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Autores / Autors	Boix Sales, Clara ; Ibáñez Martínez, María ; Zamora, T. ; Sancho Llopis, Juan Vicente ; Niessen, Wilfried M. A. ; Hernández Hernández, Félix
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IDENTIFICATION OF NEW OMEPRAZOLE METABOLITES IN WASTEWATERS AND SURFACE WATERS

C. Boix[1], M. Ibáñez[1], T. Zamora[1], J.V. Sancho[1], W.M.A. Niessen[2] and F. Hernández[1]*

[1]Research Institute for Pesticides and Water, University Jaume I, Avda. Sos Baynat, E-12071 Castellón, Spain.

[2]hyphen MassSpec, de Wetstraat 8, 2332 XT Leiden, the Netherlands.

*Corresponding author *felix.hernandez@uji.es* Tel +34 964 387366, Fax +34 964 387368

ABSTRACT

Omeprazole is one of the world-wide most consumed pharmaceuticals for treatment of gastric diseases. As opposed to other frequently used pharmaceuticals, omeprazole is scarcely detected in urban wastewaters and environmental waters. This was corroborated in a previous research, where parent omeprazole was not detected while four transformation products (TPs), mainly resulting from hydrolysis, were found in effluent wastewaters and surface waters. However, the low abundance of omeprazole TPs in the water samples together with the fact that omeprazole suffers an extensive metabolism, with a wide range of excretion rates (between 0.01-30%), suggests that human urinary metabolites should be investigated in the water environment. In this work, the results obtained in excretion tests after administration of a 40 mg omeprazole dose in three healthy volunteers are reported. Analysis by liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF MS) reported low concentrations of omeprazole in urine. Up to twenty-four omeprazole metabolites (OMs) were detected and tentatively elucidated. The most relevant OM was an omeprazole isomer, which obviously presented the same exact mass (*m/z* 346.1225), but also shared a major common fragment at *m/z* 198.0589. Subsequent analyses of surface water and effluent wastewater samples by both LC-QTOF MS and LC-MS/MS with triple quadrupole revealed that this metabolite (named as OM10) was the compound most frequently detected in water samples, followed by OM14a and OM14b. Up to our knowledge, OM10 had not been used before as urinary biomarker of omeprazole in waters. On the contrary, parent omeprazole was never detected in any of the water samples. After this research, it seems clear that monitoring the presence of omeprazole in the aquatic environment should be focused on the OMs suggested in this article instead of the parent compound.

Keywords

Omeprazole, metabolites, urine, time-of-flight mass spectrometry, triple quadrupole mass spectrometry, water samples

1. INTRODUCTION

Environmental contamination by pharmaceuticals (both human and veterinary medicines) is an issue of general concern. They are emerging pollutants widely distributed in the environment, which can enter through different routes (Zuccato et al. 2005). Once a pharmaceutical is administered, it can be excreted unchanged or as metabolites in the urine or faeces, reaching the aquatic environment commonly throughout sewage waters (Besse et al. 2012; González Alonso et al. 2010; Ortiz de García et al. 2013).

Omeprazole is one of the most frequently prescribed and administered pharmaceuticals in humans for proton pump inhibition (Andersson et al. 1993; Bruni and Ferreira. 2008; José Gómez et al. 2007; Ortiz de García et al. 2013). As an example, 51,874,630 packages, under prescription, were dispensed in Spain in 2010 (http://www.msssi.gob.es/biblioPublic/publicaciones/recursos_propios/infMedic/docs/SubgruposATCvol35n4.pdf). It is known to act by irreversibly blocking the terminal stage of gastric acid secretion in the gut. This compound is reported to be metabolized by the enzyme CYP2C19 to form the 5-hydroxy metabolite whereas CYP3A4 catalyzes the sulfone formation (Kanazawa et al. 2002; Rost et al. 1995). About 80% of orally administered omeprazole dose is excreted in urine as metabolites, whereas the remainder is excreted in the faeces, mainly from biliary secretion (Andersson et al. 1993). Different percentages of omeprazole excretion (as intact parent) can be found in the literature, ranging from 0.01% (Besse et al. 2008) to 5% (Hernando et al. 2007), or even up to 30% (Ortiz de García et al. 2013). This variation has been justified based on the different enzymatic activity of each individual. Sulfonated and 5-hydroxylated compound are the major omeprazole metabolites (OMs) found in plasma (Espinosa Bosch et al. 2007; Song and Naidong. 2006), whereas in urine the 5-hydroxylated OM

is the predominant one (Petsalo et al. 2008) (**Figure 1**). The concentration of omeprazole sulfide, another OM reported in the literature, is usually too low to be determined in plasma, and it is also negligible in urine (Rezk et al. 2006).

Several analytical methods have been reported for determination of omeprazole in plasma (Kanazawa et al. 2002; Macek et al. 2007; Rost et al. 1995; Song and Naidong. 2006) while only a few articles deal with analysis of urine samples. Petsalo et al. (Petsalo et al. 2008), focused on the determination of the 3-hydroxy-, 5-hydroxy-, demethyl-, and sulfone-OMs and omeprazole itself in urine. It was not possible to detect 3-hydroxy OM, and the concentrations of omeprazole and its sulfone OM were very low. Chung et al. (Chung et al. 2004), reported the detection of four unconjugated and two conjugated OMs in horse urine by LC-MS.

The available literature on omeprazole determination highlights the application of liquid chromatography (LC) as the most appropriate analytical tool for this compound (Kanazawa et al. 2002; Petsalo et al. 2008; Song and Naidong. 2006; Ternes et al. 2001). Although some methods have made use of UV as detection technique (Rezk et al. 2006), currently mass spectrometry (MS) is the technique of choice for determination of omeprazole, particularly LC coupled to tandem MS (MS/MS), the advantages of which, short analytical run time as well as excellent selectivity and sensitivity, are widely recognized (Espinosa Bosch et al. 2007). While LC-MS/MS with triple quadrupole (QqQ) analyzer is the workhorse for quantitative analysis of pharmaceuticals, omeprazole included, in the aquatic environment (Castiglioni et al. 2004; Gracia-Lor et al. 2010; Van Nuijs et al. 2010; Zuccato et al. 2005), LC coupled to high resolution mass spectrometry (HRMS) such as Orbitrap (Calza et al. 2012; Thevis et al. 2011), FTMS (Awasthi et al. 2012) or time-of-flight MS (Ibáñez et al. 2004; Ibáñez et al. 2006) is a powerful analytical tool for investigation of metabolites and/or

transformation products (TPs) in water. These HR MS techniques are also appropriate to perform metabolism studies of pharmaceuticals within the biomedical field (Corcoran et al. 2000; Hopfgartner et al. 1999) due to the accurate-mass full-spectrum acquisitions provided by these analyzers.

Considering the high consumption of omeprazole and the reported excretion rates of up to 20% as intact omeprazole, one might expect to find this compound in urban wastewater, or even in environmental waters. Nevertheless, its detection in water samples is rarely reported. Additionally, in our previous study on omeprazole degradation (Boix et al. 2013), only four low-abundant TPs were rarely found in water samples, with omeprazole sulfide the most frequently detected. The initial hypothesis on a possible degradation of omeprazole in waters was thus discarded and a detailed study on human urinary metabolites of omeprazole was initiated. This paper pursues the detection and elucidation of urinary OMs making use of LC-QTOF MS. Subsequently, 27 surface water (SW) and 25 wastewater (WW) samples have been analyzed by LC-QTOF MS and LC-MS/MS QqQ to investigate the presence of OMs.

2. EXPERIMENTAL

2.1 Reagents and chemicals

See Supplementary Information (SI).

2.2 Instrumentation

UHPLC-QTOF MS

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole–orthogonal acceleration-TOF mass spectrometer (Q-oaTOF

124 Premier, Waters Micromass, Manchester, UK), using an orthogonal Z-spray
125 electrospray ionization (ESI) interface operating in positive and negative ion modes
126 (For further details, see SI).

127 QTOF data were acquired under MS^E mode, an approach that enables the
128 simultaneous acquisition of both parent protonated molecules and fragment ions in a
129 single injection. So, two acquisition functions with different collision energies were
130 created. The first one, the low energy (LE) function, selecting a collision energy of 4
131 eV, and the second one, the high energy (HE) function, with a collision energy ramp
132 ranging from 15 eV to 40 eV (Díaz et al. 2011; Hernández et al. 2011a; Hernández et al.
133 2011b; Plumb et al. 2006).

134 UHPLC-MS/MS QqQ

135 A Waters Acquity UPLC system was interfaced to a triple quadrupole mass
136 spectrometer (TQD, Waters) with an orthogonal Z-spray-ESI interface. Capillary
137 voltages of 3.5 and -3.0 kV were used in positive and negative ionization mode,
138 respectively (For further details, see SI).

139 2.3 Analysis of metabolites excreted in urine

140 Urine samples were collected from three healthy volunteers with different gender (1
141 male, 2 females) and origins (2 Europeans and 1 Latin-American, all living in Europe).
142 Each volunteer ingested an oral dose of 40 mg omeprazole (i.e., two 20 mg capsules).
143 Urine samples were collected before ingestion of the drug (control sample), and after 15
144 minutes, 1, 3.5, 6.5, 9, 12 and 24 hours of drug administration. Polyethylene bottles
145 were used for collecting samples, which were immediately stored at –18 ºC until
146 analysis. The study protocol was approved by an ethical committee (University Jaume I,
147 Spain).

For analysis of urine, 1 mL of sample previously centrifuged (10,000 r.p.m., 10 minutes), was two-fold diluted with Milli-Q water. After that, 50 µL were directly injected in the UHPLC-QTOF MS system.

For the determination of metabolites released from glucuronide conjugates, 1 mL of centrifuged urine was buffered with 50 µL acetic acid/ammonium acetate (pH 5.5). After being hydrolyzed overnight with 200 units of β-D-glucuronidase at 37 ºC, 50 µL of the hydrolyzed mixture were injected in the UHPLC-QTOF MS system (Hernández et al. 2004).

2.4 Retrospective QTOF MS analysis of water samples

25 wastewater samples (15 influents and 10 effluents) were collected from three different wastewater treatment plants (WWTPs) of the Valencian region (Eastern Spain), whose anonymity must be respected, from June 2008 to December 2010. 27 surface waters were also sampled from several points located in the same area in October 2010. All these samples had been used previously in different studies performed at our lab using UHPLC-QTOF MS for their analysis.

2.5 Data processing

QTOF MS data were processed using MetaboLynx XS and ChromaLynx XS application managers (Waters Micromass vs 4.1). Regarding data from triple quadrupole, TargetLynx software (also from Waters) was used (More details in ref. (Boix et al. 2013)).

3. RESULTS AND DISCUSSION

3.1 Urinary excretion of parent omeprazole

171 Omeprazole was detected at low concentration levels (not quantified) in non-
172 hydrolyzed urine from the two European volunteers approximately between 1 and 3
173 hours after administration of the drug. In the third volunteer (Latin American female),
174 the drug was not detected in any of the urine samples collected. This would be in
175 accordance with the scientific literature where different levels of excretion for
176 omeprazole have been reported (Besse et al. 2008; Hernando et al. 2007; Ortiz de
177 García et al. 2013; Zuccato et al. 2005).

178 Interestingly, the narrow window eXtracted Ion Chromatogram (nw-XIC, 0.02Da) at
179 the exact mass of omeprazole ($[M+H]^+$ *m/z* 346.1225), showed a highly abundant peak
180 at different retention time (5.88 min for omeprazole, 4.91 min for this compound,
181 OM11a) (**Figure S1a**). Although HE MS spectra of both compounds were quite
182 different (e.g. specific fragment ions: *m/z* 136 and 151 for omeprazole; *m/z* 138 and 149
183 for OM11a, **Figures S1b,c**), they shared an important common fragment at *m/z*
184 198.0589. This fact cannot be neglected, as it might lead to a false positive of
185 omeprazole even by LC-MS/MS if only the transition 346.1>198.1 (the most commonly
186 reported in the literature) is acquired (Petsalo et al. 2008; Macek et al. 2007; Song and
187 Naidong. 2006), without sufficient chromatographic separation. This is of relevance
188 taking into account the high abundance of this metabolite.

189 3.2 Elucidation of metabolites

190 **Table 1** shows the elemental composition, mass errors of the (de)protonated
191 molecule and fragments ions, and retention time for omeprazole and twenty-four OMs
192 detected in hydrolyzed and non-hydrolyzed urine. **Figure 1** shows the structures
193 suggested for the main fragment ions together with the omeprazole metabolites reported
194 in the literature. Tentative structure proposals for OMs identified in this work are given

195 in **Figure 2**. All metabolites were detected in positive-ion mode except for the OM12,
196 which was only detected in negative-ion mode.

197 Different conjugated metabolites, as cysteine (OM1), glucuronides (OM2a, OM2b,
198 and OM2c) and sulfates (OM5 and OM12) were directly detected (**Figure 2**) in the non-
199 hydrolyzed urine. After hydrolysis, a decrease of cysteine and glucuronide conjugates
200 was observed, as well as the corresponding increase in abundance of the OM11a and
201 OM7d. Additionally, a new peak appeared, corresponding to $[C_{17}H_{20}N_3O_3S]^+$ (OM11b)
202 (*m/z* 346.1225, Δ = 1.5 mDa), with the same elemental composition as omeprazole and
203 its isomeric metabolite OM11a, but eluting even earlier (4.52 min). With these data, it
204 seems that OM7d and OM11a are partly conjugated while OM11b is fully conjugated
205 prior to urinary excretion. Based on similarities in their fragmentation, OM11a and
206 OM11b would correspond to the free forms of OM2(b,c) and OM2a, respectively.

207 The compound OM4 $[C_{16}H_{18}N_3O_3S]^+$, *m/z* 332.1069 (Δ = 0.7 mDa; 3.73 min), has
208 the same exact mass as the reported demethyl-OM (Kanazawa et al. 2002; Petsalo et al.
209 2008; Rost et al. 1995). However, OM4 was not considered to match demethyl-OM, as
210 the spectrum of OM4 shows the specific loss of *m/z* 32.9799 ([$^{\bullet}$SH]) related to the
211 reduction of the sulfoxide group.

212 The loss of the thiol radical [$^{\bullet}$SH] from the protonated molecule was observed in
213 ESI positive for all OMs when the sulfoxide moiety was converted into a sulfide.
214 Initially, cyclation of omeprazole molecule rendering a terminal thiol group was
215 considered, as reported to occur under acidic conditions (Bruni and Ferreira. 2008;
216 DellaGreca et al. 2006; Weidolf and Castagnoli. 2001). However, in the MS/MS
217 spectrum of the 4-hydroxy omeprazole sulfide reference standard ($C_{16}H_{18}N_3O_2S^+$, *m/z*
218 316.1120), the loss of the thiol radical [$^{\bullet}$SH] was also observed (**Figure S2a**).
219 Therefore, the initial hypothesis of cyclisation was rejected. The [$^{\bullet}$SH] loss (32.9799

Da) from the thioether group could be explained from a homolytic cleavage and intermediate binding to the hydrogen at the imidazolyl-N to form [$^{\bullet}$SH] **(Figure S2b)**. Although the mass error was relatively high (around 4 mDa), other elemental compositions could not be assigned. A subsequent homolytic cleavage, involving the loss of a methyl radical [$^{\bullet}CH_3$] is observed as well (see **Figure S2c**). The [$^{\bullet}$SH] loss observed for many OMs suggested the reduction of the sulfoxide group to sulfide.

Regarding the position at which hydroxylation has occurred (for OM4 but also for OM3, OM7abe, OM8abc, OM10 and OM11a), it was justified according to the fragment ions observed in their spectra. So, two fragments ions at *m/z* 149.0715 ($C_8H_9N_2O^+$) and 135.0558 ($C_7H_7N_2O^+$) (see **Table 1** and **Figure 1a**) correspond to the benzimidazole ring of the original omeprazole molecule and to its demethylated fragment ion, respectively. The presence of one of these fragment ions would therefore imply that hydroxylation has taken place in the other side of the molecule, i.e., in the pyridine ring.

In the case of OM6 (and also OM11b), the hydroxylation was located in the benzimidazole ring based on the presence of the fragment ions at *m/z* 195.0770 ($C_9H_{11}N_2O_3^+$) and 150.0919 ($C_9H_{12}NO^+$), respectively (see **Table 1** and **Figure 1a**).

Five di-oxygenated OM7s $[C_{17}H_{20}N_3O_5S]^+$ (*m/z* 378.1124) and three mono-oxygenated OM8s $[C_{17}H_{20}N_3O_4S]^+$ (*m/z* 362.1175) were also observed (**Figure 2**). However, it was not possible to predict at which position the hydroxylations occurred, as the fragment ions did not provide enough information. The SO_2 loss observed for OM7b-7e indicates the presence of the sulfone group in the molecule. Regarding OM7a, the sulfoxide group was maintained (SO_2 loss not observed) therefore suggesting a double hydroxylation. A previously searched metabolite (omeprazole sulfone-N-oxide, $[C_{17}H_{20}N_3O_5S]^+$) (Hernández et al. 2011b) might also be one of the OM7s. However, no

characteristic fragmentation from an N-oxide (involving losses of O, $OH^{\bullet}$ and H_2O) was observed (Chen et al. 2005).

Other reported OMs, like 5-hydroxy omeprazole (Hernández et al. 2011b; Kanazawa et al. 2002; Petsalo et al. 2008; Rezk et al. 2006; Rost et al. 1995; Song and Naidong. 2006), 3-hydroxy omeprazole (Kanazawa et al. 2002; Petsalo et al. 2008) and omeprazole sulfone (Kanazawa et al. 2002; Petsalo et al. 2008; Rezk et al. 2006; Rost et al. 1995) matched with the exact mass of metabolites 8 (a, b,c). Based on the structure proposed for these metabolites according to the observed fragmentation, none of them could be assigned to omeprazole-sulfone (OM8a,b are sulfides and OM8c is a sulfoxide). Moreover, 3- and 5- hydroxy omeprazole could only be related with OM8c. However, after injecting the 5-hydroxy omeprazole reference standard, its retention time and mass spectra were not in agreement with OM8c. Therefore, omeprazole-sulfone and 5-hydroxy omeprazole were discarded as candidates for the group of OM8 metabolites, while 3-hydroxy omeprazole could still be a plausible candidate for OM8c.

It is worth to mention the detection of OM13 $[C_{16}H_{16}N_3O_4S]^+$ (*m/z* 346.0862, ΔmDa= 1.3). This compound shared the same nominal mass than omeprazole (346) and its isomers OM11a and OM11b, but it had different exact mass and retention time. The potential of HR MS allowed differentiating these compounds with different elemental compositions. In this case, the elemental composition for OM13 suggested a demethylation (fragment ion at *m/z* 135.0558), sulfoxide-reduction (loss of *m/z* 32.9799, [$^{\bullet}$SH]) and subsequent hydroxylation and oxidation (fragment ions at *m/z* 212.0381 and 150.0555) from parent omeprazole (**Table 1, Figure 2**).

Two isomers, OM14a and OM14b ($[C_{16}H_{18}N_3O_2S]^+$, *m/z* 316.1220), eluting at 4.52 and 5.01 min, respectively, are the result of sulfoxide reduction (loss of *m/z* 32.9799, [$^{\bullet}$SH]) and demethylation reactions in the omeprazole structure. OM14b presented two

fragment ions at *m/z* 136.0762 ($C_8H_{11}NO^+$) and 149.0715 ($C_8H_9N_2O^+$) showing that demethylathion occurred in the pyridine ring. The metabolite OM14b corresponds to the already reported 4-hydroxy omeprazole sulfide (Hernández et al. 2011b), and the identity was confirmed with the reference standard available at our laboratory. Regarding the metabolite OM14a, the fragment ions at *m/z* 135.0558 ($C_7H_7N_2O^+$) and 150.0919 ($C_9H_{12}NO^+$) indicated that the compound was demethylated at the benzimidazole group.

As illustrative example, the elucidation process for the two most abundant metabolites, OM10 and OM11a, is discussed in detail in SI.

Up to our knowledge, some of the metabolites reported in this paper have never been investigated in water samples. Reference standards are not commercially available for most of these compounds and therefore their identity, although strongly supported by our QTOF MS accurate-mass data, could not be unequivocally confirmed.

3.3 Retrospective analysis of water samples by UHPLC-QTOF MS

After the study performed on urinary metabolites of omeprazole, the presence of the 24 identified metabolites was investigated in water samples (**Table S1**). To this aim, a total of 52 samples (15 influent wastewater (IWW), 10 effluent wastewater (EWW) and 27 surface water (SW)) previously analyzed by UHPLC-QTOF MS were retrospectively re-examined, this is, without the need of additional sample injections.

Retrospective analysis was made by performing eXtract Ion Chromatograms using narrow mass windows (± 10 mDa) at the metabolites exact *m/z*-values (nwXICs). Confirmation of the identity of the compounds detected was based on the accurate *m/z* of the (de)protonated molecule and at least two fragment ions, together with the agreement in retention time (deviation lower than ±2.5%). Both the fragment ions and

retention times, used as references were derived from the metabolism experiments. Up to nine OMs were detected in the water samples, oppositely to parent omeprazole that was not found in any of the samples (**Table 2**). OM10 was the most frequently detected compound, as it was present in 21 out of 25 WW, and in 11 out of 27 SW samples analyzed. OM7c, OM7d, OM11a, OM13, OM14a, and OM14b were also found in all types of water matrices, while OM5 was only detected in SW (21%) and EWW (10%) samples. Finally, OM7e was found in 3 SW samples (11%).

3.4 UHPLC-MS/MS analysis

In order to confirm the presence of OMs in the water samples, the same water extracts were re-analyzed by UHPLC-(ESI)-MS/MS with triple quadrupole, applying the same LC conditions used in the UHPLC-QTOF measurements in metabolism experiments. The higher sensitivity of triple quadrupole was expected to facilitate the detection of those OMs that were present at low concentrations in the water samples. The 24 OMs resulting from the metabolism study and parent omeprazole were included in the method. For each compound, two transitions were selected based on fragment ions observed in the QTOF experiments (**Table S1**). Apart from the presence of a chromatographic peak at the two transitions acquired, the identity of the findings was confirmed by calculating the peak area ratio between the quantification "Q" and the confirmation "q" transitions, which was compared with that of the "reference compound" (urine vials with the highest concentration of the OM). A finding was considered positive when experimental ion ratios were within the tolerance range (Commission Decision 2002). The agreement in retention time (deviation lower than ±2.5%) with the "reference compound" was also required.

Up to 14 OMs were detected and their identity confirmed by QqQ (**Table 2**). Eleven of these OMs (OM4, OM5, OM7c, OM7d, OM7e, OM8b, OM10, OM11a, OM13,

OM14a, and OM14b) were found in all types of water matrices analyzed. Similar to QTOF analysis, unchanged omeprazole was not found in any sample. Nevertheless, it is interesting to note that one of its isomers, OM11a, was detected in 80% IWW, 90% EWW and 30% SW samples. This fact reveals the importance of good chromatographic separation to avoid confusion between these two compounds as they share one of the transitions (346.1 > 198.1). Therefore, in order to reduce false positives of omeprazole the acquisition of additional specific transitions for each compound (see **Table S1**) and satisfactory chromatographic separation is of high relevance. This compound might be the same detected in wastewater by (Gomez-Ramos et al. 2011), after performing a XIC at the exact mass of omeprazole. OM10 was the most abundant in terms of MS arbitrary units, being consistent with the results of QTOF analysis. This compound was the most frequently detected metabolite in WW (100%) and SW (48%). Five of the OMs (OM3, OM4, OM8a, OM8b, and OM8c) were only found by LC-MS/MS analysis, due to its higher sensitivity compared to QTOF.

It is noteworthy that up to eight OMs were detected in 90-100% of effluent wastewater samples. These OMs were also present in influent wastewater, although some of them at lower frequency. Surely, the higher complexity and strong signal suppression due to matrix effects in the influent made the detection of OMs more problematic in comparison with effluent wastewater. It is also relevant the detection of up to seven OMs in around 30% of the surface water samples.

As an illustrative example, **Figure 3** shows selected UHPLC-MS/MS chromatograms for the most abundant OMs (OM5, OM10, OM7c,d,e, OM14a,b) detected in an effluent wastewater. As can be seen, experimental ion ratios did not exceed the maximum deviation allowed for any of the OMs detected, with all deviations being below 15%. OM10 and OM14b presented the highest responses, with average

areas of 10,000 and 8,000 a.u., respectively. This might reveal that they were the most relevant compounds in terms of concentration, but however quantification could not be performed due to the lack of reference standards.

CONCLUSIONS

In this work, urinary omeprazole metabolites have been investigated by UHPLC-QTOF MS. A total of twenty-four OMs were identified in urine samples of three volunteers who participated in this study, while parent omeprazole was present only at very low concentrations. OM11a was the most abundant compound. This OM is an omeprazole isomer and shares the fragment ion at *m/z* 198.0589 commonly used for the determination of omeprazole in LC-MS/MS methods. The loss of [$^{\bullet}$SH] radical deduced by TOF MS spectra in most of the OMs detected, has been crucial for justifying and suggesting possible chemical structures.

After UHPLC-QTOF MS analysis of 52 water samples, nine OMs were detected in surface water and wastewater samples, with OM10 being the most frequently found in wastewater (84% of the samples) and in surface water (41%). The results were confirmed by UHPLC-MS/MS analysis using a triple quadrupole analyzer, which superior sensitivity allowed to detect up to fourteen OMs. Unchanged omeprazole was not found in any sample; nevertheless, its isomer OM11a was detected in several samples.

In the light of the results obtained in the present work, it seems evident that monitoring unchanged omeprazole is not the best option to investigate the impact of this widely consumed pharmaceutical in the aquatic ecosystem. Instead, it is recommended to focus the research on the most abundant OMs identified in this work, i.e., those

named as OM5, OM7c, OM7d, OM10, OM11a, OM14a, and OM14b, when monitoring omeprazole in urban wastewater and also in surface water. Obviously, it would be necessary to perform absolute configuration on the relevant compounds by NMR to subsequently enable synthesis of reference compounds to perform quantitative studies, but that is beyond the present study. In addition to the OMs proposed in this paper, omeprazole sulfide -a transformation product resulting from hydrolysis of omeprazole that has been previously reported (Boix et al. 2013)- should also be included to have a realistic overview of the omeprazole impact on the aquatic ecosystem.

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525 **FIGURE CAPTIONS**

526 **Figure 1.** (a) Structure of omeprazole and some important fragment ions (b)
527 Omeprazole metabolites reported in the literature

528 **Figure 2.** Suggested structures for urinary OMs detected by UHPLC-QTOF MS after
529 omeprazole oral administration

530 **Figure 3.** UHPLC-MS/MS chromatograms for the omeprazole metabolites (a) OM5, (b)
531 OM10, (c) OM7c-7e, (d) OM14a, and (e) OM14b, in effluent wastewater

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