)	0.22	4.3	2	5.40 (108)	0.21	3.8
	10	11.52 (115)	0.54	4.7	3	5.14 (103)	0.23	4.3
R-FLX	1	1.051 (105)	0.018	1.7	1	4.93 (99)	0.21	4.2
	5	4.93 (99)	0.21	4.2	2	5.25 (105)	0.10	2.0
	10	11.33 (113)	0.50	2.7	3	5.19 (104)	0.23	4.4

3.3. Optimization of Sample Preparation Step

In order to get the highest recoveries of **ATE** and **FLX**, different SPE and d-SPE approaches were used. The choice of the best sorbent that gives an acceptable recovery for analytes with different physicochemical properties plays a crucial role in method development applied in SPE. In first step, the polarity of FLX and ATE was determined using the ALOGPs 2.1 program. This algorithm enables to calculate the octanol/water coefficient (log P) on the basis of SMILE structure. The results for FLX and ATE are presented in **Supplementary Materials, Table S2**. ATE and FLX exhibit both basic character, however ATE's log P value is lower than 1 (0.53±0.26), what is typical for hydrophilic compounds, whereas FLX (4.16 ±0.26) is high lipophylic compound (log P>3). For this reason, they have affinity for different types of sorbents. Three SPE cartridges, including Polymeric RP (Strata-X), HLB (Oasis) and silica gel modified with NH2 groups (Merck), were investigated in this work. The experiment was conducted using 50 mL of ultrapure water,

INF and EFF, which were spiked at 10 ng mL^{-1} level of each analyte. The recoveries were calculated according to following equation: $\%\ Recovery = ((C_{spiked\ and\ extracted} - C_{non\ spiked})/(C_{spiked\ before\ extraction} - C_{non\ spiked}) * 100\%$, where $C_{spiked\ and\ extracted}$ is concentration of analytes in spiked samples after extraction, $C_{spiked\ before\ extraction}$ is concentration of analytes in spiked samples before extraction and $C_{non\ spiked}$ is concentration of analytes in non-spiked samples. The recovery calculated in that way represents the loss arising from extraction step, excluding any losses by instrumental variations (e.g. matrix effects in ionization chamber). The results of SPE recoveries are presented in Fig 1. The results from NH2 cartidges were excluded due to the very low recoveries (5-36%).

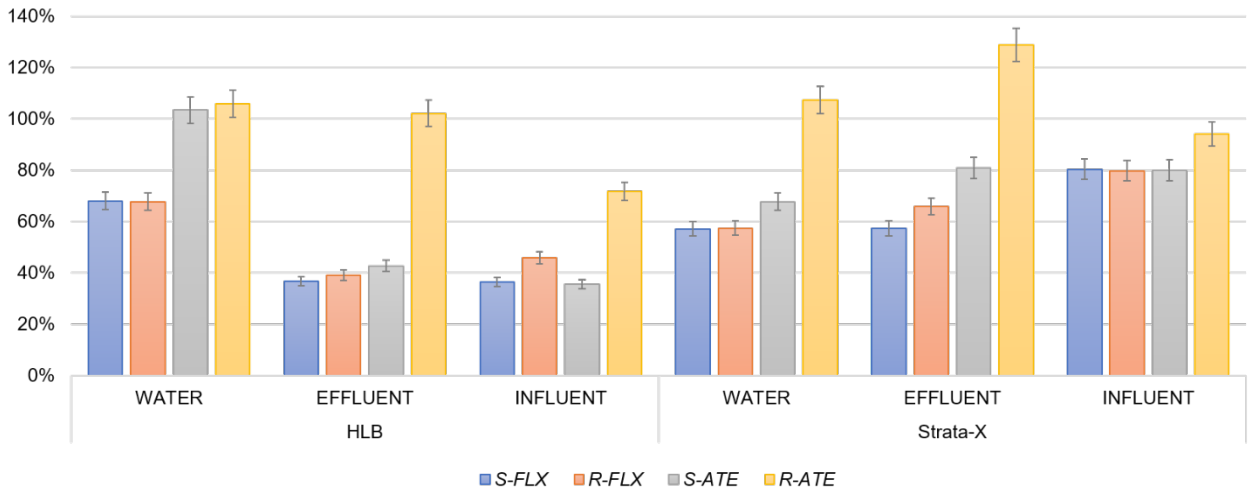


Fig 1. Effect on SPE sorbents on recovery of analytes in ultrapure water, influent and effluent samples spiked with ATE and FLX at 10 ng mL^{-1}.

The recoveries higher than 100% were obtained for *S*-ATE (Oasis HLB) and *R*-ATE (Oasis HLB and Strata-X) extracted from ultrapure water, whereas the recoveries of *S*-FLX and *R*-FLX were in the range of 50-70%. In case of real samples, lower recoveries (<50%) were obtained where the SPE was performed using Oasis HLB SPE columns. For this reason, further experiments were evaluated by using Strata-X SPE columns. Due to basic character of the analyzed compounds, the experiments were conducted under the pH of 8.0. However, to reduce the labor consumption of sample preparation step and the amount of solvents released to environment, d-SPE procedure was developed. Three different sorbents, including Polymeric RP, silica gel

modified with C18 group and silica gel modified with NH2 groups, were used in order to get the highest recoveries. Since ATE has higher affinity to hydrophilic sorbents than hydrophobic ones, it was decided to combined NH2 sorbent with Polymer RP or C18 sorbent. The experiment was conducted using 45 mL of ultrapure water, INF and EFF, which were spiked at 10 ng mL^{-1} level of each analyte and 200mg of sorbent. First d-SPE procedure was generally based on SPE procedure. The recoveries were calculated as previously. The lowest recoveries of ATE were obtained in case of mixture of C18 and NH2 sorbents (1:1). Thus, this approach was excluded from further experiments. To develop more environmentally friendly (greener) sample preparation procedure, it was decided to reduce the amount of solvent used during desorption process. Hence, two difference volumes of extraction solvents applied to desorb the analytes from the sorbent were used: 3 mL (1,5 mL of methanol and 1,5 mL of 5% ammonia solution in methanol) and 6 mL (3 mL of methanol, 1,5 mL of mixture of acetone, methanol and ethyl acetate (2:2:1 v/v) and 1,5 mL of 5% ammonia solution in methanol). Extracts were obtained from 45 mL of ultrapure water spiked at 10 ng mL^{-1} level of each targeted analyte. The results are presented in Fig.2.

High recoveries were obtained for FLX desorbed from Polymeric RP-NH2 sorbent, whereas in the case of ATE, the recoveries were lower than 30%. A significant increase in recovery values was observed for ATE desorbed from Polymeric RP sorbent with 6 mL of solvents. A slight decrease (up to 5%) was noticed for FLX desorbed from Polymeric RP sorbent with 6 mL of solvents in comparison with Polymeric RP-NH2 mixture. Hence, Polymeric RP sorbent was chosen as a preferred one, and 6 mL of solvents were decided to use as a minimum required to receive satisfactory results.

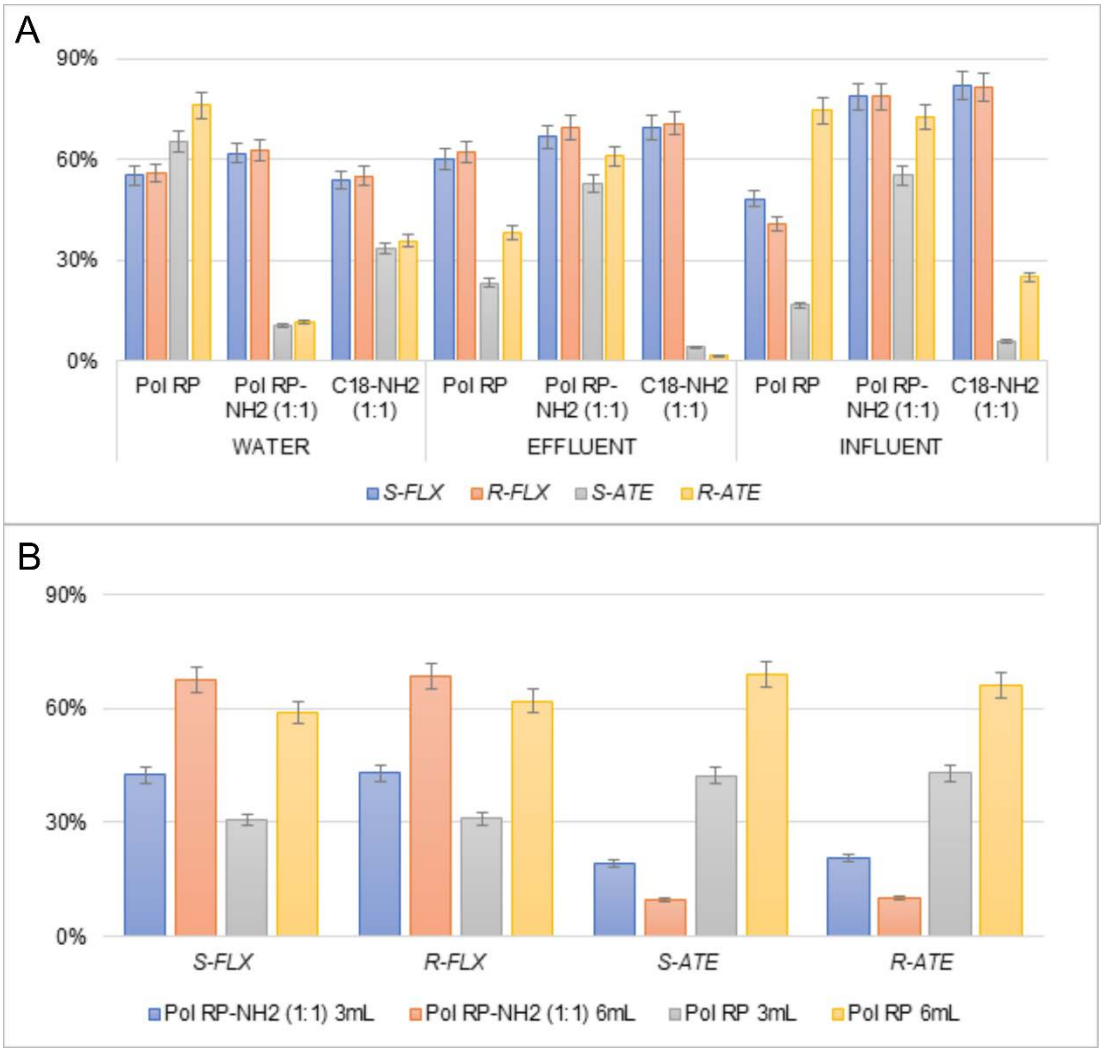


Fig 1. d-SPE optimization results presented as recoveries: a) selection of type of sorbent; b) optimization of extraction solvents volume.

3.4. Matrix effect on method performance

Due to the complexity of wastewater sample matrix, LC-MS response may be subjected to signal enhancement or suppression caused by the presence of interferents in the samples that affects analyte ionization. Therefore, the evaluation of matrix effects (%ME) is crucial to perform the reproducible and accurate quantitative analysis. In this study, %ME was evaluated by comparing the signal intensity of spiked sample extract (A_{spiked}) with response of standard in water ($A_{solvent}$) at the same concentration, according to following equation $\%ME=((A_{spiked}- A_{real\ sample})/A_{\ solvent})-1)*100\%$. The method was carried out with INF and EFF

sample by adding 10 ng mL^{-1} concentration of FLX and ATE. The obtained calculation provided information whether there was ionization enhancement (%ME> 0%) or ionization suppression (%ME<0%).

As it was expected, %ME in INF where higher than those in EFF samples. High signal suppression (-79% and 81%) was observed for ATE and FLX added to raw wastewater, while the relatively small (10-24%) ion enhancement was showed in EFF. The highest signal enhancement was obtained for *S*-ATE (24%), what may be related to the characteristics of matrix components.

3.5. Quantitative Analysis of Atenolol and Fluoxetine

ATE and FLX are thought to be among the most frequently detected pharmaceuticals in environmental samples [17]. ATE is often used to treat high blood pressure (hypertension) and congestive heart failure, whereas FLX belongs to a group of selective serotonin reuptake inhibitors (SSRIs), used to treat depression. Both depression and hypertension are civilization diseases that affect millions of people around the world [18]. Due to this fact, they are often found in wastewater INF and EFF samples as well as in sludge samples [15,19–21]. During these studies, samples of INF and EFF were collected in different seasons in order to monitor the presence of ATE and FLX at enantiomeric level. The developed and validated chiral LC-MS method was used for the analysis and the positive confirmation of all enantiomers. The transition ratio between the precursor ion m/z and the second most abundant fragment was based on European Commission Decision 2002/657/EC. In the case of these studies, the elution order of enantiomers was obtained from the literature [1,15] as *S* and *R*, respectively. The obtained results are presented in Table 4. Due to chirality of studied compounds, enantiomeric fraction (EF) was evaluated using following equation: $EF= (E_1/(E_1+E_2))$, where E_1 and E_2 are the fractions of the first and second eluting enantiomer respectively. Considering the racemate, the EF value should be 0.5, whereas in case of enantiopure compound the value of EF is 1.0 or 0 [22]. The EF values are presented in Table 5.

All concentrations of determined ATE and FLX enantiomers were in the range of 0.4-7.2 µg L^{-1}. The highest concentration was for *R*-ATE detected in INF samples collected in the summer, whereas the lowest concentrations of all enantiomers (0.4-0.7 µg L^{-1}) were found in EFF samples gathered in autumn. The concentration of *S*-FLX was generally higher than *R*-FLX in both treated and untreated wastewater. The same situation was observed in Sweden as well as in

the UK [15,19]. The *S*-FLX is considered to be more toxic to aquatic organism, therefore its presence in environmental waters raises concerns [19,20]. ATE was found to be season dependent, because the enrichment of *R*-ATE (EF= 0.3-0.4) was noticed in samples collected in spring and summer, whereas the slight increase in enrichment of S-ATE (EF= 0.53-0.63) was observed in samples collected in autumn and spring. ATE is sold as a drug in both racemate and S-enantiomer form, hence the reason of R-enrichment is presently unknown. Probably it is attributed to many factors, such as wastewater content or operational condition of WWTP. According to literature, ATE is mainly detected in INF and EFF samples as a racemate [19,20,23,24]. Due to this fact, further investigation should be performed to confirm the season dependence of ATE in wastewater in Poland.

The removal efficiency of ATE enantiomers ranges between 75 and 85% in all seasons, except from summer. The same situation can be observed for FLX enantiomers, where removal efficiency do not exceed 10%. In other cases, the slightly higher removal of *R*-FLX was observed. However, no significant changes in EF values of ATE and FLX after wastewater treatment were observed.

310 **Table 4**. Sample analysis results presented as concentration at [µg L^{-1}] level

	Spring		Summer		Autumn		Winter	
	INF	EFF	INF	EFF	INF	EFF	INF	EFF
S-ATE	4.1084±0.0052	0.803±0.025	3.08±0.28	2.65±0.12	2.506±0.024	0.646±0.081	4.34±0.67	0.6517±0.0068
R-ATE	5.105±0.081	1.148±0.026	7.2±1.2	5.25±0.37	2.244±0.074	0.418±0.064	3.708±0.083	0.690±0.028
S-FLX	1.0181±0.0022	0.798±0.052	2.81±0.45	2.58±0.37	0.97±0.13	0.704±0.044	1.55±0.25	0.8880±0.0097
R-FLX	1.55±0.72	0.568±0.032	1.46±0.17	1.43±0.31	0.64±0.13	0.485±0.044	1.55±0.72	0.534±0.012

311 **Table 5.** Enantiomeric factors of ATE and FLX calculated for different seasons

	Spring		Summer		Autumn		Winter	
	INF	EFF	INF	EFF	INF	EFF	INF	EFF
ATE	0.45	0.41	0.30	0.34	0.53	0.61	0.54	0.49
FLX	0.40	0.48	0.66	0.64	0.60	0.59	0.50	0.62

312

3.5. Assessment of greenness of developed methods

To evaluate a 'green' character of developed methods, GAPI and Analytical GREEnness (AGREE) calculator were applied. The GAPI is an index to "green assessment" of analytical protocol in terms of the amount and type of waste, environmental hazard, chemical health as well as energy requirements. The results of this assessment are presented in pictorial form covering all stages of the methodology, from sampling to final determination [13,25]. The second tool, Analytical GREEnness calculator, is a new assessment approach proposed by Pena-Pereira et al. [26]. The evaluation criteria of AGREE were taken from the twelve principles of green analytical chemistry and transformed into 0-1 range**.** The higher average score the method receives, the greener it is. In these studies, SPE-LC-MS/MS method was compared with d-SPE- LC-MS/MS method to assess the effect of sample preparation change on greenness of method. In addition, the greener procedure was compared to one reported previously in literature [19]. The results from GAPI and AGREE tools are presented in Fig 3.

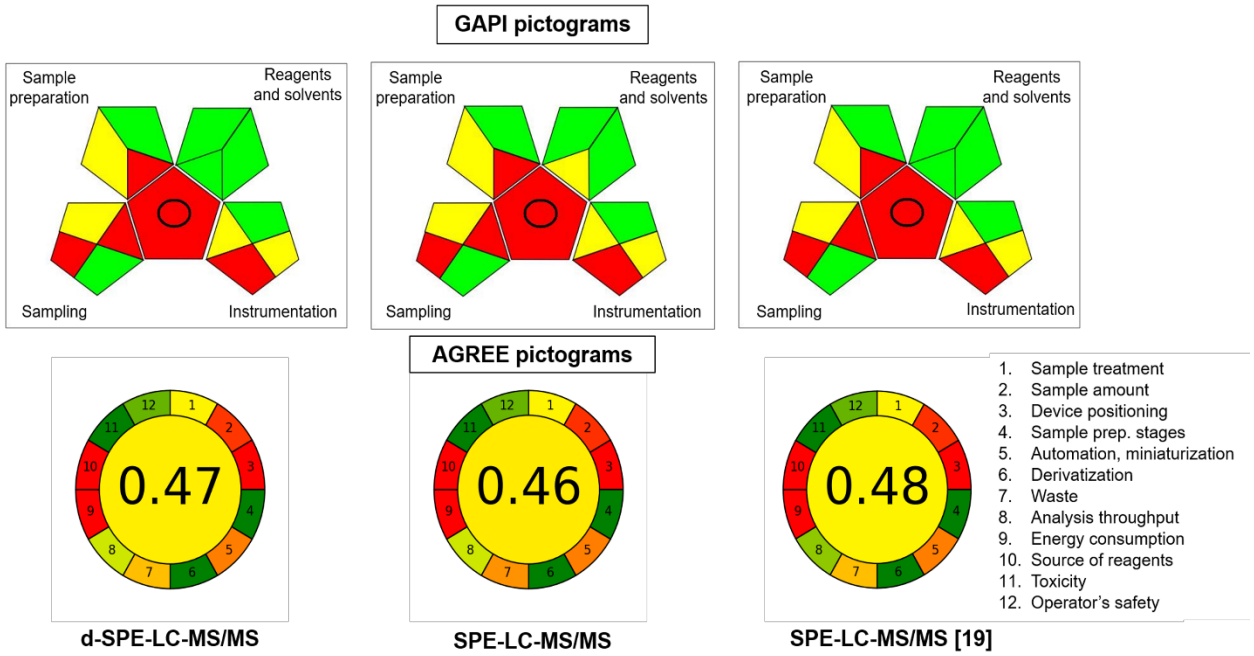


Fig. 3. Greenness assessment of developed methods for chiral separation using GAPI and AGREE tools

LC-MS is generally not environmentally friendly technique due to a large amount of solvent used and high energy consumption. Nevertheless, the use of GAPI tool allowed to compare

different methods and select the greener approach for this research. In the case of d-SPE-LC-MS/MS method, there is a significant difference in the 'Reagent and Solvents' part. Meaning, to perform d-SPE extraction the amount of solvents required is much smaller. According to the scores obtained in AGREE tool (both around 0.50), there is no significant difference between these two approaches, however, the final score of d-SPE-LC-MS/MS method (score= 0.47) is slightly higher than SPE-LC-MS/MS method (score= 0.46). Both methods have the same strong drawbacks: off-line sampling, high energy consumption and use of reagents from non-green sources. On the other hand, the use of a vancomycin packed column for the chiral separation allows to avoid the derivatization step, thus prevents the release of hazardous substances into the environment. Moreover, water and methanol, that are considered as green solvents, were used both in sample preparation and analysis steps. Still, two parameters differ these methods: the amount of sample required and amount of waste generated which is smaller in case of d-SPE. Hence, it was concluded that the d-SPE-LC-MS/MS method is marginally greener than the method with SPE extraction in a sample preparation step.

In comparison with the method developed by Evans et al. [19], there is no significant difference between its GAPI pictogram and d-SPE approach pictogram. According to AGREE results, the method reported earlier obtained a better score. Despite similar advantages and disadvantages in terms of greenness, the method from literature has higher analysis throughput, which slightly influence the final result. However, the method reported in this paper is newly developed, therefore extensive research should be carried out in order to broaden the range of determining analytes as a part of future studies.

3.6. Environmental Application of RP-LC-MS/MS Method

The first method developed in this studies was applied to determine 6 pharmaceuticals in INF and EFF samples collected in different seasons. All selected compounds were found in INF samples, whereas only KET was not detected in EFF samples, expect from those gathered in winter. Detection of profens (IBU, KET) in winter EFF is connected with flu and cold season. Due to the fact that KET and IBU belong do NSAIDs group, they are generally easily available and often taken to reduce the fever. No significant difference was observed in the occurrence of ATE, FLX and OME in both INF and EFF samples collected in various seasons. This is probably related

to long-term treatment with these compounds and explains the constant release of them into the environment. The last analyte, OFL, was found in every INF and EFF sample. OFL is useful antibiotic for the treatment of a numerous of bacterial infections, so its presence in wastewater is often confirmed [27].

4. Summary

The presented studies show the occurrence of six frequently prescribed pharmaceuticals in wastewater samples and chiral separation of ATE and FLX. Both analytes were monitored seasonally at the enantiomeric level. The enantiomeric compositions of analysed compounds presented racemic to weakly enantioselective, with the highest EF value (0.66) for FLX detected in the summer. It was also noticed that the content of ATE enantiomers in wastewater may be seasonal dependent, however, further investigations to confirm it are still required. In order to reduce the solvent consumption and time-consuming of sample preparation step, d-SPE protocol was developed. Due to the trend of working in accordance with the idea of a sustainable environment, the evaluation of environmental impact of these methods was performed. The assessment of greenness of proposed methods was carried out using two different tools: GAPI and AGREE. In both cases, the results indicates that using d-SPE instead of SPE has a slightly lower impact on the environment. Moreover, both final scores of AGREE were relatively high (around 0.50), which can be interpreted as quite good results as the categories of this tool are very strict and demanding. However, further research to develop a faster, cheaper and more environmentally friendly procedure for chiral separation should be performed.

5. Funding

The research is funded by the Scientific and Technological Research Council of Turkey (TUBITAK) within the TUBITAK-BIDEB 2219-International Postdoctoral Research Scholarship Programme.

6. Acknowledgments

Authors would like to thank Hubert Byliński for providing samples from the wastewater treatment plant and Wojciech Wojnowski for his helpful advice in the use of greenness assessment tools.

7. Literature

[1] R. Ma, B. Wang, S. Lu, Y. Zhang, L. Yin, J. Huang, S. Deng, Y. Wang, G. Yu, Characterization of pharmaceutically active compounds in Dongting Lake, China: Occurrence, chiral profiling and environmental risk, Sci. Total Environ. 557–558 (2016) 268–275. doi:10.1016/j.scitotenv.2016.03.053.

[2] B. Petrie, J. Youdan, R. Barden, B. Kasprzyk-Hordern, Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry, J. Chromatogr. A. 1431 (2016) 64–78. doi:10.1016/j.chroma.2015.12.036.

[3] J. Jose, J. Sandra Pinto, B. Kotian, A. Mathew Thomas, R. Narayana Charyulu, Comparison of the regulatory outline of ecopharmacovigilance of pharmaceuticals in Europe, USA, Japan and Australia, Sci. Total Environ. 709 (2020) 134815. doi:10.1016/j.scitotenv.2019.134815.

[4] A. Küster, N. Adler, Pharmaceuticals in the environment: Scientific evidence of risks and its regulation, Philos. Trans. R. Soc. B Biol. Sci. 369 (2014). doi:10.1098/rstb.2013.0587.

[5] M. Liu, H. Yin, Q. Wu, Occurrence and health risk assessment of pharmaceutical and personal care products (PPCPs) in tap water of Shanghai, Ecotoxicol. Environ. Saf. 183 (2019) 109497. doi:10.1016/j.ecoenv.2019.109497.

[6] L. Charuaud, E. Jardé, A. Jaffrézic, M. Liotaud, Q. Goyat, F. Mercier, B. Le Bot, Veterinary pharmaceutical residues in water resources and tap water in an intensive husbandry area in France, Sci. Total Environ. 664 (2019) 605–615. doi:10.1016/j.scitotenv.2019.01.303.

[7] NACWA.ORG, (n.d.). https://www.nacwa.org/ (accessed May 25, 2020).

[8] P. Schröder, B. Helmreich, B. Škrbić, M. Carballa, M. Papa, C. Pastore, Z. Emre, A. Oehmen, A. Langenhoff, M. Molinos, J. Dvarioniene, C. Huber, K.P. Tsagarakis, E. Martinez-Lopez, S.M. Pagano, C. Vogelsang, G. Mascolo, Status of hormones and painkillers in wastewater effluents across several European states—considerations for the EU watch list concerning estradiols and diclofenac, Environ. Sci. Pollut. Res. 23 (2016) 12835–12866. doi:10.1007/s11356-016-6503-x.

[9] C. Adams, Y. Wang, K. Loftin, M. Meyer, Removal of Antibiotics from Surface and Distilled Water in Conventional Water Treatment Processes, J. Environ. Eng. 128 (2002) 253–260. doi:10.1061/(ASCE)0733-9372(2002)128:3(253).

[10] A. Bahlmann, W. Brack, R.J. Schneider, M. Krauss, Carbamazepine and its metabolites in wastewater: Analytical pitfalls and occurrence in Germany and Portugal, Water Res. 57 (2014) 104–114. doi:10.1016/J.WATRES.2014.03.022.

[11] M. Eichelbaum, Side effects and toxic reactions of chiral drugs: a clinical perspective., Arch. Toxicol. Suppl. 17 (1995) 514–521. doi:10.1007/978-3-642-79451-3_44.

[12] M.S. Kostich, A.L. Batt, J.M. Lazorchak, Concentrations of prioritized pharmaceuticals in effluents from 50 large wastewater treatment plants in the US and implications for risk estimation, Environ. Pollut. 184 (2014) 354–359. doi:10.1016/j.envpol.2013.09.013.

[13] J. Płotka-Wasylka, A new tool for the evaluation of the analytical procedure: Green Analytical Procedure Index, Talanta. 181 (2018) 204–209. doi:10.1016/j.talanta.2018.01.013.

[14] M. Li, X. Liang, X. Guo, X. Di, Z. Jiang, Enantiomeric separation and enantioselective determination of some representive non-steroidal anti-inflammatory drug enantiomers in fish tissues by using chiral liquid chromatography coupled with tandem mass spectrometry, Microchem. J. 153 (2020) 104511. doi:10.1016/j.microc.2019.104511.

[15] A.R. Ribeiro, L.H.M.L.M. Santos, A.S. Maia, C. Delerue-Matos, P.M.L. Castro, M.E. Tiritan, Enantiomeric fraction evaluation of pharmaceuticals in environmental matrices by liquid chromatography-tandem mass spectrometry, J. Chromatogr. A. 1363 (2014) 226–235. doi:10.1016/j.chroma.2014.06.099.

[16] P. Konieczka, J. Namieśnik, Quality assurance and quality control in the analytical chemical laboratory: a practical approach, 2nd ed, Anal. Bioanal. Chem. 411 (2019) 5–6. doi:10.1007/s00216-018-1461-4.

[17] A. Szymonik, A. Lach, Pharmaceuticals in surface and drinking water, Proceddings of ECOpole, DOI:10.2429/Proc.2013.7(2)096. 7 (2013) 23–26. doi:10.2429/proc.2013.7(2)096.

[18] Z. Li, Y. Li, L. Chen, P. Chen, Y. Hu, H. Wang, Prevalence of depression in patients with hypertension: A systematic review and meta-analysis, Med. (United States). 94 (2015). doi:10.1097/MD.0000000000001317.

[19] S.E. Evans, P. Davies, A. Lubben, B. Kasprzyk-Hordern, Determination of chiral pharmaceuticals and illicit drugs in wastewater and sludge using microwave assisted extraction, solid-phase extraction and chiral liquid chromatography coupled with tandem mass spectrometry, Anal. Chim. Acta. 882 (2015) 112–126. doi:10.1016/J.ACA.2015.03.039.

[20] L. Duan, Y. Zhang, B. Wang, S. Deng, J. Huang, Y. Wang, G. Yu, Occurrence, elimination, enantiomeric distribution and intra-day variations of chiral pharmaceuticals in major wastewater treatment plants in Beijing, China, Environ. Pollut. 239 (2018) 473–482. doi:10.1016/j.envpol.2018.04.014.

[21] S. Evans, J. Bagnall, B. Kasprzyk-Hordern, Enantiomeric profiling of a chemically diverse mixture of chiral pharmaceuticals in urban water, Environ. Pollut. 230 (2017) 368–377. doi:10.1016/j.envpol.2017.06.070.

[22] E. Sanganyado, Z. Lu, W. Liu, Application of enantiomeric fractions in environmental forensics: Uncertainties and inconsistencies, Environ. Res. 184 (2020) 109354. doi:10.1016/j.envres.2020.109354.

[23] E. Sanganyado, Z. Lu, Q. Fu, D. Schlenk, J. Gan, Chiral pharmaceuticals: A review on their environmental occurrence and fate processes, Water Res. 124 (2017) 527–542. doi:10.1016/j.watres.2017.08.003.

[24] B. Petrie, K. Proctor, J. Youdan, R. Barden, B. Kasprzyk-Hordern, Critical evaluation of monitoring strategy for the multi-residue determination of 90 chiral and achiral micropollutants in effluent wastewater, Sci. Total Environ. 579 (2017) 569–578. doi:10.1016/j.scitotenv.2016.11.059.

[25] W. Wojnowski, J. Namieśnik, J. Płotka-Wasylka, Dispersive liquid-liquid microextraction combined with gas chromatography–mass spectrometry for in situ determination of biogenic amines in meat: Estimation of meat's freshness, Microchem. J. 145 (2019) 130–138.

doi:10.1016/j.microc.2018.10.034.

[26] F. Pena-Pereira, W. Wojnowski, M. Tobiszewski, AGREE – Analytical GREEnness metric approach and software, Anal. Chem. (2020) acs.analchem.0c01887. doi:10.1021/acs.analchem.0c01887.

[27] B. Shao, X. Sun, J. Zhang, J. Hu, H. Dong, Y. Yang, Determination of ofloxacin enantiomers in sewage using two-step solid-phase extraction and liquid chromatography with fluorescence detection, J. Chromatogr. A. 1182 (2008) 77–84. doi:10.1016/j.chroma.2007.12.073.

Supplementary material

Analysis of chiral pharmaceutical residues in influent and effluent samples at racemic and enantiomeric level using liquid chromatography-tandem mass spectrometry

Aysegul Dogan[1,2*], Dagmara Kempińska-Kupczyk[2], Paweł Kubica[2], Agata Kot-Wasik[2]

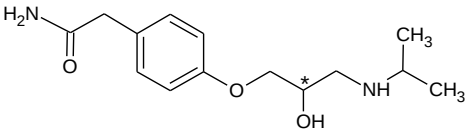
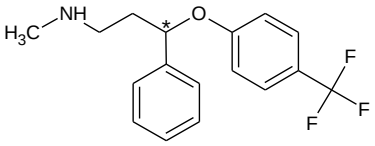
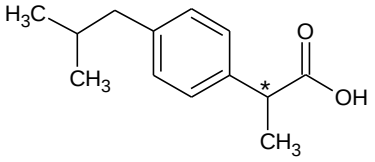
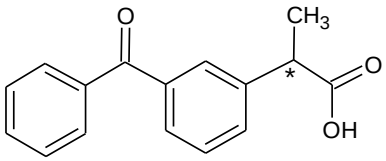
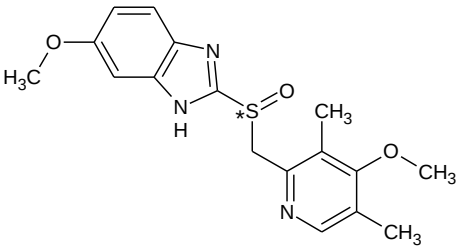
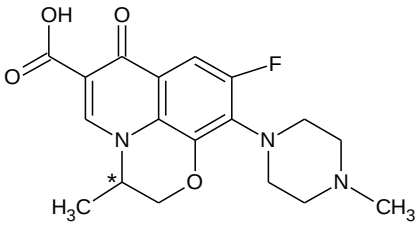
[1]*Department of Analytical Chemistry, Faculty of Pharmacy, Hacettepe University, 06100, Sihhiye, Ankara, Turkey.*

[2]*Department of Analytical Chemistry, Faculty of Chemistry, Gdańsk University of Technology, 11/12 G. Narutowicza Street, 80-233 Gdańsk, Poland*

Author for correspondence e-mail: *ayseguld@hacettepe.edu.tr*

Table S1

Analytes selected for analysis.

Compound	Indication	Molecular weight (g/mol)	pKa	Structure
Atenolol (ATE)	β-blocker	266	9.6	
Fluoxetine (FLX)	seretonin reuptake inhibitor	309.3	9.8	
Ibuprofen (IBU)	NSAID	206.28	5.3	
Ketoprofen (KET)	NSAID	254.28	4.45	
Omeprazole (OME)	proton pump inhibitor	345.4	4.77-9.29	
Ofloxacin (OFL)	antibiotic	361.4	5.97-9.28	

* represents chiral centre

Table S2

Lipophilicity descriptors calculated using the ALOGPs 2.1 program

Descriptor	A LOGPs	ACLOG P	A LOG P	M LOGP	XLOG P2	XLOG P3	Log P Av.	Log S
Analyte								
ATE	0.57	0.41	0.67	0.93	0.46	0.16	0.53±0.26	-2.41
FLX	4.09	3.96	4.03	4.15	4.65	4.05	4.16±0.25	-4.41