Degradation of duloxetine: Identification of transformation products by UHPLC-ESI(+)−HRMS/MS, in silico toxicity and wastewater analysis

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Abstract
Duloxetine (DUL), an antidepressant drug, has been detected in surface water and wastewater effluents, however, there is little information on the formation of its transformation products (TPs). In this work, hydrolysis, photodegradation (UV irradiation) and chlorination experiments were performed on spiked distilled water, under controlled experimental conditions to simulate abiotic processes that can occur in the environment and wastewater treatment plants (WWTPs). Eleven TPs, nine from reaction with UV light and two from chlorine contact, were formed and detected by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry, and nine of them had their chemical structures elucidated upon analyses of their fragmentation patterns in MS/MS spectra. The formation and degradation of the TPs were observed. The parent compound was completely degraded after 30 min in photodegradation and after 24 hr in chlorination. Almost all TPs were completely degraded in the experiments. The ecotoxicity and mutagenicity of the TPs were predicted based on several in silico models and it was found that a few of these products presented more ecotoxicity than DUL itself and six TPs showed positive mutagenicity. Finally, wastewater samples were analyzed and DUL and one TP, possibly formed by chlorination process, were detected in the effluent, which showed that WWTP not only did not remove DUL, but also formed a TP.

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Keywords:
High resolution mass spectrometry
Duloxetine
Degradation processes
Transformation products
Wastewater analysis
In silico toxicity

Introduction
Pharmaceutical compounds have been found in drinking water, groundwater, surface waters and treated wastewater since many of these compounds cannot be completely removed by conventional treatment systems (Writer et al., 2013; Subedi and Kannan, 2015). There is concern about the effects of these compounds on the environment and human health and several studies have already been carried out on the ecotoxicity and environmental risks of drugs, studies that can be found in the literature (Taylor and Senac, 2014; Acuña et al., 2015; Silva et al., 2015). Nevertheless, a pharmaceutical compound in the environment may undergo complex interactions with biotic and abiotic factors, forming

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transformation products (TPs) which may have different physical and chemical properties from the original compound, changing their ecotoxicity or persistence in the environment (El Najjar et al., 2013; Carpinteiro et al., 2017; Yin et al., 2017).

Another concern is that there are no regulatory laws for safe concentrations of drugs in waters. Currently, in the European Union, the Water Framework Directive (Directive 2013/39/EU, 2013) monitors some pharmaceutical compounds in the aquatic environments and in the United States, the Unregulated Contaminant Monitoring Regulation monitored several drugs in drinking water (US EPA, 2017a).

Duloxetine (DUL) is a selective serotonin-norepinephrine reuptake inhibitor (SNRI) antidepressant drug, mostly used to treat major depression, generalized anxiety disorders, fibromyalgia, and stress urinary incontinence. DUL had sales in the order of $5 billion in the United States under the brand name Cymbalta and was the seventh bestselling prescription drug in 2013 (Cymbalta Sales Data. https://www.drugs.com/stats/cymbalta Accessed September 17, 2018). In Portugal, DUL was the ninth most prescribed antidepressant in 2015, resulting in a defined daily dose (60 mg) of more than 7.4 million (DGS, 2016).

Only few publications reported DUL in wastewater or surface waters. One work has reported the presence of the compound in effluent of wastewater treatment plant (WWTP) and surface water located downstream of the WWTP in the order of ng/L (Schultz and Furlong, 2008). Fick et al. (2011) performed a study that detected DUL in the effluent from 1 to 14 ng/L with an average removal efficiency of 29% in WWTP. Then it is to be expected that this drug is found in the environmental matrices.

There are a few studies on the degradation of DUL, but most are focused on its stability for pharmaceutical use and none of them focuses on the formation of products in the environment and in the wastewater treatment plant (Sinha et al., 2009; Chhalotiya et al., 2010; Kumar et al., 2012; Datar and Waghmare, 2014; Chadha et al., 2016). However, to the best of our knowledge, to date only one study has been published on the TPs formed by DUL, this paper showed that seven TPs were formed from DUL by gamma radiolysis in humic acid solutions to simulate real environmental conditions (Santoke et al., 2012). There is little information about the behavior of the compound with chlorine, and that would be relevant since chlorination may be present in the disinfection step in WWTP. Elucidating TPs in degradation processes such as photodegradation and chlorination is an important step to understand their pathways and potential risks to the aquatic environment.

An essential tool to identify TPs is high resolution mass spectrometry (HRMS). High resolution mass spectrometers, such as orbitrap, time-of-flight (TOF) or fourier-transform ion cyclotron resonance (FTICR), which provide high resolution and high mass measurement accuracy to elucidate the chemical structure by elemental compositions, fragmentation patterns and isotopic distributions could be used (Bletsou et al., 2015; Beretsou et al., 2016).

Another essential point to consider is the toxicity of the TPs in environment and human health. Most of these compounds, however, do not have analytical reference standards to evaluate the toxicity in vitro or in vivo. In addition, these methods are often expensive and time-consuming. As an alternative, in silico methods, based on computational models to simulate and estimate the toxicity can be useful for toxicity prediction (Wilde et al., 2016; Trawiński and Skibiński, 2017). There are several methods for predicting in silico toxicity in which each presents advantages and limitations such as quantitative structure–activity relationships (QSAR) model, which uses molecular descriptors of chemical to predict toxicity. A significant tool to give reliability in the results of the QSAR model is the applicability domain (AD), which means the limitations of the model and predictions outside the AD may not be considered as a result (Worth et al., 2004).

The objective of this work was to elucidate the TPs that may occur in environment and WWTP through abiotic processes: hydrolysis, chlorination and photodegradation, under controlled experimental conditions by ultra-high-performance liquid chromatography (UHPLC) coupled to quadrupole time-of-flight mass spectrometer (QTOF/MS). The formation/degradation of TPs was also analyzed by their respective time profile. After elucidating the TPs, toxicity was predicted by in silico tools, to estimate mutagenicity and ecotoxicity and in addition, method validation was carried out to accurately measure the concentration of DUL and finally, the TPs were investigated in wastewater samples.

1. Materials and methods

1.1. Standards and reagents

Duloxetine hydrochloride of high purity grade (>98%) was purchased from TCI Chemicals (Zwijndrecht, Belgium). Methanol, acetonitrile, formic acid and water, LC-MS grade were supplied from Fisher Scientific (Hampton, USA). Sodium hypochlorite 10% (W/V) solution was supplied by Panreac (Barcelona, Spain) and sodium hydroxide (NaOH) 0.1 mol/L was obtained from Honeywell (Seelze, Germany). L(+)-Ascorbic acid was purchased from Merck (Darmstadt, Germany). Milli-Q ultrapure system from Millipore (Barnstead International, Dubuque, USA) was used to obtain ultrapure water. Nylon membrane filter (0.45 μm) was purchased from Teknokroma (Barcelona, Spain). Stock solutions of 1000 mg/L duloxetine hydrochloride were prepared and stored at –80°C in ultrapure water.

1.2. Degradation experiments

In chlorination, hydrolysis and photodegradation experiments, distilled water was spiked with 4 mg/L of the pharmaceutical compound. The high concentration was used to facilitate the identification of the products. The pH of samples was adjusted to 7.5 with 0.1 mol/L NaOH and was not buffered to avoid influences on the formation of TPs with buffering agents. The reason for pH 7.5 is that it is a close value to be found in effluents and surface waters. Samples were carried out at room temperature and in the dark room. For the identification of the TPs, the spiked samples were compared to blanks (distilled water).
In the chlorination experiments, sodium hypochlorite (5 mg/L free chlorine) solution was added to 20 mL of sample and when removing the aliquot, a quenching agent (ascorbic acid at concentration of 600 mg/L) was added to neutralize chlorine.

In photodegradation, a photoreactor with 100 mL of sample cooled by water circulation was used. The lamp was a 450 W medium-pressure mercury-vapor lamp (Hanovia, UK) with irradiance around 0.37 W/cm and with the total irradiated energy being 40%–48% in the ultraviolet range and 40%–43% in the visible region. The details of the photoreactor have been published elsewhere (Pinto da Costa et al., 2015).

The hydrolysis experiment was performed to evaluate the stability of DUL in distilled water. In all experiments the solutions were stirred with a magnetic bar. Aliquots of 0.1 mL volume were withdrawn in ambers glass vials with inserts in certain times: hydrolysis at 0 hr, 1 hr, 3 hr, 6 hr, 1 day, 2 days, 4 days; chlorination at 0 min, 1 min, 15 min, 30 min, 1 hr, 2 hr, 3 hr, 4 hr, 1 day and photodegradation at 0 min, 5 min, 10 min, 15 min, 30 min, 45 min 1 hr, 1.5 hr, 2 hr and were stored for further analyses at −80°C. Samples were directly injected in UHPLC-QTOF/MS.

1.3. Toxicity assessment by in silico tools

The toxicity assessments of the identified TPs of DUL were performed using in silico tools: Ecological Structure Activity Relationships (ECOSAR, 2.0, Washington DC, USA), Toxicity Estimation Software Tool (T.E.S.T, version 4.2.1 Washington DC, USA), VEGA KNN/read-across and VEGA SARpy/IRFMN (VEGA Platform, version 1.1.4, Milan, Italy http://www.vega-qsar.eu). These tools are based on QSARs and SA methods freely available to users and uses SMILES in the canonical form of chemical structure for predictions. QSARs are mathematical models which estimate the toxicities based on the difference of the physicochemical characteristics of chemicals and their biological activities. In ECOSAR, the methodology is built on a linear mathematical relationship between logKow, estimated if no experimental value is available, and log of toxicity values. If the toxicity result of the chemical compound has different classes, we will consider the value of higher toxicity, in other words, of lower concentration. In ECOSAR, 96-hr fish LC50, 48-hr D. magna LC50 and 96-hr green algae EC50 endpoints were predicted. The tool does not provide a value for AD in prediction toxicity. However, the software shows the logKow cut-off for each class, which means that it is the point at which a compound is no longer soluble. Therefore, if the logKow of the compound is higher than logKow cut-off, then no effects at saturation are expected (US EPA, 2017b).

T.E.S.T was used for the prediction of mutagenicity by the Ames test endpoint, in which positive results indicated a mutagenic potential and ecotoxicity by 96-hr F. minnow LC50, 48-hr D. magna LC50 and T. pyriformis IG50 endpoints. The consensus method was used based on average of toxicity prediction QSAR methods, were: Hierarchical clustering, FDA, nearest neighbor, single model and group contribution. Details of molecular descriptors and QSARs methods are documented in User’s Guide for T.E.S.T. (US EPA, 2016). The consensus method consists of the AD for each QSAR method to use in the prediction and if it results in only one QSAR method, the consensus method will be outside the AD.

VEGA SARpy/IRFMN performs the prediction of the structural alerts for mutagen present in the compound and the AD of predictions is based on the Applicability Domain Index (ADI), which can result in values from 0 to 1. The ADI is established by other models in which the majority is calculated by similar compounds found in the training set and test set of the model. Values between 1 and 0.9, the predicted compound is inside the AD, values between 0.9 and 0.65, the compound could be out the AD and values below 0.65, the compound is out of the AD of the model. VEGA KNN/read-across uses the same AD, based on the ADI. In this work, a prediction greater than 0.65 was considered within the AD and models in which most of the results are outside the AD will not be shown.

1.4. Wastewater sampling and sample preparation

Raw wastewater (influent) and effluent samples were collected from WWTP of Lisbon in amber bottles and stored in thermal boxes until arrival at the laboratory. The WWTP serves approximately 800,000 people and receives effluents from several hospitals in the region. The primary treatment is by decantation, the secondary treatment uses biofiltration and tertiary treatment consists of chlorination and UV irradiation processes. For solid phase extraction (Oasis HLB 200 mg, 6 mL, Waters), 250 mL of samples were previously filtered by nylon membrane (0.45 μm) and the pH was adjusted to 3 with acetic acid (2%). The samples were run in solid phase extraction cartridges in a stream of 5–8 mL/min previously conditioned with 5 mL of methanol and 5 mL of ultrapure water. Then, the cartridges were dried with nitrogen flow, eluted with 5 mL of methanol and 2 mL of acetic–methanol ratio of 1:1. For the reconstitution, 0.1 mL of water–methanol ratio of 4:1 was used, followed by storage at −80°C for further analysis. The method validation can be found in Appendix A Section S1 and Table S1.

1.5. Instrumentation

For identification purposes, an UHPLC Ultimate 3000 RSLCnano system (Thermo Fischer Scientific, Germany) coupled with a quadrupole time-of-flight (QTOF) Impact II mass spectrometer with an electrospray ion source (Bruker Daltonics, Germany) was used. The chromatographic column was a Kinetex 1.7 μm C18 100 Å, 150 × 2.1 mm ( Phenomenex, USA). The mobile phase consisted of H2O acidified with 0.1% formic acid (A) and the organic phase was acetonitrile (B) at concentration of 600 mg/L) was added to neutralize chlorine.

The gradient used was (%B): 0 min (20%); 1.5 min (30%); 10 min (75%); 13 min (100%) and volume injection of 10 μL. The gradient used was (%B): 0 min (20%); 1.5 min (30%); 10 min (75%); 13–17 min (100%) and 18–25 min (5%).

Samples were analyzed in broadband collision-induced dissociation (bbCID) and in product ion scan mode. In bbCID, low collision energy and high collision energy are applied simultaneously to generate fragments and precursor ions in a single run. The product ion scan mode (MS/MS) was used to confirm the fragment ions. Analysis was performed in positive electro-spray ionization (ESI+) mode operating in the high-resolution mode over the range m/z 50–1000 with...
acquisition rate of 3 Hz. The mass spectrometer optimized parameters were as follows: ion spray voltage, 2.5 kV; end plate offset, –500 V; nebulizer gas (N2), 2.8 bars; dry gas (N2), 8 L/min; dry heater, 200°C. 20 μL of sodium formate 10 mmol/L was used for internal calibration on the high-precision calibration mode (HPC).

1.6. Data processing

Compass Data Analysis (Bruker Daltonics, Germany) was performed to analyze fragmentation patterns in order to identify possible TPs by the visual comparison of the chromatograms of the experimental samples with the blank samples (non-spiked). The suspected TP was analyzed in product ion scan mode (MS/MS mode) to obtain the structural information. Moreover, the elemental formulas were estimated by mass error of less than 5 ppm and the isotopic patterns were analyzed as to determine the presence of chlorine in the molecule, since the 35Cl and 37Cl have identifiable isotopic distributions.

2. Results and discussion

2.1. Degradation experiments

In the hydrolysis experiments, DUL showed stability over time in 4 days and no TP was detected. The non-detection of TPs may be attributed to the fact that was performed at room temperature. In the literature, one study reported that the DUL was degraded in 5%–16% at 80°C, with an acid solution, in 4 hr (0.5 mol/L HCl, 9 hr) and four TPs were detected (Datar and Waghmare, 2014). These TPs may have been formed by the high temperature of the experiment or/and by the presence of the acid in the solution. Another study showed that when the temperature was 60°C for 15 days, DUL also gave rise to 10 TPs (Sinha et al., 2009). Both works used HPLC as separation technique, being therefore not possible to identify the TPs because no mass measurement was available.

In the photodegradation experiment, DUL produced nine TPs, seven were elucidated through fragmentation patterns obtained from the MS/MS spectra analyses. The probable chemical structures assigned to these products may have been formed by different reactions. Nevertheless, two isomers of DUL, called DUL-ISO1 and DUL-ISO2 did not have their structures identified by the fact that they present fragmentation patterns similar to the DUL ones.

The time profile showing the formation and degradation of TPs is presented in Fig. 1a and b. As it can be seen, DUL was completely degraded after 30 min by UV irradiation (DUL-UV). Most of TPs formed had their maximum signal intensity in the first 10 min from the beginning of the experiment and after 45 min in contact with UV light, all TPs were degraded. The pathways for the formation of TPs were proposed (Fig. 2). Santoke et al. (2012) observed that DUL degraded about 72% under gamma irradiation and several TPs were identified using a TOF mass spectrometer. In comparison to our study only one TP identified by Santoke et al. (2012) has the same molecular mass, that is, TP-330 in this study, but with a different structure. It should be noted, however, that in the work of Santoke et al. (2012) there was no identification of the compound by fragmentation of the precursor ion and, moreover, gamma irradiation, rather than UV irradiation, was used to elucidate the mechanisms of TPs formation.

The contact of chlorine with DUL resulted in complete removal of the drug in 24 hr of reaction (DUL-CL) and two TPs were detected and identified (Fig. 1c). One TP detected, TP-332, had at least one chlorine atom attached to the molecule, probably bound to one of the aromatics rings. The MS spectra enabled to confirm the presence of chlorine by means of the 35Cl and 37Cl isotopic distribution. The most abundant TP was TP-332. The chromatograms of elucidated TPs can be found in Appendix A Fig. S1 and details on the identification are below.

2.2. Structure elucidation of TPs

2.2.1. DUL, DUL-ISO1 and DUL-ISO2

In MS/MS spectrum, two DUL isomers with similar fragmentation patterns were detected, only the m/z intensities varied (Appendix A Fig. S2). As such, and unfortunately, analysis of the fragmentation patterns, that were too similar, did not enable to identify the isomers formed.

The analysis of the fragmentation patterns in the MS/MS of DUL (Appendix A Fig. S3) showed that the parent ion lost methylamine (CH3NH2) to give m/z 267 (C15H13OS+) which in turn lost ethylene (C2H4) to give the fragment ion at m/z 239 (C15H12O+). This latter fragment ion lost one water (H2O) molecule at m/z 221 (C15H12S+) and loss of thiophene (C4H4S) giving m/z 183 (C13H11O2S+). Since the TPs chemical structures are similar to DUL it was to be expected that the TPs formed would have similar losses.

2.2.2. TP-312A, TP-312B and TP-312C

These TPs, although with the same molecular mass, have different fragmentation patterns observed in their MS/MS spectra (Fig. 3). Analysis of the MS/MS spectrum of TP-312A (Fig. 3a) led us to propose that it has been formed by an oxidation on the DUL aliphatic chain. Indeed, the MS/MS spectrum showed the neutral loss of CH3NH2 at m/z 281 (C15H12O2S+) and it may be concluded that the oxidation did not occur on the methyl group. The fragment ion m/z 197 (C13H9O5) may be attributed to the elimination of C4H4S together with CH3NH. It may therefore be concluded that oxidation also did not occur in thiophene. The elimination of H2O at m/z 263 (C15H12O2S+) from m/z 281 resulted, probably, from an ether group present in the TP molecule, this loss occurred also on the DUL structure. The most intense peak at m/z 255 (C15H12O2S+) was due to the elimination of both CH4NH2 and C4H4S, the latter one probably originating from the naphthalene or thiophene ring. The formation of the fragment ion m/z 253 (C15H12OS+) reinforces the hypothesis of oxidation on α-carbon with the loss of N-methylformamide (C4H7N0). TP-312B appears to have been formed by an epoxidation on naphthalene. This hypothesis is supported by the identification of fragment ions at m/z 253 (C13H10O2S+) and m/z 228 (C12H9N0), in its MS/MS spectrum (Fig. 3b). The former fragment ion indicated the neutral loss of C4H4N, possibly ethylmethylamine from the aliphatic chain with the amine group. The latter fragment ion, m/z 228, implied the loss of
C₄H₄S, the thiophene group, suggesting that no reaction occurred in this group. Furthermore, only one loss of H₂O has been identified at m/z 263 (C₁₇H₁₁OS⁺) from m/z 281 (C₁₇H₁₃O₂S⁺) suggesting it to be related to the ether group of the TP structure, meaning that no hydroxylation occurred. To reinforce our assumption for the structure of this TP, studies of UV/chlorine degradation have identified the epoxidation process in carbamazepine (Wang et al., 2016; Zhou et al., 2016). Furthermore, hydroxylation of naphthalene occurring in this TP structure was proposed by the fact that only one loss of H₂O was detected. This loss could be related to the ether group of the compound, same occurred on the structure of the DUL, the most likely position for hydroxylation being the 1-position.

TP-312A. The fragment ion m/z 269 (C₁₆H₁₂O₂S⁺) supported this hypothesis by loss of a C₂H₅N radical from the parent ion, only possible with imine. According to the literature, the formation of imines and nitriles from amides by UV radiation and soft X-rays has been confirmed (Johnson et al., 2011). Furthermore, hydroxylation of naphthalene occurring in this TP structure was proposed by the fact that only one loss of H₂O was detected. This loss could be related to the ether group of the compound, same occurred on the structure of the DUL, the most likely position for hydroxylation being the 1-position.

Fig. 1 – Time profile of DUL and TPs by (a) and (b) photodegradation and (c) chlorination. A is the peak area of the respective TP and A₀ is the peak area of DUL at t = 0 min.

C₄H₄S, the thiophene group, suggesting that no reaction occurred in this group. Furthermore, only one loss of H₂O has been identified at m/z 263 (C₁₇H₁₁OS⁺) from m/z 281 (C₁₇H₁₃O₂S⁺) suggesting it to be related to the ether group of the TP structure, meaning that no hydroxylation occurred. To reinforce our assumption for the structure of this TP, studies of UV/chlorine degradation have identified the epoxidation process in carbamazepine (Wang et al., 2016; Zhou et al., 2016).

The analysis of the fragmentation patterns in the MS/MS spectrum of TP-312C (Fig. 3c), suggested a TP structure bearing an imine group, possible originating from the amide group of TP-312A. The fragment ion m/z 269 (C₁₆H₁₂O₂S⁺) supported this hypothesis by loss of a C₂H₅N radical from the parent ion, only possible with imine. According to the literature, the formation of imines and nitriles from amides by UV radiation and soft X-rays has been confirmed (Johnson et al., 2011). Furthermore, hydroxylation of naphthalene occurring in this TP structure was proposed by the fact that only one loss of H₂O was detected. This loss could be related to the ether group of the compound, same occurred on the structure of the DUL, the most likely position for hydroxylation being the 1-position.

Fig. 2 – Proposed pathway of TPs formation.

Fig. 3 – MS/MS spectra and proposed structures of (a) TP-312A, (b) TP-312B and (c) TP-312C.
because it is more stable and studies found in the literature indicated that the hydroxylation process may occur in compounds with aromatic rings (Vinu and Madras, 2011; Yuzawa et al., 2012) The loss of C4H4S, was identified by fragmentation from the parent ion resulting in m/z 228 \((C_{15}H_{13}OS)^{+}\), proving that there was no hydroxylation of this group. The fragment ion m/z 202 \((C_{14}H_{11}NO)^{+}\) resulting from m/z 228 by the loss of C2H2, probably occurred from the aromatic group. Another fragment ion that confirms a TP structure resulting from the formation of 1-naphthol was that at m/z 161 \((C_9H_7O_2)^{+}\), formed by losses of thiophene and the aliphatic amine from the parent ion.

2.2.3. TP-328A and TP-328B

Two TPs with the same molecular mass were identified but with different MS/MS spectra. In the MS/MS spectrum of TP-328A (Appendix A Fig. S4), neutral losses of two H2O molecules were identified, one at m/z 310 \((C_{15}H_{13}O_{2}S)^{+}\) from the parent ion and the other at m/z 234 \((C_{14}H_{11}NO)^{+}\) from m/z 252 \((C_{15}H_{13}O_2N)^{+}\). The former H2O loss justifies the presence of one OH group on the TP structure that might be due to hydroxylation, probably in β-carbon because it is more stable. The other H2O loss may result from the presence of an ether group. Another hydroxylation probably formed the 1-naphthol, since no other H2O loss was detected.

Analysis of the MS/MS spectrum of TP-328B suggests a structure formed by hydroxylation and epoxidation processes. The loss of CH3NH2 at m/z 297 \((C_{15}H_{13}O_{2}S)^{+}\) was identified in the MS/MS spectrum (Appendix A Fig. S5), arguing for no reactions on the methyl group and no imine formation. Hydroxylation probably occurred on β-carbon and, elimination from the parent ion of C3H7N, resulting in m/z 269 \((C_{15}H_{11}O_{2}S)^{+}\), suggests the fragmentation of the aliphatic amine group (N-Methylthanolamine), corroborating our hypothesis. The fragment ion at m/z 213 \((C_{15}H_{13}O_2)^{+}\), the most intense peak in the spectrum, showed the neutral losses of C3H7S and CH3NH2, which may indicate that there was no reaction on the thiophene group. The m/z 185 \((C_{15}H_{13}O_2)^{+}\) fragment ion could be due to the loss of CO from fragment ion m/z 213 \((C_{15}H_{13}O_2)^{+}\), probably involving the oxygen formed by epoxidation.

2.2.4. TP-330

Based on the MS/MS spectrum of this TP (Appendix A Fig. S6), losses of two H2O molecules at m/z 294 \((C_{16}H_{14}NOS)^{+}\) from the parent ion were detected. We attribute the first loss of H2O to the presence of OH on the naphthalene, whereas the second H2O molecule loss could occur from the ether group by hydrogen rearrangement, in a process similar to the one that occurred in DUL. Two hydroxylation processes on the naphthalene group and formation of imine from TP-312A are proposed for the formation of TP-330. The peak at m/z 251 \((C_{15}H_{11}OS)^{+}\) showed the losses of two H2O molecules and of C3H7N from parent ion. The loss of C3H7N suggests that hydroxylation did not occur on β-carbon. Indeed, if hydroxylation had occurred on β-carbon C3H7N should have been eliminated, which was not the case. In theory, hydroxylation could occur on the methyl or α-carbon groups if there was no formation of imine. It should be noted that, on the α-carbon, it would be unlikely because it forms a hemiaminal group, which would be unstable and corroborating this assumption, in the MS/MS spectrum losses of CH3NH2O or H2O + CH3N at the methyl group, were not detected. The eliminations from the parent ion of two H2O molecules and C3H7N giving rise to m/z 237 \((C_{15}H_{13}OS)^{+}\) are in agreement with the formation of the imine, C3H7N representing the loss of the aliphatic imine. The fragment ion at m/z 223 \((C_{15}H_{11}S)^{+}\) showed the elimination of CO from m/z 251 fragment ion, which may argue for a TP structure where 1-naphthol has been formed.

2.2.5. TP-348

Analysis of neutral losses in the MS/MS spectrum of this TP showed the loss of two water molecules, one from the parent ion to give fragment ion m/z 312 which, in turn, lost one H2O molecule giving rise to fragment ion m/z 294 \((C_{16}H_{12}NOS)^{+}\) (Appendix A Fig. S7). In view of these observations it seems likely that two hydroxylation reactions, one on β-carbon of aliphatic chain and the other on the naphthalene ring might have been responsible for the TP structure. The third loss of H2O could be from an ether group. In theory, hydroxylation at the α-carbon of the aliphatic chain could occur, but it forms an unstable compound. Hydroxylation on the methyl group was not considered by the fact that elimination of CH3NH2O or CH3N were not found in the MS/MS spectrum. Two hydroxylations and dehydrogenation of naphthalene were considered to occur since, after losing one H2O molecule, a naphthol group might have been formed. The hydroxylations are proposed to have been formed at the 1,2 positions of naphthalene in agreement with McConkey et al. (2002) that identified the formations of hydroxyl groups in these positions by degrading naphthene with natural sunlight. The fragment ion at m/z 235 \((C_{16}H_{12}S)^{+}\), could be formed by the loss of the CH3NH2, 3 H2O and CO molecules from m/z 312 \((C_{16}H_{12}NOS)^{+}\). The loss of the CO molecule may confirm the hypothesis of a TP structure where formation of the naphthol group occurred.

2.2.6. TP-290

In the MS/MS spectrum (Appendix A Fig. S8), three consecutive losses of H2O were identified, one from the parent ion to m/z 272 \((C_{15}H_{11}NOS)^{+}\), another from this one to m/z 254 \((C_{15}H_{11}NO)^{+}\) and the other from the latter ion to m/z 236 \((C_{15}H_{11}NOS)^{+}\). One of these losses probably involves OH at the ether group after hydrogen rearrangement. The structure proposed is a molecule where the sulfur atom has been replaced by one oxygen atom to form an alcohol group being the other OH attached to the methyl group. The peak at m/z 211 \((C_{15}H_{11}O)^{+}\) showed the neutral losses of H2O and C3H7NO, either both directly from the parent ion, or H2O from the parent ion and C3H7NO from m/z 272. The latter loss may indicate that OH was bound to methyl. In theory it could be bound to the α-carbon of the aliphatic chain, but if that was so, a hemiaminal group might have been formed, which would result in a less stable compound. The loss of CO at m/z 183 \((C_{15}H_{11}O)^{+}\) from m/z 211, suggests a structure bearing a 1-naphthol group.

2.2.7. TP-332

The MS spectrum of this TP shows (Appendix A Fig. S9), based on the isotopic contribution of chlorine \(^{35}Cl/^{37}Cl\), that its structure bears one chlorine atom. This chlorine appears to be attached to the aromatic ring corroborated by the loss of C3H7ClO from the parent ion giving rise to m/z 155 ion.
Table 1 – Transformation products (TPs) of duloxetine (DUL) after degradation process identified by their fragmentation patterns in the MS/MS spectra.

<table>
<thead>
<tr>
<th>TP code</th>
<th>Retention time (min)</th>
<th>Structure</th>
<th>Theoretical mass [M + H]^+</th>
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<th>Mass error (ppm)</th>
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(continued on next page)
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UV: Photodegradation; CL: Chlorination.
C₈H₁₃NS⁺ (Appendix A Fig. S10). The neutral loss from the parent ion of C₈H₉N at m/z 273 (C₁₅H₁₀ClOS⁺) corroborates that there was no reaction on the aliphatic amine. The fragment ion at m/z 217 (C₁₀H₈O₃Cl⁺) showed the losses of both C₄H₄S and CH₃NH₂ from the parent ion. This means that the chlorine is bound to the aromatic ring, possibly on the 1-position, forming 1-chloronaphthalene group, considering that the 1-position is more reactive than 2-position, which can be explained by the high stability of the resonance structure for the reactive intermediate (arenium ion) (Sorrell, 2005).

2.3. Summary of fragmentation patterns

Table 1 shows the retention time, fragmentation patterns, mass error and DBE of the TPs detected after the degradation processes.

2.4. Toxicity prediction

Ecotoxicity of DUL and their identified TPs were predicted by QSAR models from ECOSAR and from T.E.S.T (Details of these models were described in Section 1.6). The DUL-ISO1 and DULISO2 toxicity predictions were not performed because the chemical structures were not elucidated. Most of the results across presented predictions that may be outside the AD (0.9 > ADI ≥ 0.65). The QSAR analysis for mutagenicity resulted in one TP with positive mutagenicity prediction for all methods, TP-330, which was formed by UV irradiation. All TPs with positive mutagenicity prediction were formed and completely degraded in less than 90 min in contact with UV light.

2.5. Wastewater analysis

Samples from a wastewater effluent were analyzed by UHPLC-Q-TOF/MS in MS full scan mode to detect the TPs previously elucidated by the laboratory experiments. In MS/MS mode, DUL showed poor fragmentation at low concentrations.

<table>
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<th>Compound</th>
<th>Fish LC₅₀ 96 hr (mg/L)</th>
<th>Relative potency a</th>
<th>D. magna LC₅₀ 48 hr (mg/L)</th>
<th>Relative potency a</th>
<th>Green algae EC₅₀ 96 hr (mg/L)</th>
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<th>T. pyriformis IGC₅₀ (mg/L)</th>
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<td>1.00</td>
<td>1.00</td>
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<td>1.41</td>
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<td>0.08</td>
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<td>0.731</td>
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<td>0.892</td>
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a Prediction out of applicability domain.
MS mode, DUL was quantified (Appendix A Fig. S11a) at concentrations of 95.5 ng/L in the influent and 79.6 ng/L in the effluent, removal of 17% and TP-332 was detected in the effluent (Appendix A Fig. S11b). This TP was probably formed in the chlorination process of the WWTP and presented more ecotoxicity than DUL in the in silico prediction. The TPs of this work were identified under controlled conditions. In wastewater samples they might have been degraded, not being detected due to low concentrations or having not even been formed. Moreover, complex interactions with biotic and abiotic processes may occur. It would be necessary to analyze more effluents samples from different sources such as hospitals wastewater.

3. Conclusions

In the present work, the experiments showed that DUL reacts with chlorine and under UV irradiation, producing 11 TPs under controlled laboratory conditions, nine of them were elucidated and to the best of our knowledge, this is the first work that identifies the TPs of DUL by processes that may occur in the environment and in wastewater treatment plants. UHPLC Q-TOF/MS showed to be a powerful tool to elucidate TPs by analyzing the fragmentation patterns in MS/MS mode.

It could be concluded that DUL was completely degraded in both processes. In the hydrolysis process the drug presented stability over 4 days and no TP was detected. In photodegradation all TPs have been degraded after 45 min of experiment and in chlorination, TP-290 was degraded until the end of the experiment (after 24 hr) and TP-332 was partially eliminated.

QSARs assessments revealed six TPs with positive results for mutagenicity. Concerning ecotoxicity prediction, only TP-332 showed to be more toxic than the parent compound for all endpoints. In wastewater samples, it was concluded that DUL was not totally removed at WWTP and the TP-332 was formed, possibly during chlorination process. For future research, the possible formation of these TPs should be investigated in studies of emerging contaminants. Another fact to be considered is to verify the toxicity of these TPs by in vitro and in vivo assays.

Acknowledgments

The authors are grateful to Águas de Portugal for providing samples. This work was supported by Brazilian Federal Agency Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for PhD grants (No. 99999.000845/2014-00) and Fundação para a Ciência e a Tecnologia (FCT) Portugal (Projects UID/MULTI/00612/2013, PEst-OE/QUI/UI0612/2013 and LISBOA-01-0145-FEDER-022125).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2019.02.025.

References