UHPLC-QTOF MS SCREENING OF PHARMACEUTICALS AND THEIR METABOLITES IN TREATED WASTEWATER SAMPLES FROM ATHENS

M. Ibáñez[1], V. Borova[2], C. Boix[1], R. Aalizadeh[2], R. Bade[1], N.S. Thomaidis[2]and F. Hernández[1]

[1]Research Institute for Pesticides and Water, University Jaume I, 12071, Castellón, Spain

[2]Department of Chemistry, National and Kapodistrian University of Athens, 157 84 Athens, Greece

* Author for correspondence: E-mail: felix.hernandez@uji.es

Tel: +34 964 387366; FAX: +34 964 387368

ABSTRACT

After consumption, pharmaceuticals are excreted as parent compounds and/or metabolites in urine and faeces. Some are not completely removed during wastewater treatments, forcing sewage treatment plants (STPs) to apply alternative technologies to guarantee quality of treated water. To monitor the removal efficiency of STPs, not only unchanged compounds and metabolites have to be taken into account, but also formation of possible transformation products (TPs). In this work, QTOF MS has been used for screening metabolites/TPs of pharmaceuticals in effluent wastewater from Athens. A customised database was built with the exact masses of metabolites reported in literature for the parent drugs found in an initial screening. Additionally, TPs identified in previous degradation experiments performed at our laboratory were included. Up to 34 metabolites/TPs were detected for omeprazole, venlafaxine, clindamycin, clarithromycin, clopidogrel or dipyrone, among others. Seven corresponded to TPs whose reference standards were available at our lab, seven were TPs previously identified in laboratory degradation experiments, eight were TPs tentatively identified by QTOF MS without reference standards, and twelve TPs were discovered after using the common fragmentation pathway approach. Tentative identification of TPs was supported by prediction of their chromatographic retention time based on the use of advanced chemometric QSRR models.

KEYWORDS: screening, pharmaceuticals, metabolites/transformation products, treated wastewater, QTOF MS

1. INTRODUCTION

Nowadays, the presence of pharmaceuticals in the environment is a matter of concern because of their wide consumption and potential negative effects on water quality and living organisms. After consumption, pharmaceuticals are excreted and disposed into the sewer systems, finally entering sewage treatment plants (STPs) as the unaltered parent compound or as free or conjugated metabolites. Primary and secondary treatments are commonly applied in STPs, whereas some of them use additional disinfection/oxidation processes, such as ozonation, ultraviolet light (UV) or chlorination. During these treatments, pharmaceuticals can be removed but also transformed into different transformation products (TPs), in many cases unknown, which may be released into the aquatic environment through effluent wastewater. Although rather limited, reported data show that some TPs are as, if not more, hazardous than the parent compound, producing negative effects on humans and wildlife [1-3]. However, the ecotoxic, mutagenic and other potential harmful effects of TPs are mostly unknown and need to be investigated [1].

For all these reasons, it is important to investigate the possible presence of human metabolites of pharmaceuticals and also their possible TPs in effluent wastewaters. Only with a comprehensive approach giving the maximum information possible on the presence of these compounds, would it be possible to estimate the overall contribution of chemicals to the aquatic environment, as well as their potential impact, long-term toxicological effects on living organisms and the combined effect of exposure to multiple compounds [4]. As many of the TPs are still unknown, the analytical task is a challenge as not only the reported TPs must be detected and identified, but also new/unreported TPs must be discovered.

High resolution mass spectrometry (HRMS) instruments, such as Orbitrap and Time-of-Flight (TOF), have been reported for the analysis of pharmaceuticals in water samples [5-6]. These are also the preferred analytical tools for the identification and elucidation of TPs, thanks to the sensitive accurate-mass full-spectrum measurements provided by these analyzers [7-9].. Hybrid analyzers, such as QTOF MS, allow data acquisition under different collision-induced dissociation conditions within the same single injection i.e. MS^E mode [10-11] or broadband collision-induced dissociation (bbCID), obtaining additional useful information on accurate masses of both (de)protonated molecules and fragment ions. This is valuable for identification/elucidation purposes, and may allow the tentative identification of the compound detected even when the reference standard is not available [12].

The initial objective of this paper was to perform a broad investigation on the presence of TPs/metabolites of pharmaceuticals in effluent wastewaters from Athens using LC-QTOF MS. This work is the result of a collaborative work, where two research teams from the University of Athens and University Jaume I of Castellon have participated. After an initial screening for parent pharmaceuticals performed at Athens, the sample vials were shipped to Castellón, where the presence of a large number of metabolites/TPs was investigated. Specific TPs previously identified in degradation experiments performed at our laboratory for pharmaceuticals as omeprazole (also metabolites), venlafaxine, gemfibrozil, ibuprofen, irbesartan and ofloxacin, were monitored. Additionally, a home-made database was widened to around 450 compounds, including theoretical exact masses of metabolites/TPs reported in the literature.Special emphasis was placed upon the degradation products of those pharmaceuticals identified in Athens wastewater after the initial screening. Prediction of chromatographic retention time was an additional tool to support the identity of the compound found in the samples. The

newly detected compounds were then searched and verified in effluent samples with the QTOF MS system used in Athens, as a further proof of their wide presence. This work is a proof of concept study for the power of retrospective analysis offered by LC-HRMS instruments in the quest for new compounds in the environment.

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), sodium hydroxide (NaOH) and formic acid (HCOOH) were acquired from Scharlau (Barcelona, Spain). Leucine enkephalin, used as lock mass in XEVO G2 QTOF, was purchased from Sigma-Aldrich (St. Louis, MO, USA).Ethyl acetate (EtAc) (LC–MS grade, 99.9 % purity, Lichrosolv) and hydrochloric acid (37%) were purchased from Merck (Darmstadt, Germany) whereas 2-propanol of LC-MS grade was from Fisher Scientific (Geel, Belgium).Ammonium hydroxide solution was prepared using ammonia (25%), which was obtained, from Panreac (Barcelona, Spain). Ammonium acetate and ammonium formate, all LC-MS grade, were purchased from Fluka, Sigma–Aldrich (Germany).

The empty solid phase extraction polypropylene tubes (6 mL) as well as the cartridge materials WCX, ZT and WAX, were obtained from Phenomenex (Torrance, USA). The Isolute ENV+ material and the frits (20 μm, 6 mL) were from Biotage (YstradMynach, UK). Glass fiber filters (GFF, pore size 0.7 μm) used in wastewater filtration were obtained from Millipore (Cork, Ireland). Syringe filters (RC) 4 mm and pore size 0.2 μm were obtained from Phenomenex (Torrance, CA, USA).

2.2 Sample collection and treatment

Twenty four hour flow proportional composite samples of secondary treated wastewater samples were collected from the STP of Athens (Greece), in October 2014, during seven consecutive days, from 6/10 to 12/10. Athens and its metropolitan area have a population of 3,737,550 inhabitants, according to the census of 2011, but the number of people estimated based on number of house connections is 4,562,500. The STP of Athens is designed with primary sedimentation, activated sludge process with biological nitrogen and phosphorus removal and secondary sedimentation. The most important physical and chemical characteristics of the wastewater collected in this period as well as the flow rates are summarized in **Table S.1**.

Wastewater samples were collected in 1.5 L bottles and directly transferred to the laboratory in chilled conditions after sampling. There they were filtered immediately on GF filters (GFF, 0.7 μm) and stored at -20°C until analysis. The applied analytical method was derived from Kern et al., with modifications [3]. Briefly, 200 mL of the 24-h composite effluent samples were defrosted and adjusted to pH 6.5±0.2. The cartridges used for solid phase extraction (SPE) consisted of four different SPE cartridge materials, Strata X (200 mg) and a mixture of Strata WAX (100 mg), Strata WCX (100 mg) and IsoluteENV+ (150 mg), in order to achieve sufficient enrichment for a broad range of compounds (neutral, acidic, basic). The cartridges were conditioned with 5 mL methanol and 10 mL water. Next, 200 mL of the sample were passed through the cartridge at a flow rate of 10 mL min^{-1} using a VARIAN (Vac Elut SPS 24) vacuum manifold. After drying of the SPE material by passing air for 1 h, the analytes were sequentially eluted with 2 x 2 mL of basic methanol and ethyl acetate solution (v/v, 50:50, +2% v/v ammonia), followed by 2 mL of an acidic methanol and ethyl acetate solution (v/v, 50:50, +1.7% v/v formic acid). The extracts were evaporated to 100 μL under a constant,

gentle stream of nitrogen at 40 °C and reconstituted with a mixture of water:methanol (v/v, 50:50), to a volume of 1000 μL. The extracts were filtered through a 0.2 μm RC syringe filter and collected in 1.5 mL vial and kept at -20°C for further analyses at the Research Institute for Pesticides and Water, University Jaume I (Spain). Finally, 25 μL of the diluted extract were directly injected in the UPLC-QTOF MS system under MS^E mode.

2.3 Instrumentation

2.3.1 University of Athens (UoA)

A UHPLC system (DionexUltiMate 3000 RSLC, Thermo FisherScientific, Germany) interfaced to a QTOF mass spectrometer (MaxisImpact, Bruker Daltonics, Bremen, Germany) was used for the screening of samples. The chromatographic separation was performed on an Acclaim™ RSLC 120 C_{18} analytical column (2.1i.d. × 100 mm length, 2.2μm particle size) from Thermo Fisher Scientific (Dreieich, Germany) preceded by a guard column of the same packaging material, ACQUITY UPLC BEH C18 1.7 μm, VanGuard Pre-Column, Waters (Ireland), thermostated at 30˚C. The QTOF MS system was equipped with an electrospray ionization source (ESI), operating in positive and negative ionization modes. The mobile phases in positive ionization mode (PI) were (A) MeOH with 5 mM ammonium formate and 0.01 % v/v HCOOH and (B) an aqueous solution with 10% of MeOH, 5 mM ammonium formate and 0.01% HCOOH. For negative ionization mode (NI), the mobile phases were (A) MeOH with 5 mM ammonium acetate and (B) an aqueous solution with 10% of methanol and 5 mM ammonium acetate. For more details, see Supplementary Information, Section S.2.1.

2.3.2 University Jaume I (UJI)

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), using an orthogonal Z-spray-ESI interface operating in both positive and negative ionisation modes. The chromatographic separation was performed using an Acquity UPLC BEH C_{18}analytical column (2.1i.d. × 100 mm length, 1.7μm particle size) from Waters, at a flow rate of 300 μL/min. The mobile phases used were (A) H_2O with 0.01% HCOOH and (B) MeOH with 0.01% HCOOH. See Supplementary Information, Section S.2.2, for additional details.

2.4 General strategy

A QTOF screening was performed in both laboratories (UoA-Greece and UJI-Spain) for the analysis of the same treated wastewater samples.

Firstly, the UoA laboratory built a database containing pharmaceuticals. Target compounds included in the database were screened in the samples based on retention time, mass accuracy, isotopic pattern and MS/MS fragments. The extracted ion chromatogram (EIC or XIC, depending on the manufacturer) of all the (de)protonated compounds were produced together with the applied detection settings:± 5 ppm for mass accuracy, isotopic fit <200 mSigma and ± 0.2 min for the retention time tolerance. Smart Formula Manually algorithm (Bruker) and MS/MS fragment ions, in bbCID function, were further evaluated when needed.

In a second step, the UJI laboratory applied a general screening for investigating the metabolites/TPs of pharmaceuticals, based on two home-made databases (described below) depending on whether the reference standards were available at the lab (target

screening) or not (suspect screening). Parent compounds of the metabolites were also included.

2.5 Retention time prediction

A retention time (t_R) prediction approach, based on a previous artificial neural network (ANN) method developed in the laboratory of UJI [13] was implemented to aid in the tentative identification of metabolites. Canonical simplified molecular line entry system strings (SMILES) were created using ChemSpider (Royal Society of Chemistry, UK) and/or ChemSketch (ACD Labs) freeware for 544 compounds and, from these, 16 molecular descriptors (as ANN inputs) were generated including the number of double and triple bonds (nDB or nTB), the number of carbon and oxygen atoms (nC or nO), the number of 4–9 membered rings (nR04-nR09), unsaturation index (UI), hydrophilic factor (Hy), Moriguchi and Ghose–Crippen logP (MlogP and AlogP respectively) as well as with software predicted logKow data [14]. Prediction of t_R (as the designated single output) *via* neural networks was performed using Alyuda NeuroIntelligence 2.2 (Cupertino, CA). Further information regarding the optimisation and development of the ANN method can be found in the supporting information (SI, Section S.3). A second retention time prediction approach based on a non linear k-nearest neighbourhood clustering classification (kNN), genetic algorithm selection techniques (GA) and Support Vector Machines (kNN-GA-SVM) modeling was applied by the laboratory of UoA for further identification of metabolites in the UoA retrospective screening. The application of the kNN-GA-SVM modeling technique was selected among others which have been developed in UoA, as it showed the highest statistical confidence and accuracy for the prediction of retention time of 1851 compounds in positive ionization mode [13]. Five molecular descriptors were used including the LogD (at pH=3.6), complementary

information content index (CIC1), eigenvalue sum from Z weighted distance matrix that is accounting for the presence of heteroatoms and multiple bonds, atomic polarizabilities and logP estimated by Ghose–Crippen (AlogP) [15]. These molecular descriptors were selected by GA and introduced to SVM for predicting t_R. Further information regarding the optimization and development of the SVM model can be found in the supporting information (SI, Section S.4).

2.6 Database used for metabolites of pharmaceuticals

Firstly, a customized database was compiled containing 12 metabolites for which reference standards were available at IUPA lab (10,11-dihydroxy carbamazepine and carbamazepine 10,11-epoxide; N-desmethyl clarithromycin; clopidogrel carboxylic acid; enalaprilat; losartan carboxylic acid; 5-hydroxy omeprazole; 4-aminoantipyrine, 4-formyl and 4-acetamido antipyrine; clofibric acid and fenofibric acid). Those compounds identified in experiments performed at our laboratory under laboratory-controlled conditions were also added (note that parent compounds were also included):

i) TPs of pharmaceutical omeprazole (5 from hydrolysis, 5 sunlight photodegradation and 7 chlorination) and its metabolites (24) [10, 16] (see **Tables S.5** and **S.6** in Supplementary Information, respectively),

ii) TPs obtained in experiments with surface water and wastewater treatment plant activated sludge for venlafaxine, gemfibrozil, ibuprofen, irbesartan and ofloxacin (22 in total plus 3 found after applying the common fragmentation pathway strategy) [17] (**Table S.7** in Supplementary Information)

Reference standards for the TPs identified in degradation experiments were only available for 1-hydroxy-, 2-hydroxy- and α-hydroxy ibuprofen; O- and N-desmethyl venlafaxine; and 4-hydroxy omeprazole sulphide. For the rest of the tentatively identified

TPs, information on their fragment ions was obtained from degradation experiments data. The sample vial obtained in previous laboratory degradation experiments with the highest concentration of the (tentatively) identified TP was injected in the batch of wastewater samples to check the retention time.

A second home-made database was also compiled, including theoretical exact masses of metabolites reported in the literature (**See Table S.8** in Supplementary Information). Special emphasis was put in the metabolites of the pharmaceuticals reported in the initial screening by UoA, when this information was available. This database is being continuously updated, and while performing this work, it contained almost 450 compounds (corresponding to 100 parents).

3. RESULTS AND DISCUSSION

3.1 Initial screening of parent pharmaceuticals

Initially, a QTOF target screening was applied by the UoA, looking for parent pharmaceuticals. A database containing more than 150 pharmaceuticals was compiled.

45 pharmaceuticals were identified in the effluent wastewater samples (see **Table 1**), including antibiotics (azithromycin, clarithromycin, clindamycin, trimethoprim), pharmaceuticals used to treat high blood pressure (irbesartan, valsartan, losartan, telmisartan, diltiazem, propranolol, metoprolol, celiprolol, atenolol, eprosartan), direct renin inhibitor approved for the treatment of hypertension (aliskiren), antidepressants (citalopram, venlafaxine, mirtazapine, clomipramine), non-steroidal anti-inflammatory drugs (diclofenac), used to reduce cholesterol levels (fenofibrate, gemfibrozil), diuretics (furosemide), analgesics (gabapentin, tramadol), used to treat epileptic seizures (carbamazepine, oxcarbazepine, lamotrigine), antiviral drugs (amantadine, rimantadine), antiretroviral drug (atazanavir), antipsychotic (clozapine), antifungal (climbazole,

fluconazole), used to prevent myocardial infarction (clopidogrel), local anesthetic and class-1b anti-arrhythmic drug (lidocaine), used for joint and muscular pain (meclofenamic acid, niflumic acid), antipsychotics (quetiapine, amisulpride, sulpiride) and even for treatment of moderate-to-severe Alzheimer's disease (memantine), used to treat arrhythmia (propafenone, sotalol, verapamil), anti-diabetic drug (vildagliptin, sitagliptin), a first-generation antihistamine (diphenhydramine) or second-generation antihistamine (cetirizine). Levamisole was also identified. This compound was initially used as anthelmintic and immune modulatory; however, it is being now increasingly used as a cutting agent in cocaine. The identity of all parent compounds was confirmed by means of reference standards.

Almost all of the above indicated parent compounds had been detected and quantified in effluent samples collected in Athens during previous years (April 2011 and 2012) using LC-(QqQ) MS/MS instrumentation [18-19]. However, some of the reference standards were not available at the moment of analysis and therefore were not included in the developed method. This was the case of diltiazem, oxcarbazepine, quetiapine, clindamycin, losartan, irbesartan, telmisartan, amantadine and memantine/rimantadine. Thus, the current study also provided information of their first investigation in wastewater samples of Athens.

3.2 Screening of metabolites/TPs of pharmaceuticals

In a second step, the sample vials were shipped to UJI where a QTOF target screening was applied for metabolites/TPs of pharmaceuticals, independently of the availability of reference standard. The (de)protonated molecule was searched in the sample extracts by performing automated narrow-window extracted ion chromatograms (nw-XICs) for all compounds included in the database. Due to the narrow mass window employed (150

ppm), only one chromatographic peak was commonly observed and subjected to subsequent investigation. Characteristic isotopic pattern and fragment ions, typically in the HE function, were then further evaluated. UHPLC was a valuable tool for selecting perfectly almost co-eluting fragment ions that would correspond to the same precursor, minimizing in this way spectrum interferences that would complicate the identification process. The metabolites and TPs found in the analysis of the seven effluent wastewater samples are shown in **Table 2**. Some examples are discussed below to illustrate the strategy applied in the different cases under study.

3.2.1 TPs/metabolites for which reference standards are available: information on retention time and fragment ions known

Some compounds, for which reference standards were available, were identified in the samples. This was the case of 4-acetamido aminoantipyrine and 4-formyl aminoantipyrine, two metabolites of the widely used analgesic metamizole (parent compound was not detected).These compounds have been frequently reported by IUPA research group in Spanish wastewater samples in previous studies [11,20] at relatively high concentration levels [20]. Other detected compounds were carbamazepine and its TP 10,11-dihydroxy carbamazepine, clopidogrel carboxylic acid, losartan carboxylic acid, desmethyl-clarithromycin and fenofibric acid.

3.2.2 TPs/metabolites identified in laboratory experiments: reference standards not available but information on retention time and fragment ions known.

This situation occurred for several compounds, for which degradation and/or metabolism studies were previously performed at our laboratory. Among these, omeprazole can be

emphasized, a widely consumed pharmaceutical that, however, is not commonly detected in wastewaters. Three metabolites, OM10, OM14b (4-hydroxy-omeprazole sulphide) and OM7d were identified in all effluent samples with abundant signals that might reveal high concentrations. The results are in agreement with previous studies [16], where these three compounds (together with OM14a, which however was not found in the samples from the present work), were the most frequently detected in Spanish effluent wastewater. These compounds were also found in influent wastewater collected from Italy and Spain and analysed by both Orbitrap and QTOF MS [21].

Another compound was irbesartan, for which two transformation products (IB3a and IB3b) were identified in all the seven samples. Valsartan, venlafaxine and its TP VB1a (O-desmethyl venlafaxine), as well as the TP GSWB1 of gemfibrozil were also present in the samples. These results are in close agreement with those obtained in previous studies [17, 21].

In these cases, the presence of the TPs/metabolites was confirmed using the sample vial with the highest concentration of the (tentative) analyte from previous laboratory degradation experiments. This allowed the confirmation of the retention time and the main fragment ions observed.

As an example, **Figure 1** shows the nw-XICs (150 ppm mass window) for $[M+H]^+$(or $[M-H]^-$) in LE function, and the main fragments in HE function for three metabolites of omeprazole (OM10, OM14b, OM7d), two TPs of irbesartan (IB3a, IB3b), one TP of venlafaxine (VB1a) and one TP of gemfibrozil (GSWB1) in an effluent wastewater sample from Athens.

3.2.3 Reference standard not available: Information on experimental retention time and fragment ions unknown - Tentative identification.

The accurate-mass full-spectrum acquisition QTOF MS data allowed detecting and tentatively identifying a notable number of TPs in the samples under study, regardless of the availability of the reference standards.

Figure 2 shows the tentative identification of clindamycin sulfoxide in an effluent wastewater sample. The LE spectrum in ESI positive of the chromatographic peak detected at 6.10 min, showed an abundant signal at *m/z* 441.1821 (**Figure 2a, bottom**), with the isotope profile of a chlorine atom. This could correspond to the protonated molecule of clindamycin sulfoxide ($C_{18}H_{34}N_2O_6SCl$, expressed as protonated molecule, with a mass error of -1.1 ppm in relation with its theoretical exact mass). The HE spectrum showed three fragment ions at *m/z* 377.1837 ($C_{17}H_{30}N_2O_5Cl$, corresponding to the loss of CH_3SOH), *m/z* 126.1280 ($C_8H_{16}N$) and 164.0710 ($C_9H_{10}NO_2$), all with mass errors below 2 ppm (**Figure 2a, top**). The structure of these fragment ions was justified on the basis of their measured accurate masses, and all were compatible with the structure of the candidate TP. Moreover, the fragment ions *m/z* 377 and 126 are in accordance with the scientific literature [22-23]. All these data strongly support the tentative identification of the compound as clindamycin sulfoxide. In a similar way, 10 TPs were tentatively identified: norcitalopram, α-hydroxymidazolam, two TPs of propafenone (5-hydroxy and N-desisopropyl), a metabolite of climbazole, one metabolite of verapamil, and 14-hydroxy-clarithomycin.

3.2.4 Common fragmentation pathway with the parent compound or with other metabolites/TPs

The presence of additional chromatographic peaks in the extracted ion chromatograms (nw-XICs) from HE acquisition at *m/z* fragments corresponding to the

parent compound may alert the investigator to the presence of potential drug metabolites [24]. This strategy assumes that many metabolites/TPs share the fragmentation pathway with the parent compound. It can be applied not only for the fragmentation pathway of the parent compound but also for other metabolites/TPs identified in the samples.

The use of MS^E acquisition mode is of great help when using this strategy, as it gives information on the fragmentation of compounds in the collision cell without precursor ion selection. MS^E mode involves the sequential acquisition of accurate mass data at low and high collision energy. At low energy (LE), fragmentation is minimized and the most abundant ion corresponds normally to the parent molecule (adducts in some cases). However, at high collision energy (HE), fragmentation of the molecule is favoured. So, both (de)protonated molecule and fragment ion data are enabled in a single injection without the need of selecting the precursor ion.

An illustrative example of positive findings using this strategy is outlined below. The XIC at the *m/z* corresponding to the protonated molecule of carbamazepine (*m/z* 237.1028) showed a chromatographic peak at the expected retention time of the parent pharmaceutical (8.80 min), but also another one at 7.04 min (**Figure 3a, bottom**). Moreover, XICs of the fragment ions of carbamazepine (*m/z* 192.0813, 194.0970 and 179.0735) indicated that both compounds shared these three fragment ions **(Figure 3a, middle)**. Therefore, the peak at 7.04 min was treated as a potential metabolite/TP. After investigating the LE function at that retention time **(Figure 3a, top)**, the accurate mass was assigned to *m/z* 255.1138 **(Figure 3b, bottom)**, which corresponded to the elemental composition $[C_{15}H_{15}N_2O_2]^+$ (error 1.2 ppm). According to this molecular formula, this metabolite would contain two hydrogen atoms and one oxygen more than carbamazepine. After searching in the METLIN database [25] for metabolites of carbamazepine, one possible metabolite was found: 10-hydroxycarbazepine. In addition,

spectra of this compound at different cone voltages were available in the same database, and perfectly fitted with our experimental TOF mass spectra. According to the literature, 10-hydroxycarbazepine is not a true metabolite of carbamazepine but the major pharmacologically active component after oxcarbazepine ingestion [26]. In any case, all experimental data and information reported in the literature strongly support that the compound detected in effluent wastewater was 10-hydroxycarbazepine [25, 27]. Obviously, for ultimate confirmation, the reference standard would be required.

Another interesting case was related to diltiazem, a pharmaceutical used alone or together with other medicines to treat severe chest pain (angina) and/or high blood pressure (hypertension). In the last few years, its use as an adulterant or cutting agent has increased considerably. The reference standard was not available for the UJI, but its tentative identification was supported by the presence of the protonated molecule (*m/z* 415.1692) and two important fragment ions at *m/z* 178.0327 (C_9H_8NOS) and 150.0377(C_8H_8NS) (see peak at 8.56 min in **Figure 4 (a-c)**), which were in agreement with data provided by UoA and reported in the literature [25, 28].Up to six additional chromatographic peaks sharing both masses (see **Figure 4 (d-g)**) were observed. The LE spectrum of all these possible related compounds was investigated, and they were the result of demethylation (2 compounds), double demethylation (1), deacetylation (1) or a combination of these processes (3). According to the literature, N-desmethyl diltiazem is reported to be the major metabolite. Reference standards should be acquired and injected to unequivocally confirm the identity of these TPs. Similarly, 1 metabolite/TP of clarithromycin and 3 of quetiapine were found.

To sum up, the presence of 34 TPs/metabolites was confirmed in the samples: seven of which corresponded to TPs for which reference standards were available at our lab; seven corresponded to TPs previously identified in laboratory degradation

experiments (for two of them the reference standard was available); eight were tentatively identified by QTOF MS without the use of reference standards, and twelve additional TPs were discovered after using the common fragmentation pathway approach.

3.2.5 Retrospective analysis and retention time prediction using ANN and SVM models

A retrospective analysis was then performed at UoA and the presence of all the 19 tentatively identified TPs was confirmed. Tandem MS spectra were similar to that found at UJI. Prediction of t_R by ANN and SVM models was applied for all 19 tentatively identified metabolites (**Table 2**) in the laboratories of UJI and UoA, respectively, to further support identification. As in previous works on t_R prediction at UJI [13, 29], a window of ±2 minutes was used as a means of added confidence in tentative identification. UoA also applied prediction of t_R for these compounds using a SVM model [13]. **Table 3** shows the results of the applied methods. For both methods, all but one of the metabolites were found within a ±2 minute window, with most within ±1 minute. The one metabolite outside of this window, O-desalkylquetiapine carboxylic acid, was only outside for the ANN model, whereas for the SVM model it was within a ±1 min window.

Although one metabolite fell outside the 2 minute window, it is important to note that t_R prediction is not absolutely precise, and, for ANN method, more than 90% of all compounds used in the optimization were found within 2 minutes (**Table S.3, in Supplementary Information**). These results prove the value of retention time prediction in suspect analysis, particularly for metabolites and TPs for which standards are difficult or impossible to obtain. Also the comparison of retention time models is important as

they may give additional identification evidence of the presence of doubtful TPs, such as O-desalkylquetiapine carboxylic acid.

CONCLUSIONS

UHPLC-QTOF MS has been shown as a powerful technique for screening and identification of metabolites and transformation products of pharmaceuticals. Operating the instrument under MS^E acquisition mode it is feasible to simultaneously obtain full-spectrum accurate-mass data at low and high collision energy. The combination of these two datasets is very useful for identification and elucidation purposes, as LE MS spectra usually show the (de)protonated molecule, while HE MS spectra are richer in fragment ions. With all the information provided by this technique (accurate mass, isotopic distribution, and MS data at LE and HE), and the efficient chromatographic separation offered by UHPLC, it is feasible to identify compounds in complex environmental matrices, by searching for target analytes (parent or metabolites) on the basis of a compound database. In this work, analysis of treated Greek urban wastewater has allowed the detection and identification of 34 TPs/metabolites of pharmaceuticals. Some reported metabolites, such as 4-formyl- and 4-acetamido-aminoantipirine, fenofibric acid and clopidogrel carboxylic acid, for which reference standards were available at our lab, were found. In addition, metabolites identified in previous degradation experiments performed under laboratory controlled conditions, such as those of the widely but seldom detected in water, omeprazole (omeprazole OM7d, omeprazole OM10 or 4-hydroxy omeprazole sulphide) or desmethyl-venlafaxine, amongst others, have also been found. With a good knowledge of the technique, and after appropriate treatment of all MS data provided, there is a high reliability in the identification of suspected candidates, even without reference standards. Thus, clindamycin sulfoxide, 14-hydroxy-clarithromycin, desmethyl citalopram or norquetiapine have been tentatively identified in the samples. Ultimate confirmation would require the injection of reference standards, which should be acquired only in those cases where QTOF experimental data strongly support their

presence in the samples. Finally, but no less important, is the fact that QTOF under MS^E mode also allowed applying the common fragmentation pathway approach between parent and metabolites. Following this strategy, up to six metabolites of diltiazem, three of quetiapine and one metabolite of oxcarbazepine were detected.

Data reported in this work are of relevance to estimate the contribution of wastewaters in terms of the discharge of metabolites and TPs from commonly consumed drugs to the aquatic environment.

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FIGURE CAPTIONS

Figure 1. Narrow-Window eXtracted Ion Chromatograms (nw-XICs) at 150 ppm mass window for $[M+H]^+$ (or $[M-H]^-$) in LE function, and main fragments in HE function for TPs OM10, OM14b, OM7d, IB 3a, IB3b, VB1a and GWSB1 in the effluent wastewater 4.

Figure 2. Detection and identification of clindamycin sulfoxide in an effluent wastewater. (a) nw-XICs at 150 ppm mass window for $[M+H]^+$ in LE function and main fragments in HE function. (b) LE (low energy) and HE (high energy) TOF mass spectra for the sample. Possible structures assigned.

Figure 3. Detection and identification of 10-hydroxycarbazepine in an effluent wastewater. (a) nw-XICs chromatograms at 150 ppm mass window for *m/z* 237.103, corresponding to the ion $[M+H]^+$ of carbamazepine in LE function, and *m/z* 192.081, 194.097 and 179.074 corresponding to its main fragments in HE function. nw-XIC chromatogram for *m/z* 255.1138, corresponding to a possible metabolite. (b) Combined LE and HE spectrum of potential metabolite.

Figure 4. Detection and identification of metabolites of diltiazem. nw-XICs at 150 ppm mass window for (a) *m/z* 415.169 in LE function, corresponding to the ion $[M+H]^+$ of diltiazem, and (b) *m/z* 178.033 and (c) 150.037 corresponding to its main fragments in HE (8.56 min). 150 ppm-XICs in LE at (d) *m/z* 401.154 (corresponding to two demethylated metabolites; 7.01 and 8.55 min), (e) 387.121 (didesmethylated metabolites; 7.15 and 8.55 min), (f) 373.159 (deacethylated; 7.60 min) and (g) 359.143 (two combinations of deacethylation and demethylation; 5.97 and 7.83 min).

Table 1. Parent pahrmaceuticals identified in effluent wastewater samples by the UoA. All compounds were confirmed with reference standard.

	1	**2**	**3**	**4**	**5**	**6**	**7**
Aliskiren	√	√	√	√	√	√	√
Amantadine	√	√	√	√	√	√	√
Amisulpride	√	√	√	√	√	√	√
Atazanavir	√	√	√	√	√	√	√
Atenolol	√	√	√	√	√	√	√
Azithromycin	√	√	√	√	√	√	√
Carbamazepine	√	√	√	√	√	√	√
Celiprolol	√	√	√	√	√	√	√
Cetirizine	√	√	√	√	√	√	√
Citalopram	√	√	√	√	√	√	√
Clarithromycin	√	√	√	√	√	√	√
Climbazole	√	√	√	√	√	√	√
Clindamycin	√	√	√	√	√	√	√
Clopidogrel	√	√	√	√	√	√	√
Clozapine	√	√	√	√	√	√	√
Diclofenac	√	√	√	√	√	√	√
Diltiazem	√	√	√	√	√	√	√
Diphenhydramine	√	√	√	√	√	√	√
Eprosartan	√	√	√	√	√	√	√
Fluconazole	√	√	√	√	√	√	√
Furosemide	√	√	√	√	√	√	√
Gabapentin	√	√	√	√	√	√	√
Irbesartan	√	√	√	√	√	√	√
Lamotrigine	√	√	√	√	√	√	√
Levamisole	√	√	√	√	√	√	√
Lidocaine	√	√	√	√	√	√	√
Losartan	√	√	√	√	√	√	√
Meclofenamic acid	d	d	d	d	d	d	d
Memantine	√	√	√	√	√	√	√
Metoprolol	√	√	√	√	√	√	√
Mirtazapine	d	d	d	d	d	d	d
Niflumic acid	√	√	√	√	√	√	√
Oxcarbazepine	d	d	d	d	d	d	d
Propranolol	√	√	√	√	√	√	√
Propafenone	√	√	√	√	√	√	√
Quetiapine	d	d	d	d	d	d	d
Sitagliptin	√	√	√	√	√	√	√
Sotalol	√	√	√	√	√	√	√
Sulpiride	√	√	√	√	√	√	√
Telmisartan	√	√	√	√	√	√	√
Trimethoprim	√	√	√	√	√	√	√
Valsartan	√	√	√	√	√	√	√
Venlafaxine	√	√	√	√	√	√	√
Verapamil	√	√	√	√	√	√	√
Vildagliptin	√	√	√	√	√	√	√

d: detected (only (de)protonated molecule was observed at the expected retention time)
√: confirmed ((de)protonated molecule and at least one fragment ion were observed at the expected retention time).

Table 2.Metabolites/TPs of pharmaceuticals found in effluent wastewater samples by the UJI.

	Compound	1	2	3	4	5	6	7
Reference standards	**Clarithromycin**	√	√	√	√	√	√	√
	Desmethyl-clarithromycin*	√	√	√		√	√	√
	Carbamazepine	√	√	√	√	√	√	√
	10,11-dihydro-10,11-dihydroxy carbamazepine*	√	√	√	√	√	√	√
	Clopidogrel	√	√	√	√	√	√	√
	Clopidogrel carboxylic acid *	√	√	√	√	√	√	√
	Dypirone/metamizol							
	4-acetamido antipyrine *	√	√	√	√	√	√	√
	4-formyl aminoantipyrine *	√	√	√	√	√	√	√
	Fenofibrate							
	Fenofibric acid*	√	√	√	√	√	√	√
	Losartan		√	√	√	√	√	√
	Losartan carboxylic acid *	√	√	√	√	√	√	√
Degradation experiments	**Omeprazole**							
	Omeprazole OM7d	√	√	√	√	√	√	√
	Omeprazole OM10	√	√	√	√	√	√	√
	Omeprazole OM14b=4-hydroxy-omeprazole sulphide*	√	√	√	√	√	√	√
	Irbesartan	√	√	√	√	√	√	√
	Irbesartan IB3a	√	√	√	√	√	√	√
	Irbesartan IB3b	√	√	√	√	√	√	√
	Valsartan	√	√	√	√	√	√	√
	Venlafaxine	√	√	√	√	√	√	√
	VB1a=O-Desmethylvenlafaxine *	√	√	√	√	√	√	√
	Gemfibrozil							
	Gemfibrozil GSWB1	√	√	√	√	√	√	√
Tentative identification	**Climbazole**	√	√	√	√	√	√	√
	BAY g 5919	√	√	√	√	√	√	√
	Clindamycin	√	√	√	√	√	√	√
	Clindamycin sulfoxide	√	√	√	√	√	√	√
	Clarithromycin	√	√	√	√	√	√	√
	14-hydroxy-clarithromycin	√	√	√	√	√	√	√
	Citalopram	√	√	√	√	√	√	√
	Norcitalopram	√	√	√	√	√	√	√
	Midazolam							
	α-hydroxymidazolam	√	√	√	√	√	√	√
	Propafenone	√	√	√	√	√	√	√
	5-hydroxy-propafenone	√	√	√	√	√	√	√
	N-desisopropyl-propafenone	√	√	√	√	√	√	√
	Verapamil	√	√	√	√	√	√	√
	Met D617 verapamil	√	√	√	√	√	√	√
Common fragmentation pathway	**Clarithromycin**	√	√	√	√	√	√	√
	Met 590 clarithromycin	√	√	√	√	√	√	√
	Cetirizine	√	√	√	√	√	√	√
	Met 295 cetirizine	√	√	√	√	√	√	√
	Diltiazem	√	√	√	√	√	√	√
	N-Desmethyl diltiazem	√	√	√	√	√	√	√
	O-Desmethyl diltiazem	√	√	√	√	√	√	√
	N,N-Didesmethyl diltiazem	√	√	√	√	√	√	√
	O-Deacetyl diltiazem	√	√	√	√	√	√	√
	O-Deacetyl-N-desmethyl diltiazem	√	√	√	√	√	√	√
	O-Deacetyl-O-desmethyl diltiazem	√	√	√	√	√	√	√

	Oxcarbazepine	√	√	√	√	√	√	√
	10-hydroxycarbazepine	√	√	√	√	√	√	√
	Quetiapine	√	√	√	√	√	√	√
	Norquetiapine	√	√	√	√	√	√	√
	Quetiapine N-oxide	√	√	√	√	√	√	√
	O-desalkyl quetiapine carboxylic acid	√	√	√	√	√	√	√

*Confirmed with reference standard available

Table 3. Comparison of the t_R prediction models for all tentatively identified metabolites (from Table 2), together with sample and predicted t_R, between the two laboratories.

Compound	Samples t_R (min) UJI	Predicted t_R (min) UJI	Inaccuracy in predicted t_R (min) UJI	Samples t_R (min) UoA	Predicted t_R (min) UoA	Inaccuracy in predicted t_R (min) UoA
BAY g 5919	9.45	11.24	1.79	9.08	8.28	0.80
Clindamycinsulfoxide	6.1	6.23	0.13	5.18	3.55	1.63
14-hydroxy-clarithromycin	8.65	8.82	0.17	7.32	8.63	-1.31
Norcitalopram	7.87	7.89	0.02	6.63	6.29	0.34
α-hydroxymidazolam	8.99	8.47	-0.52	8.43	8.98	-0.55
5-hydroxy-propafenone	7.93	8.19	0.26	6.62	7.03	-0.41
N-desisopropyl-propafenone	7.20	5.95	-1.25	5.96	5.80	0.16
Met D617 verapamil	7.38	7.78	0.40	6.09	6.21	-0.12
N-Desmethyldiltiazem	8.69	7.52	-1.17	5.83	6.35	-0.52
O- Desmethyldiltiazem	7.01	7.06	0.05	7.30	6.48	0.82
N,N-Didesmethyldiltiazem	7.15	7.22	0.07	5.93	5.73	0.20
O-Deacetyldiltiazem	7.60	6.63	-0.97	6.51	6.20	0.31
O-Deacetyl-N-desmethyl diltiazem	7.83	6.48	-1.35	5.21	5.47	-0.26
O-Deacetyl-O-desmethyl diltiazem	5.97	6.23	0.26	6.67	5.72	0.95
10-hydroxycarbazepine	7.04	6.22	-0.82	5.96	6.20	-0.24
Norquetiapine	8.48	6.82	-1.66	7.37	6.47	0.90
Quetiapine N-oxide	8.76	8.44	-0.32	7.58	8.41	-0.83
Met 590 clarithromycin	8.05	8.06	0.01	6.68	6.85	-0.17
O-desalkyl Quetiapine carboxylic acid	8.91	12.53	3.62	7.70	7.38	0.32

FIGURES

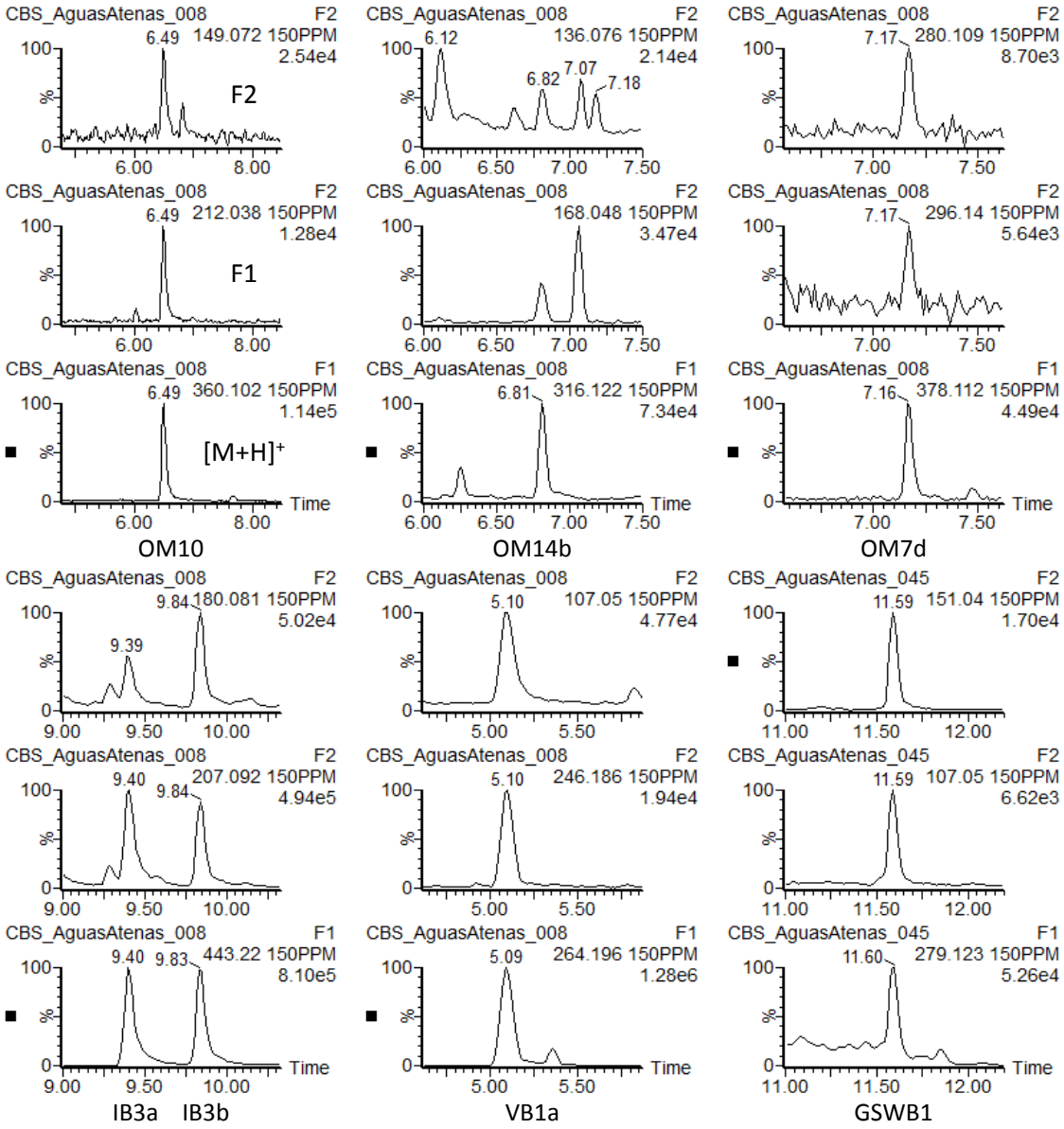
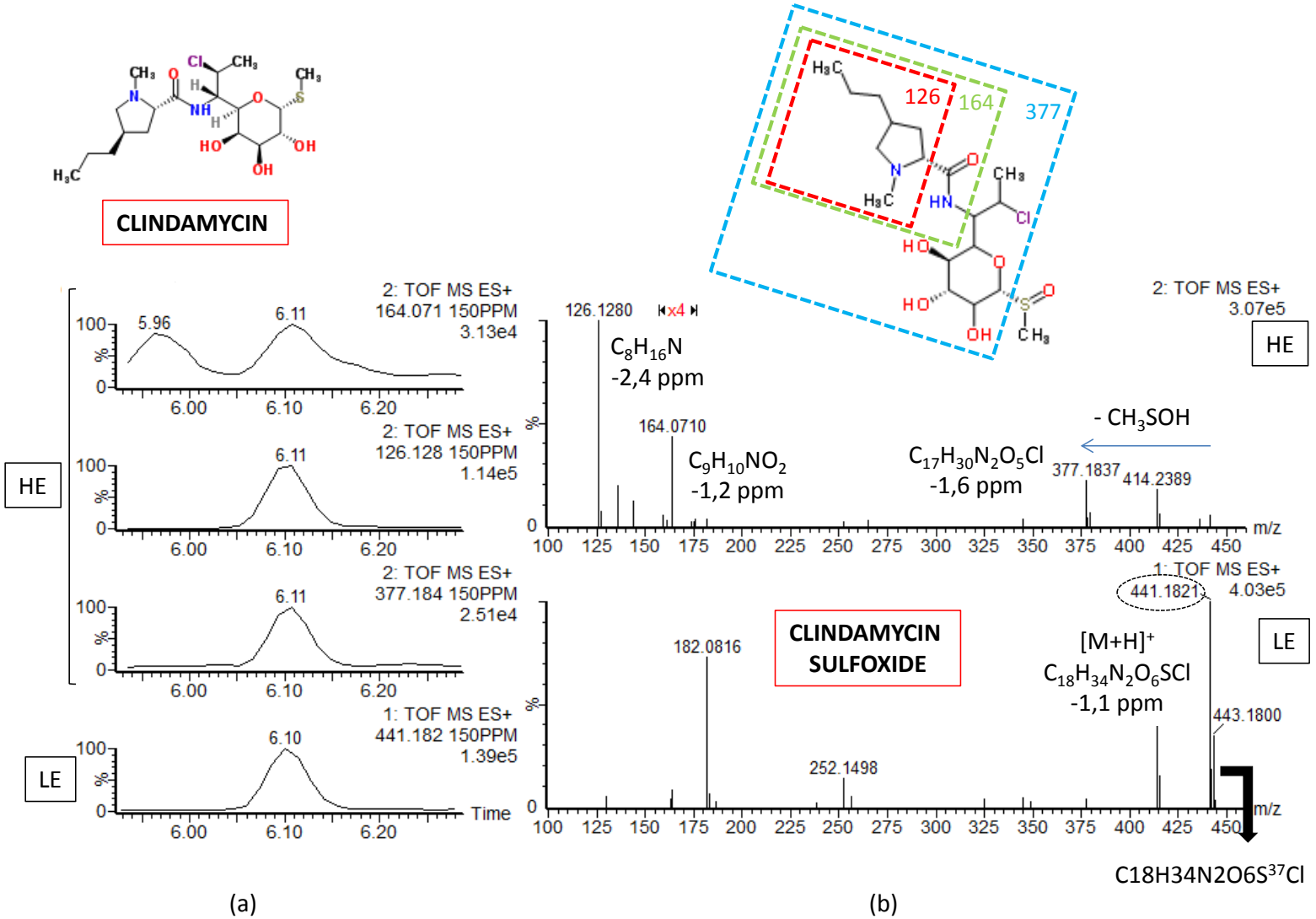


Fig 1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126
164
377
HE
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3.13e4
5.96
6.11
2: TOF MS ES+
126.128 150PPM
1.14e5
6.11
2: TOF MS ES+
377.184 150PPM
2.51e4
6.11
LE
1: TOF MS ES+
441.182 150PPM
1.39e5
6.10
Time
6.00
6.10
6.20
126.1280
x4
C8H16N
-2,4 ppm
164.0710
C9H10NO2
-1,2 ppm
C17H30N2O5Cl
-1,6 ppm
- CH3SOH
377.1837
414.2389
2: TOF MS ES+
3.07e5
HE
m/z
CLINDAMYCIN
SULFOXIDE
182.0816
252.1498
[M+H]+
C18H34N2O6SCl
-1,1 ppm
441.1821
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LE
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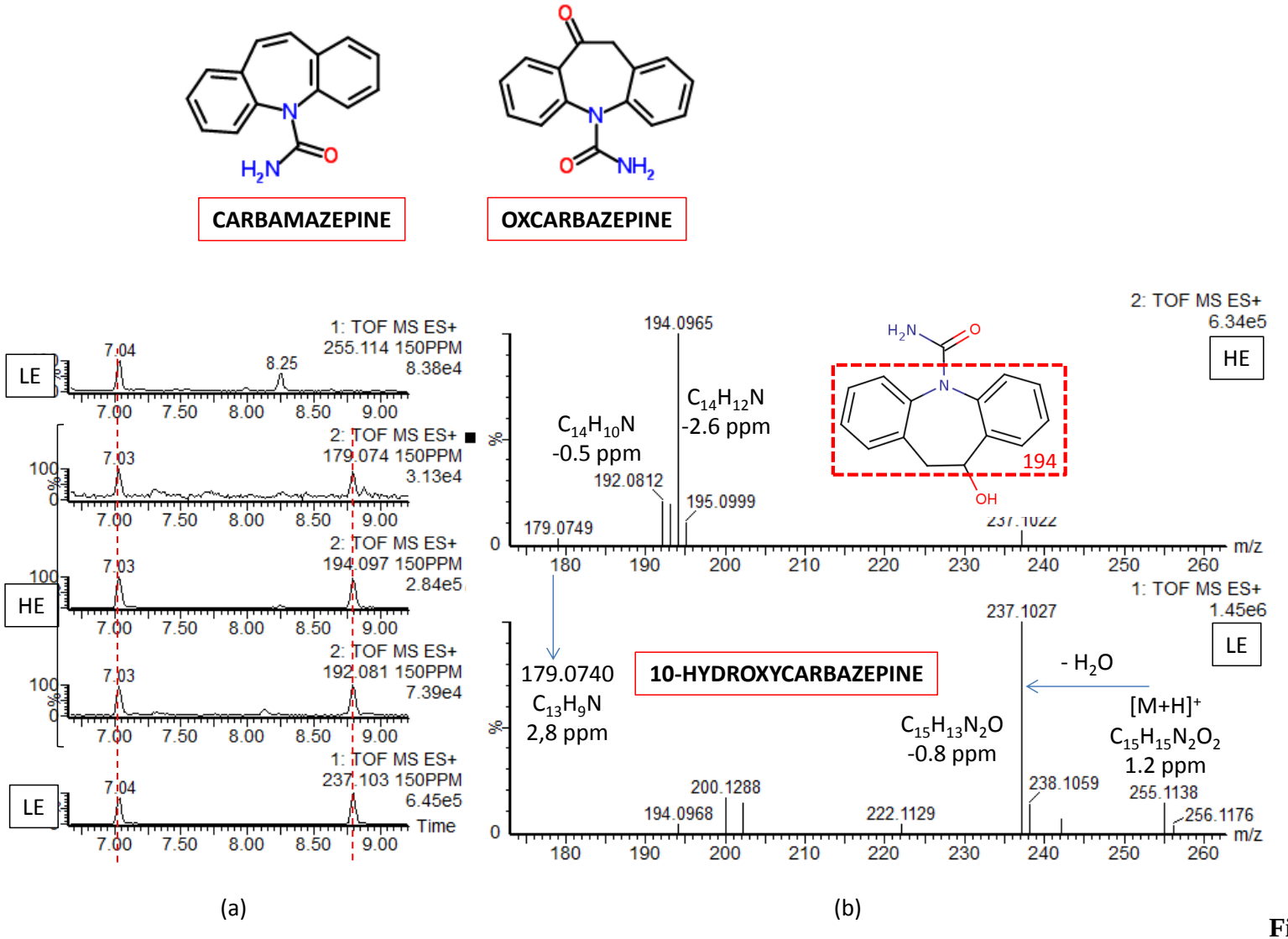
(a)
(b)

Fig 2


CARBAMAZEPINE
OXCARBAZEPINE
LE
HE
LE
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8.38e4
2: TOF MS ES+
179.074 150PPM
3.13e4
2: TOF MS ES+
194.097 150PPM
2.84e5
2: TOF MS ES+
192.081 150PPM
7.39e4
1: TOF MS ES+
237.103 150PPM
6.45e5
7.04
8.25
7.03
7.04
Time
2: TOF MS ES+
6.34e5
HE
194.0965
C14H12N
-2.6 ppm
C14H10N
-0.5 ppm
192.0812
195.0999
179.0749
194
OH
m/z
1: TOF MS ES+
1.45e6
LE
237.1027
179.0740
C13H9N
2,8 ppm
10-HYDROXYCARBAZEPINE
- H2O
[M+H]+
C15H15N2O2
1.2 ppm
C15H13N2O
-0.8 ppm
200.1288
194.0968
222.1129
238.1059
255.1138
256.1176

(a) (b)

Fig 3

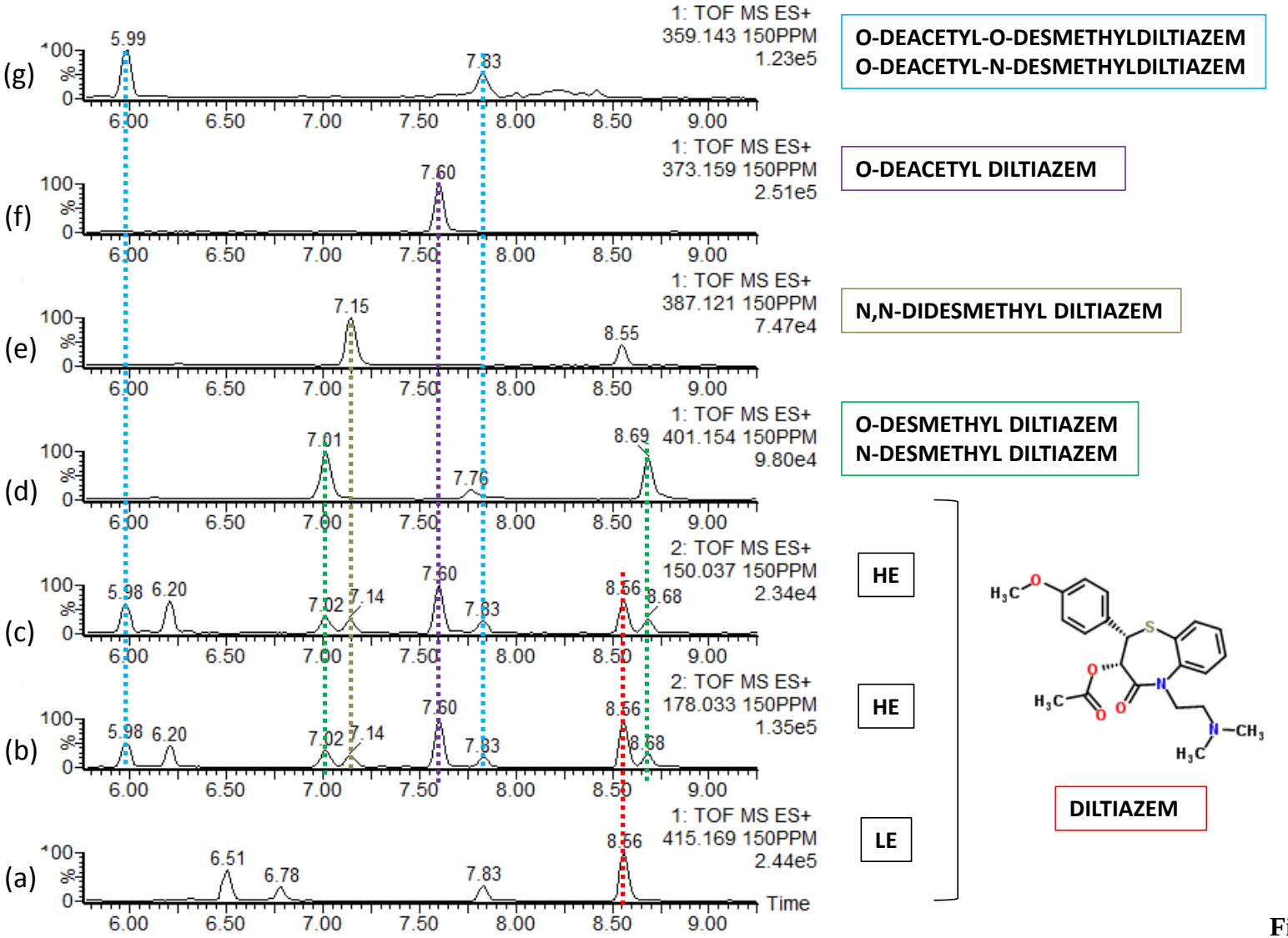
(g)
1: TOF MS ES+
359.143 150PPM
1.23e5
5.99
7.83
O-DEACETYL-O-DESMETHYLDILTIAZEM
O-DEACETYL-N-DESMETHYLDILTIAZEM
(f)
1: TOF MS ES+
373.159 150PPM
2.51e5
7.60
O-DEACETYL DILTIAZEM
(e)
1: TOF MS ES+
387.121 150PPM
7.47e4
7.15
8.55
N,N-DIDESMETHYL DILTIAZEM
(d)
1: TOF MS ES+
401.154 150PPM
9.80e4
7.01
7.76
8.69
O-DESMETHYL DILTIAZEM
N-DESMETHYL DILTIAZEM
(c)
2: TOF MS ES+
150.037 150PPM
2.34e4
5.98
6.20
7.02
7.14
7.60
7.83
8.56
8.68
HE
(b)
2: TOF MS ES+
178.033 150PPM
1.35e5
5.98
6.20
7.02
7.14
7.60
7.83
8.56
8.68
HE
(a)
1: TOF MS ES+
415.169 150PPM
2.44e5
6.51
6.78
7.83
8.56
LE
Time
6.00
6.50
7.00
7.50
8.00
8.50
9.00
100
%
0
DILTIAZEM

Fig 4