

Trace Analysis of Polar Pharmaceuticals in Wastewater by LC–MS–MS: Comparison of Membrane Bioreactor and Activated Sludge Systems

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Abstract

In order to assess the efficiency of wastewater treatment plants in removing pharmaceuticals from wastewater, sensitive and reliable methods are necessary for trace analysis of these micropollutants in the presence of a highly complex matrix. In this study, conventional activated sludge (CAS) and membrane bioreactor (MBR) treatment systems are compared in eliminating pharmaceuticals in wastewater. The pharmaceuticals investigated include aceclofenac, carbamazepine, diclofenac, enalapril, and trimethoprim. Analysis is performed using a liquid chromatograph with hybrid linear ion-trap mass spectrometer equipped with a polar reversed-phase column to achieve good separation and minimize matrix effects. To pre-concentrate the samples, the use of two types of solid-phase extraction packing materials in tandem assures good recoveries of all the target analytes. In the influent, the concentration of these compounds ranges from 0.09 to 1.4 µg/L. Diclofenac shows resistance to degradation in the CAS but is amenable to degradation in the MBR. Trimethoprim and enalapril are only slightly eliminated in the CAS but are reduced by more than 95% in the MBR. Carbamazepine removal is negligible, while aceclofenac is only 50% reduced in CAS and MBR. In general, these results indicate that MBR has a higher efficiency in removing some polar pharmaceuticals in wastewater.

Introduction

The presence of certain pharmaceuticals in ground and surface waters (1,2) is a serious environmental problem because these compounds are biologically active and could potentially affect non-target and susceptible species. Pharmaceutical residues in the environment have the potential to elicit deleterious effects in some organisms such as crustaceans (3) and amphipods (4). Because most pharmaceuticals are relatively polar, their adsorption to soil or particulates could be of little importance; hence, most of these compounds are mobile in the

environment. Persistent polar pharmaceuticals may reach drinking water sources and may become a serious problem in places that depend highly on recycled water, such as in France (1), United States (5), and Australia (6).

The occurrence of pharmaceuticals in the environment indicates incomplete removal of these drugs from municipal wastewater treatment plants (WWTP). In addition, agricultural runoffs may also contribute to pharmaceutical pollution of water resources due to irrigation with WWTP effluent (7). The most widely used process for wastewater treatment is through conventional activated sludge (CAS) systems, which utilizes the flocculent suspension of microbial mass (8). In this process, wastewater is mixed with the bacterial population in an aeration tank, which is then transported to a sedimentation tank where the flocculated biomass settles while the effluent goes on to the next step. Membrane bioreactor (MBR) is a system that combines the biological treatment of microorganisms and the membrane separation process, which replaces the secondary clarifiers into a single step (9,10). The influent or feed water is mixed with the biomass, and this mixture is filtered through the membrane, separating the biomass from the treated water. There are several advantages of using MBR. The main benefit of MBR over CAS is that the amount of suspended solids remaining in the effluent of MBR is much lower than in CAS, resulting in a better quality treated water (9). The low turbidity of the effluent water makes it more amenable to further treatment (10). Another benefit of MBR results from its inherently high sludge age, which allows for slow-growing bacteria to develop (9), leading to enhanced degradation of some compounds, such as trimethoprim, as observed in previous work (11). A recent study reported the elimination of six acidic drugs in two MBR systems with different sludge retention times and compared it to the removal in CAS (12). It was found that the MBR performed better over the CAS. The study on MBR application for wastewater treatment followed by more advanced methods is also available (13). Earlier publications reported varying removal efficiencies of several pharmaceuticals in MBR (14–17).

The aim of this work is to evaluate the relative efficiencies of a

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full-scale CAS in a municipal WWTP and a pilot-scale MBR installed within the plant, in eliminating the five polar pharmaceuticals listed in Table I. This WWTP system is unique because the influent water is split between the CAS and MBR, hence a direct comparison of the overall removal efficiencies can be made. The model pharmaceuticals selected in this study were chosen because they are known to be present in surface waters (except aceclofenac, which has not been detected before) and represent a wide range of usage and chemical properties. Aceclofenac is included in this study because of its potential to be present in the environment, considering that it has a similar structure to diclofenac, which is persistent in the environment. To date, no literature has been found on the detection of aceclofenac in either wastewater or surface water.

One of the techniques commonly used to detect and quantify pharmaceutical compounds is liquid chromatography–tandem mass spectrometry (LC–MS–MS) (18,19). Under selected reaction monitoring (SRM) mode in LC–MS–MS, the sensitivity and selectivity of the analytical method is significantly improved. The capability of an instrument to perform tandem MS is especially useful when analyzing samples with complex matrices, such as sludge, wastewater, and WWTP effluent. This is because in addition to the chromatographic retention time, the confidence in identifying target analytes can be increased by selecting the molecular ion as a precursor ion, and two or more product ions for monitoring to meet the ideal number of identification points. The use of an ultra-performance liquid chromatograph (UPLC) equipped with a triple quadrupole mass spectrometer detector operated under SRM mode has become increasingly popular in analyzing for pharmaceuticals in wastewater influent and effluent (20,21).

Experimental

Chemicals

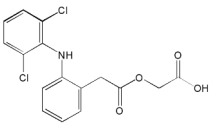
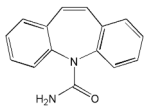
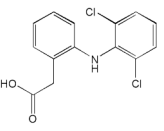
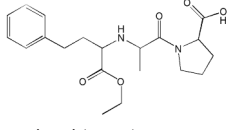
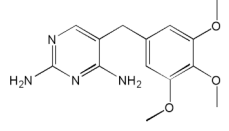
Carbamazepine and enalapril were purchased from Sigma Aldrich (Munich, Germany), diclofenac was purchased from Jescuder (Rubí, Spain), aceclofenac was obtained from Toronto Research Chemicals Inc. (Toronto, Canada), trimethoprim was obtained from Riedel de Hæen (Steinheim, Germany), and carbamazepine- d_{10} was obtained from CDN Isotopes Inc. (Quebec, Canada). Oasis HLB SPE cartridges (6 cc/200 mg) were obtained from Waters (Milford, MA). Isolute ENV+ (6 mL/200 mg) SPE cartridges were obtained from Biotage (Uppsala, Sweden). Synergi Polar RP-100 column was donated by Phenomenex (Torrance, CA).

The solvents used were HPLC-grade, purchased from Merck (Darmstadt, Germany).

Wastewater treatment plant

Samples for this study were taken from the Rubí municipal WWTP in Barcelona, Spain. This WWTP receives an average daily flow of 22,000 m³/day of municipal, hospital, and industrial wastewater. The plant employs a CAS system for the treatment of the wastewater (22). The hydraulic retention time (HRT) and the sludge retention time (SRT) were calculated to be approximately 12 h and three days, respectively. A pilot scale-MBR plant was set-up within the plant and received the same inflow as that of the CAS (22). The MBR consisted of two flat sheet membranes, each with an area of 0.106 m² and a nominal porosity of 0.4 μ m (Kubota; Osaka, Japan), submerged in a 21 L active volume of inoculated sludge. The HRT was 14 h, while the SRT was infinite because no sludge was removed from the reactor. Oxygen con-

Table I. Structures, pKas, Water Solubilities, and Uses of the Compounds

Compound	pKa*	log K _{ow} [†]	Water solubility* (mmol/L)	Use [§]
 Aceclofenac (ACF)	2.60	4.16	75	non-steroidal anti-inflammatory agent
 Carbamazepine (CBZ)	13.94	2.67	0.33	anticonvulsant
 Diclofenac (DCF)	4.18	4.06	29	non-steroidal anti-inflammatory agent
 Enalapril (ENAL)	3.17	2.43	4.8	angiotensin-converting enzyme inhibitor for treating hypertension
 Trimethoprim (TMP)	7.20	0.791	3.8	anti-infective agent, urinary, antimalarial folic acid antagonist

* Calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris.

[†] log P.

[‡] pH 7.0, 25°C.

[§] Source: <http://pubchem.ncbi.nlm.nih.gov>.

centration was maintained between 1–2 mg/L by continuous aeration. Composite samples were collected every hour for 24 h from the influent that feeds the CAS and MBR, the WWTP effluent, and the MBR effluent. Wastewater was collected four times during the entire month of July (2007).

Sample preparation

Two hundred milliliters of the influent and 400 mL each of the effluent composite samples from CAS and MBR were filtered through a 1.0- μm glass filter and a 0.45- μm nylon filter. Clogging was minimized by changing both filters several times. Duplicate samples were extracted from each sampling interval. Prior to SPE, the pH of the filtered water was adjusted to 3.0 using concentrated HCl. The extraction of the compounds was accomplished using a tandem solid-phase extraction (SPE) method consisting of an Oasis HLB cartridge from Waters (200 mg, 6 mL) placed on top of Isolute ENV+ from Biotage (200 mg, 6 mL). The cartridges were conditioned with 3 mL \times 2 methanol and 3 mL \times 2 deionized water at pH 3.0. The samples were loaded at a flow rate of 1–3 mL/min. The sorbent was dried by allowing the vacuum to run for \sim 1 min after all the water samples had passed through the cartridges. The compounds were eluted from the SPE cartridges using 2 mL methanol three times, followed by 2 mL ethyl acetate three times. The eluate was dried using nitrogen and reconstituted with 1 mL of 95:5 v/v water–acetonitrile. The samples were transferred to 2-mL vials containing 20 μL of 10 $\mu\text{g}/\text{mL}$ carbamazepine-d10 to correct for volume changes.

LC and MS

The separation of the compounds was performed on Agilent 1100 Series Chromatograph using Synergi Polar-RP column with a particle size of 2.5 μm and a dimension of 100 \times 3.00 mm. The packing material of this column is silica with an ether-linked phenyl group and a polar endcapping. This stationary phase is ideal for increasing the retention time of highly polar and aro-

matic analytes, hence improving method selectivity (manufacturer's catalog). The mobile phases used were (A) acetonitrile and (B) 10mM formic acid (adjusted to pH 2.79 with ammonia). A gradient elution was employed starting with 5% A for 1 min, ramped to 95% A within 8 min, maintained at 95% A for 3 min, and back down to 5% A in 1 min. The column was re-equilibrated at 5% A for 5 min corresponding to a total run time of 17 min using a flow rate of 400 $\mu\text{L}/\text{min}$. The sample injection volume was 10 μL . Column temperature was not controlled and was at room temperature.

The detection of the compounds was carried out in a hybrid triple quadrupole/linear ion trap MS (Applied Biosystems 4000 Q TRAP) equipped with an electrospray ionization source (TurboIonSpray) conducted in positive mode and operated using SRM. The selection of the precursor ion and the product ions as well as optimization of the declustering potential, collision energy, cell exit potential, TurboIonSpray source temperature, collision gas, and ion spray voltage were performed by continuously injecting a standard solution of 5–10 ppm of each compound directly to the mass spectrometer. Declustering potential, collision energy, and cell exit potential varied for each analyte. The rest of the MS conditions for all analytes used the following: TurboIonSpray source temperature of 700 $^{\circ}\text{C}$, ion spray voltage of 5.5 kV, curtain gas of 30 psi, ion source gas 1 & 2 of 50 psi, and collision gas set to high. Two product ions were monitored; the more intense ion was used for quantitation. The fragment ions for each compound are listed in Table II. External standard calibration made from 5.0 to 200 ng/mL in water was prepared to calculate the concentration of the compounds. Replicate samples collected for each day were analyzed in between each set of standards.

Results and Discussion

Sample preparation and analysis

Because of the wide range of polarity and acid–base properties of the analytes in this study, a tandem SPE using Oasis HLB and Isolute ENV+ was used to optimize the extraction of the five target pharmaceuticals. In an attempt to increase the recovery of polar compounds, a higher capacity of the adsorbent material for SPE was used. However, a lower amount of SPE adsorbent material was not tested for recovery. The sorbent in Oasis HLB is based on a copolymer of hydrophilic *N*-vinylpyrrolidone and hydrophobic divinylbenzene. The selection of this SPE material is based on previous work that used Oasis HLB to extract pharmaceuticals from wastewater (23,24). In a recent work by Vanderford and Snyder (2006), between 72% to 113% recoveries were reported using Oasis HLB for several pharmaceuticals, which included carbamazepine, diclofenac, enalapril, and trimethoprim (25). Isolute ENV+ sorbent is made of a copolymer of hydroxylated polystyrene and divinylbenzene. This material was included in the extraction procedure because this was found to be effective in extracting very polar compounds, such as the iodinated contrast agents (26). Methanol is a widely used extraction solvent and was adapted in the procedure. The addition of ethylacetate in the elution step improved the percent recovery

Table II. Fragment Ions Monitored in SRM Mode

Compound	Precursor ion [M+H] ⁺	Product ions	Declustering potential	Collision energy	Cell exit potential
Aceclofenac	354	215*, 214	66	31, 57	12, 32
	356	216, 214	61	61, 53	12, 18
Carbamazepine	237	194*	91	29	16
		192	91	31	12
Diclofenac	296	215,	41	29	14
		214*	41	45	16
Enalapril	377	303,	71	33	54
		234*	61	31	20
Trimethoprim	291	261,	136	35	4
		230*	136	35	6

* Ion used for quantitation.

for diclofenac. The sample pH was adjusted to 3 because it was observed that higher SPE recoveries were obtained compared to the extraction performed at pH 7. The SPE recoveries for these target analytes in spiked influent and effluent matrices ranged from < 50–182%, as shown in Table III. The CAS effluent was used for the SPE recovery instead of the MBR effluent because the CAS effluent matrix is worse than that of the MBR effluent; hence it was assumed that the recoveries in the effluent are at the worse case scenario. The wide variability in recoveries is typically observed in analyzing pharmaceuticals in wastewater and in other environmental samples due to high matrix effects and can be alleviated by using isotope dilution MS (25,27,28). Because every compound is affected differently by the matrix, resulting in varying degrees of ionization suppression and enhancement in LC–MS–MS, quantification is ideally done using a stable isotope labeled equivalent of each compound. Unfortunately, only d10-

carbamazepine was used in this study due to the cost of isotopically labeled reference compounds. Nevertheless, because several replicates of the composite samples were analyzed for each treatment system, the results of the study comparing the removal efficiencies of CAS and MBR should provide a reliable estimate of how effective these treatment processes are for the selected pharmaceuticals. The instrument detection limits were calculated as three times the signal-to-noise ratio for the five compounds ranging from 0.01 to 0.15 $\mu\text{g}/\mu\text{L}$. The method detection limits for aceclofenac and diclofenac, which is calculated as five times the signal-to-noise ratio, ranged from 0.0002–0.0025 $\mu\text{g}/\text{L}$ in influent and CAS effluent wastewater (29). The method detection limits of the other compounds were not obtained but are expected to be within the same range.

Different aqueous mobile phases were tested to improve the separation of the five pharmaceuticals. Acetonitrile was the organic mobile phase used throughout the optimization of chromatography. Other solvent systems that were investigated such as: (i) water with 0.3% HCOOH, (ii) 1mM NH_4HCO_2 –0.1% HCOOH–0.2% acetonitrile at pH 2.8, and (iii) 50mM $\text{NH}_4\text{CH}_3\text{CO}_2$ at pH 4.7 all gave unsatisfactory peak shapes of either diclofenac or enalapril (Figure 1). Peak splitting has been observed for enalapril, which is attributed to the presence of cis- and trans-conformational isomers (30). Solvent systems composed of water with 0.2% HCOOH or 10mM formic acid adjusted to pH 2.8 with ammonia resulted in acceptable peak shapes (Figure 2) and also improved the ionization efficiencies in LC–MS–MS. For this work, the aqueous mobile phase selected is 10mM formic acid adjusted to pH 2.8 with ammonia.

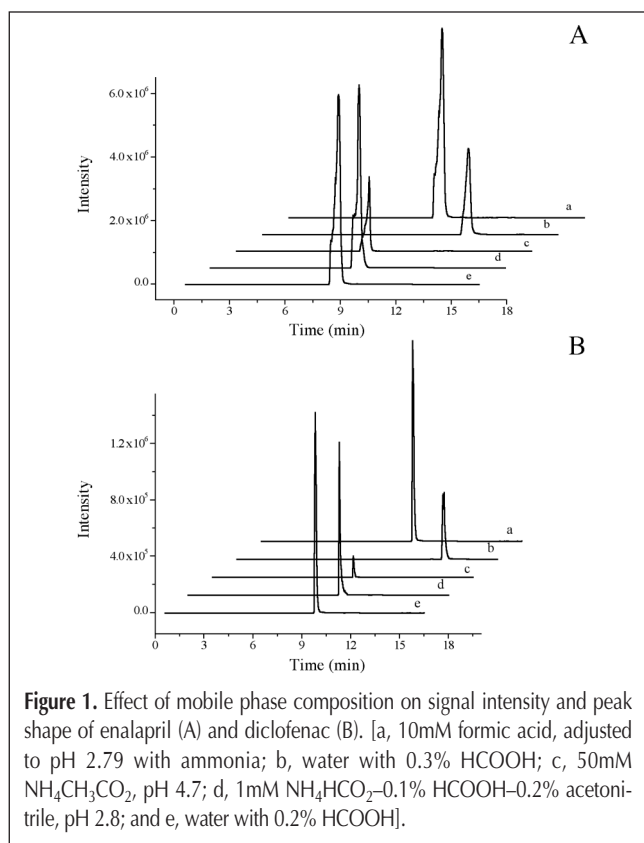


Figure 1. Effect of mobile phase composition on signal intensity and peak shape of enalapril (A) and diclofenac (B). [a, 10mM formic acid, adjusted to pH 2.79 with ammonia; b, water with 0.3% HCOOH; c, 50mM $\text{NH}_4\text{CH}_3\text{CO}_2$, pH 4.7; d, 1mM NH_4HCO_2 –0.1% HCOOH–0.2% acetonitrile, pH 2.8; and e, water with 0.2% HCOOH].

Table III. SPE Recoveries of the Analytes in the Influent Spiked at 1 $\mu\text{g}/\text{L}$ and 10 $\mu\text{g}/\text{L}$, and Effluent Spiked at 2 $\mu\text{g}/\text{L}$ Matrices at pH 3 ($n = 3$)

Compound	Influent (1 $\mu\text{g}/\text{L}$)*	Influent (10 $\mu\text{g}/\text{L}$)*	Effluent*
Aceclofenac	102 \pm 20	< 50	100 \pm 10
Carbamazepine	122 \pm 9	182 \pm 20	87 \pm 7
Diclofenac	74 \pm 10	< 50	58 \pm 30
Enalapril	< 50	123 \pm 30	69 \pm 8
Trimethoprim	nd [†]	< 50	< 50

* Recovery \pm RSD (%).
[†] nd = not detected.

Removal of pharmaceuticals in biological treatment systems

The wastewater treatment technology has been advancing in an effort to provide a cleaner effluent to the environment, particularly in places where water is scarce and water re-use is significant for domestic water supply. Pharmaceutical residues are continuously released into the environment through WWTP discharges from both human and animal use. Due to the constant exposure of biota to low levels of pharmaceutical residues in receiving waters, concerns over the potential long-term effects of these bioactive chemicals in the ecosystem can not be ignored. Elimination of these compounds at point and non-point sources is key to preventing their potential long-term adverse effects to the ecosystem.

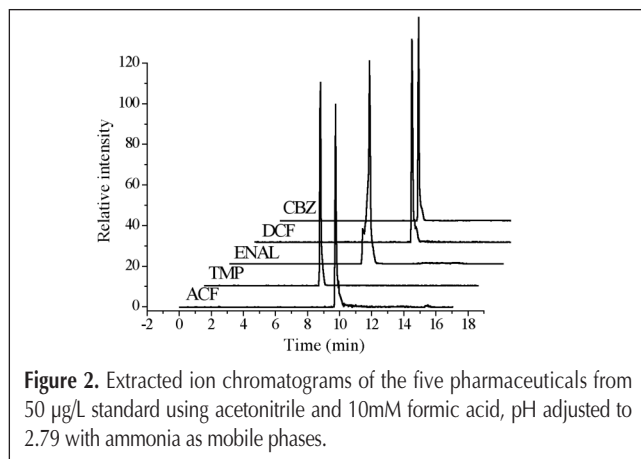


Figure 2. Extracted ion chromatograms of the five pharmaceuticals from 50 $\mu\text{g}/\text{L}$ standard using acetonitrile and 10mM formic acid, pH adjusted to 2.79 with ammonia as mobile phases.

The compounds investigated in this study were chosen because they have been shown to be persistent in the environment except for aceclofenac. Carbamazepine was reported at WWTP influent concentrations as high as 2,000 ng/L in Germany (31,32), 360 ng/L in Spain (33), and 369 ng/L in Canada (34). Diclofenac and trimethoprim were detected at concentrations ranging from 50–500 ng/L and 40–650 ng/L, respectively, in WWTP influents from Croatia and Spain (35). Diclofenac was observed in WWTP influents at concentrations of 1,900 ng/L in Germany (36) and 251 ng/L in Japan (12). Trimethoprim was present at 290 ng/L in raw influent water in Switzerland (37) and at relatively high concentrations from 2100–7900 ng/L in the US (38). Enalapril was observed at 35 ng/L in the influent and 0.85 ng/L in the effluent of a WWTP in Nevada, USA (25).

In this study, the concentrations of the five compounds at the influent and effluent of the CAS and MBR are shown in Table IV. There were only seven samples analyzed for the influent and MBR effluent because of breakage of the sample containers collected on July 20, while a total of eight samples were analyzed for the CAS effluent. All five compounds were detected in the influent at parts-per-trillion (ppt) levels. The amount of carbamazepine detected in the wastewater is similar to that previously reported in the literature (22), but diclofenac concentration was ~80% lower than those observed from previous work. While most of the compounds investigated in this study remained detectable in the effluent during treatment by either CAS or MBR systems (Figure 3), the performance of the MBR technology in reducing the concentration of the majority of pharmaceuticals from wastewater is generally better than that of the CAS.

The relatively constant concentrations of carbamazepine in the influent and effluent of CAS and MBR alike indicates that this

pharmaceutical is hardly eliminated in either of these wastewater treatment systems. This observation is consistent with findings from previous studies (32,34,39). It has been suggested that in order to eliminate carbamazepine in wastewater, the addition of a tertiary treatment involving advanced oxidation process is necessary to achieve up to 92–99% reduction (36).

Aceclofenac is slightly susceptible to biological elimination in CAS and MBR (about 50% reduction observed). At present, there is no literature available to compare this behavior with other water treatment systems. Nonetheless, diclofenac, which is one of the metabolites of aceclofenac (40,41), has negligible elimination (only 8%) in CAS, but was more amenable to biodegradation in MBR (78% reduction). It is possible that aceclofenac released in wastewater may undergo biotransformation to diclofenac, although no study has been reported showing this conversion in WWTPs. Better removal of diclofenac (~80%) was observed for MBR with SRT of 65 days relative to another MBR with an SRT of 15 days (12). The authors pointed out that diclofenac seem to have a very slow rate of microbial degradation. The slightly lower rate of diclofenac removal in the MBR studied may be attributed to a lesser amount of active sludge volume used. Further, sludge characteristics may also play an important role in pharmaceutical removal (42). For further elimination of diclofenac in wastewater, ozonation could be employed (36).

Trimethoprim was slightly reduced in CAS by ~29% and was effectively eliminated by up to 97% in MBR. This compound has been observed to have a higher biodegradation rate in CAS systems that include a nitrification process (43). Nevertheless, trimethoprim remained detectable in systems that use a combination of nitrification and denitrification as secondary treatment followed by sand filtration as a tertiary treatment (37). The SRTs in these systems were not mentioned, though. The elimination of trimethoprim via sludge sorption can be considered negligible because of its high water solubility and very low log K_{ow} . Hence, the high removal efficiency of trimethoprim in the MBR can be attributed to the much higher SRT in MBR compared to CAS, which translates to a more diverse microbial population in MBR.

Elimination of enalapril was as high as 79% in CAS and 95% in MBR. A combination of biodegradation and sludge sorption may have contributed to the removal of enalapril. In an earlier work, the presence of enalapril in the particulates obtained from filtering surface water indicates the sorption property of enalapril to solids (44). Unlike most pharmaceuticals commonly

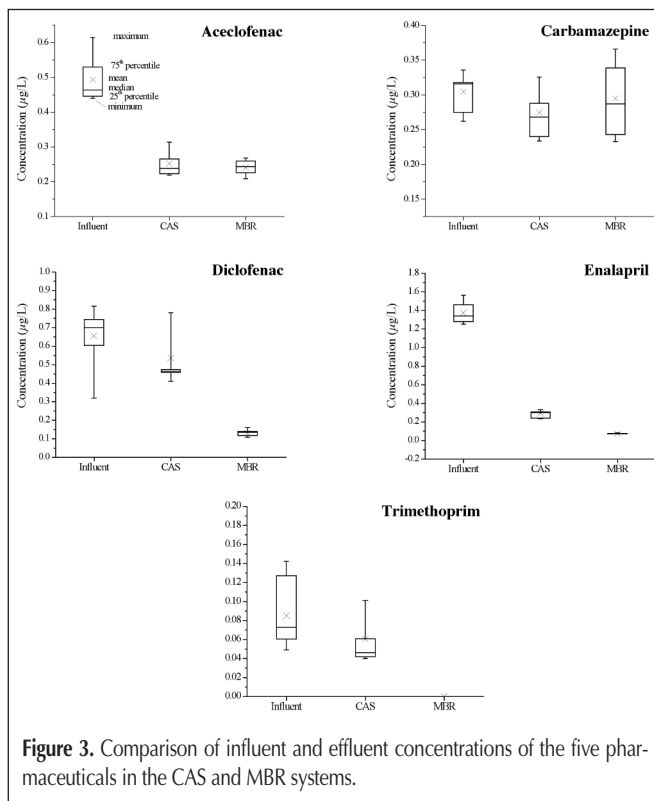


Figure 3. Comparison of influent and effluent concentrations of the five pharmaceuticals in the CAS and MBR systems.

Table IV. Average Concentrations of the Five Pharmaceuticals in the Influent, CAS Effluent, and MBR Effluent Collected in July (2007)

Compound	Influent (µg/L)*	CAS effluent (µg/L)†	MBR Effluent (µg/L)*
Aceclofenac	0.49 ± 0.06	0.25 ± 0.03	0.24 ± 0.02
Carbamazepine	0.31 ± 0.03	0.28 ± 0.03	0.30 ± 0.05
Diclofenac	0.7 ± 0.2	0.5 ± 0.2	0.13 ± 0.02
Enalapril	1.4 ± 0.1	0.29 ± 0.04	0.073 ± 0.004
Trimethoprim	0.09 ± 0.04	0.06 ± 0.02	< 0.013

* $n = 7$, sample container was broken.

† $n = 8$.

detected in surface waters, enalapril is susceptible to photodegradation under UV (45), and under simulated sunlight radiation which results in > 90% photolysis after 40 h of exposure (46). Therefore, the concentration of enalapril may be potentially lowered in treatment systems where UV disinfection is included.

Conclusion

This study demonstrates the need for sensitive and reliable analytical methods for investigating the occurrence and fate of pharmaceuticals in wastewater treatment systems. While there were only five pharmaceuticals included in this study, it is clear that their varying chemical behavior result in a large variability in recoveries during sample preparation and analysis. It is now well-documented in the literature that while LC-MS-MS provides the high selectivity and desired identification points for trace analysis of organic compounds, it is not free of pitfalls, especially when sample matrix is complex. Therefore, in the analysis of pharmaceuticals in wastewater, it would be ideal to compensate for matrix effects using isotope dilution MS if it is affordable. While the current study did not use this approach due to budget restrictions, reasonable conclusions can be made from the results of the analysis. It is clear from this study, that CAS is not sufficient in eliminating many pharmaceuticals in the effluent of WWTPs. On the other hand, MBR is generally more effective over the traditional biological treatment systems in reducing the concentrations of pharmaceuticals in wastewater. Among the five drugs considered, trimethoprim and enalapril were highly susceptible to elimination by MBR. The inadequate removal of some of the drugs after the MBR treatment calls for the application of tertiary treatments such as advanced oxidation process, nanofiltration, or reverse osmosis. However, these processes are expensive to operate. There is an obvious need for a more cost effective, highly efficient water treatment technology if water reuse is to continue in many parts of the world. Finally, further studies are needed to establish the limit of concentration of these drugs that can be tolerated by the ecosystem, and an assessment of the long-term effects of these pharmaceuticals at environmentally relevant conditions.

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