

1 REACTION OF DIAZEPAM AND RELATED BENZODIAZEPINES WITH CHLORINE. KINETICS,  
2 TRANSFORMATION PRODUCTS AND IN-SILICO TOXICOLOGICAL ASSESSMENT

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9 **Abstract**

10 In this work, the reaction of four benzodiazepines (diazepam, oxazepam, nordazepam and  
11 temazepam) during water chlorination was studied by means of liquid chromatography- quadrupole-  
12 time of flight - mass spectrometry (LC-QTOF-MS). For those compounds that showed a significant  
13 degradation, i.e. diazepam, oxazepam and nordazepam, parameters affecting to the reaction kinetics  
14 (pH, chlorine and bromide level) were studied in detail and transformation products were tentatively  
15 identified. The oxidation reactions followed pseudofirst-order kinetics with rate constants in the range  
16 of  $1.8\text{-}42.5\text{ M}^{-1}\text{ s}^{-1}$ ,  $0.13\text{-}1.16\text{ M}^{-1}\text{ s}^{-1}$  and  $0.04\text{-}20.4\text{ M}^{-1}\text{ s}^{-1}$  corresponding to half-life values in the range  
17 of 1.9-146 min, 1.8-87 h and 2.5-637 h for oxazepam, nordazepam and diazepam, respectively,  
18 depending of the levels of studied parameters. Chlorine and pH affected significantly the reaction  
19 kinetics, where an increase of the pH resulted into a decrease of the reaction rate, whereas higher  
20 chlorine dosages led to faster kinetics, as expected in this case. The transformation of the studied  
21 benzodiazepines occurs mainly at the 1,4-diazepine 7-membered-ring, resulting in ring opening to  
22 form benzophenone derivatives or the formation of a 6-membered pyrimidine ring, leading to  
23 quinazoline derivatives. The formation of these by-products was also tested in real surface water  
24 samples observing kinetics of oxazepam degradation slower in river than in creek water, while the  
25 degradation of the two other benzodiazepines occurred only in the simpler sample (creek water).  
26 Finally, the acute and chronical toxicity and mutagenicity of precursors and transformation products  
27 were estimated using quantitative structure-activity relationship (QSAR) software tools: Ecological  
28 Structure Activity Relationships (ECOSAR) and Toxicity Estimation Software Tool (TEST), finding that

29 some transformation products could be more toxic/mutagenic than the precursor drug, but additional  
30 test would be needed to confirm this fact.

31

32 **Keywords:** psychoactive drugs, high resolution mass spectrometry, water treatment, disinfection by-  
33 products

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## 36 1. Introduction

37 Benzodiazepines are the most prescribed psychoactive pharmaceuticals in the treatment of disorders  
38 related to the central nervous system such as the anxiety, depression, convulsion, insomnia or panic  
39 disorder (Woods et al., 1995). Besides the hypnotic, sedative and anesthetic effects, they can provoke  
40 aggressive behavior, disinhibition or physically and mentally dependence. In the period 2000-2012, an  
41 increase of 57.4% in the consumption of anxiolytic and hypnotic drugs was observed in Spain  
42 (["http://www.aemps.gob.es/medicamentosUsoHumano/observatorio/docs/ansioliticos\\_hipnoticos-](http://www.aemps.gob.es/medicamentosUsoHumano/observatorio/docs/ansioliticos_hipnoticos-2000-2012.pdf)  
43 [2000-2012.pdf](http://www.aemps.gob.es/medicamentosUsoHumano/observatorio/docs/ansioliticos_hipnoticos-2000-2012.pdf) (accessible 08.08.2015, in Spanish)," n.d.), where diazepam, marketed as Valium®  
44 among other names, is one of the most prescribed benzodiazepines. Diazepam is mainly  
45 administrated in oral doses of 2-20 mg per day being its main excretion route urine (Baker et al.,  
46 2014). Nordazepam, oxazepam, temazepam and hydroxy-diazepam are the human metabolites of  
47 diazepam, although the two first can be metabolites of other benzodiazepines too. Moreover,  
48 nordazepam, oxazepam and temazepam are also prescribed as psychoactive benzodiazepinic drugs.

49 In the same way as other pharmaceuticals, diazepam and its metabolites can enter wastewater  
50 treatment plants (WWTPs) (Calisto and Esteves, 2009; De Almeida et al., 2015; Kosjek et al., 2011;  
51 Racamonde et al., 2014), by urinary excretion or direct spills, and then they can reach surface water if  
52 not completely removed at WWTPs (Kosjek et al., 2011; Mendoza et al., 2014), which are finally a  
53 source of drinking water. A common step in drinking water production is the use of disinfection  
54 methods to reduce the level of microorganisms or contaminants in the drinking water, by using e.g.  
55 chlorine, ozone, chloramination, chlorine dioxide, H<sub>2</sub>O<sub>2</sub>, ferrate or UV radiation (Postigo and  
56 Richardson, 2014; Sharma, 2008). However, such disinfectants agents can generate a range of  
57 transformation products (TPs) which are sometimes more toxic than the precursor compound  
58 (Gonzalez-Marino et al., 2016; Postigo and Richardson, 2014). In the case of benzodiazepines,  
59 photodegradation (Calisto et al., 2011; Jakimska et al., 2014; Kosjek et al., 2011; West and Rowland,  
60 2012) and hydrolysis (Cabrera et al., 2005; Han et al., 1977) have been previously reported as well as  
61 the advanced oxidative treatments (Bautitz and Nogueira, 2010; Bautitz et al., 2012). However,  
62 despite the fact that chlorine is the most widely used disinfectant, the study of its reaction with  
63 benzodiazepines has not been performed so far.

64 The aim of this work was to study the reaction of diazepam and three related compounds (oxazepam,  
65 nordazepam and temazepam) with chlorine. For those benzodiazepines reacting in a significant  
66 degree, parameters affecting to the kinetics, such as pH, chlorine and bromide content were studied in  
67 detail. Further, TPs were tentatively identified by using liquid chromatography (LC) and high resolution  
68 mass spectrometry (HRMS) (Bautitz et al., 2012; Calisto et al., 2011; Gonzalez-Marino et al., 2016;  
69 Jakimska et al., 2014; Kosjek et al., 2011), with a quadrupole-time of flight (QTOF) system. Finally, a  
70 preliminary evaluation of the ecotoxicity of the TPs was performed using quantitative structure-activity  
71 relationship (QSAR) tools and the magnitude of the reaction was assessed in real environmental water  
72 matrices.

73

## 74 **2. Materials and method**

### 75 **2.1. Chemicals and materials**

76 All benzodiazepine standards were purchased from Cerilliant (Round Rock, TX, USA) as 1 mg mL<sup>-1</sup>  
77 solutions in methanol (MeOH) or acetonitrile (ACN). All concentrations cited here refer to the neutral  
78 species. Table 1 shows the name of the benzodiazepines considered, their abbreviation and some  
79 physico-chemical properties. Mixtures and dilutions were prepared in methanol and kept at -20 °C.

80 MeOH (LC-grade), glacial acetic acid (100%) and hydrochloric acid (37%) were obtained from Merck  
81 (Darmstadt, Germany). Potassium bromide, dipotassium hydrogen phosphate, potassium dihydrogen  
82 phosphate and ascorbic acid were supplied by Sigma-Aldrich (Steinheim, Germany) and dissolved in  
83 ultrapure water to carry out the chlorinated experiments. A solution of sodium hypochlorite (6-14%)  
84 was purchased from Sigma-Aldrich. The exact nominal free chlorine content were regularly  
85 determined by reaction with N,N-diethyl-p-phenylenediamine using photometric detection (Clesceri et  
86 al., 1998). Ultrapure water was obtained in the laboratory by purifying demineralized water in a Milli-Q  
87 Gradient A-10 system (Millipore, Bedford, MA, USA).

88

### 89 **2.2. Real samples**

90 Surface water samples were collected from a small creek, which is not affected by urban activities,  
91 and from a river after receiving the discharge of a WWTP, ca. 5 Km downstream. Both streams were

92 situated in the Northwest of Spain. All samples were collected in amber bottles and stored at 4°C until  
93 used.

94

### 95 **2.3. Chlorination experiments**

96 Chlorination experiments were carried out with 10 mL of water in 16 mL amber closed vials at room  
97 temperature (20±2°C). For preliminary test and the study of factors affecting to the reaction kinetics,  
98 ten milliliters of ultrapure water were buffered at pH 6, 7 or 8, with a KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> 0.03 M solution,  
99 and spiked with the target analytes at 0.1-0.25 µg mL<sup>-1</sup>. Also, potassium bromide was added at 0.05-  
100 0.1 µg mL<sup>-1</sup> in some experiments. These pH values and bromide concentrations, which is known to  
101 react to HBrO with chlorine, were selected as to represent typical surface waters (Gonzalez-Marino et  
102 al., 2016). After free chlorine addition at concentrations in the 0.5-10 µg mL<sup>-1</sup> range, also representing  
103 typical dosages during drinking water production (Gonzalez-Marino et al., 2016), the amber vials were  
104 manually shaken for 2-3 s. Then, 6-10 aliquots of 1 mL were collected at increasing reaction times,  
105 from time 0 s (before chlorine addition) to 120 min-96 h (depending of the reaction rate of the drug).  
106 These aliquots were transferred to 2 mL vials containing 10 µL of ascorbic acid (60 mg mL<sup>-1</sup>) as  
107 quenching agent to stop the reaction.

108 In experiments devoted to the identification of TPs (performed in triplicate), a similar procedure was  
109 carried out, but ultrapure water was buffered at pH 7, spiked with the drug at 1 µg mL<sup>-1</sup>, initial chlorine  
110 dose was set to 10 µg mL<sup>-1</sup> and bromide was either not added or added at 0.1 µg mL<sup>-1</sup>. When real  
111 samples were tested, the procedure was the same, but neither buffering nor bromide addition was  
112 performed.

113 The aliquots collected before chlorine addition (time 0 s) were used as control experiments to verify  
114 that the target analytes were stable in aqueous solution and that, therefore, dissipation of the  
115 benzodiazepine was caused by the oxidant agent and neither by hydrolysis nor other reactions. In  
116 addition, procedural blanks were carried out and used for discarding chromatographic peaks  
117 associated to chlorine, bromide, buffer or ascorbic acid reaction with impurities or contaminations  
118 during the identification of TPs (see below). All reaction samples were stored at -20°C until analysis,  
119 which was performed in less than 7 days after experiments were conducted

120

#### 121 **2.4. LC-HRMS analysis and TPs identification**

122 A LC-QTOF-MS instrument from Agilent (Wilmington, DE, USA) was used for the identification and  
123 determination of benzodiazepines and TPs. The LC was a 1200 Series liquid chromatographic system  
124 consisting of a membrane degasser, a binary high-pressure gradient pump, a thermostated LC column  
125 compartment and an autosampler. The LC was coupled to an Agilent 6520 Series Accurate Mass  
126 QTOF-MS equipped with a Dual electrospray ion source. Separation of analytes was carried out on a  
127 2.0 mm × 100 mm (particle size: 4 μm, pore size: 80 Å) Synergi Fusion RP column (Phenomenex, CA,  
128 USA). Ultrapure water (A) and MeOH (B) containing acetic acid at 0.1% were used as mobile phase.  
129 The mobile phase flow and oven temperature were set at 0.2 mL min<sup>-1</sup> and 35°C, respectively, and the  
130 gradient was: 0-10 min, 5%B; 10-12 min, 100%B; 12-12.1 min, 5%; 12.1-22 min, 5%B. The injection  
131 volume was 50 μL.

132 Nitrogen (99.999%), used as nebulizing and drying gas, was provided by a nitrogen generator (Erre  
133 Due srl, Livorno, Italy). Nitrogen (99.9995%) used for collision-induced dissociation (for MS/MS  
134 measurements) was purchased at Praxair Spain (A Coruña, Spain). Benzodiazepines and TPs were  
135 ionized in positive electrospray (ESI) with the following parameters being applied: gas temperature:  
136 350 °C; drying gas: 9 L min<sup>-1</sup>; nebulizer: 42 psi; capillary: 4000 V; fragmentor: 120 V; skimmer voltage:  
137 65 V; and octapole RF Peak: 750 V. The instrument was operated in the 2 GHz (extended-dynamic  
138 range) mode providing a FWHM resolution of ca. 4700 at m/z 113 and ca. 11,000 at m/z 980. A  
139 reference solution was continuously sprayed in the ESI source using a second nebulizer following the  
140 manufacturer specifications during the chromatographic run. Two masses (m/z 121.050873 and m/z  
141 922.009798) from the components of this reference solution were used to continuously recalibrate the  
142 Q-TOF, maintaining the mass accuracy. Instrument control, data acquisition and evaluation were  
143 performed with the Mass Hunter software (Agilent Technologies). The determination of selected  
144 benzodiazepines and the identified TPs was carried out in MS mode acquiring the scan MS spectra  
145 from 70 to 950 m/z with an acquisition rate 2 spectra/s.

146 Identification of TPs was carried out using the Mass Hunter software package (Agilent Technologies).  
147 First, the peak-picking from raw data was performed with an algorithm taking account the isotopic ion  
148 cluster for the identification of these entities, called *Find molecular feature* function, included in Mass

149 Hunter Qualitative software. The data were exported as CEF (compound exchange file) to the Mass  
150 Profile Professional software (Agilent Technologies) for the retention time and m/z value alignment,  
151 filtering and normalization. Then, the statistically significant differences were evaluated by ANOVA (p:  
152 0.05) between the control group (aliquots at time 0s) and aliquots collected at different times,  
153 excluding those features (peaks) which were not observed at least in the 3 replicates of any aliquot  
154 corresponding to a reaction time. Then, theoretical formula were produced taking into account the  
155 isotopic distribution and mass accuracy, so that the score (100 is a perfect match) corresponds to a  
156 combination of these factors. Cut-off values were set at <5 ppm mass error and >80 of score and the  
157 formula with higher score was selected. Finally, MS/MS fragmentation patterns were acquired and  
158 interpreted in order to tentatively elucidate the structure of each TP, by fragmentation of the [M+H]<sup>+</sup> ion  
159 of each TP at different collision energies (10, 20 and 40 V) with an acquisition rate of 4 spectra/s in the  
160 m/z 30-400 range.

161

## 162 **2.5. QSAR evaluation of ecotoxicity**

163 An assessment of the ecotoxicity of three studied benzodiazepines and their TPs were performed by  
164 using the US Environmental Protection Agency Toxicity Estimation Software Tool (TEST) version 4.1  
165 (<https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test> (accessible 04/07/2016),  
166 n.d.) and ECOSAR version 1.11 ([https://www.epa.gov/tsca-screening-tools/ecological-structure-  
167 activity-relationships-ecosar-predictive-model](https://www.epa.gov/tsca-screening-tools/ecological-structure-activity-relationships-ecosar-predictive-model) (accessible 04/07/2015), n.d.). These software predict  
168 toxicity of a chemical compound from the structure based on the similarity to the set of chemicals used  
169 to construct the QSAR model. In ECOSAR, toxicity values can be estimated using different linear  
170 regression models, for each chemical class, between log LC<sub>50</sub> (50% lethal concentration) from a  
171 database and log P calculated by EPISUITE KOWWIN software. When a structure could not be  
172 related to predefined chemical class, the estimations were given by neutral organics QSAR equations  
173 which represent the minimum of the toxicity and, therefore, the toxicity might be less accurate. Thus,  
174 48-hour *Daphnia magna* LC<sub>50</sub>, 96-hour fish LC<sub>50</sub> and 96-hour green algae half maximal effective  
175 concentration (EC<sub>50</sub>) were estimated as well as the chronic values defined as geometric mean of no  
176 observed effect concentration and the lowest observed effect concentration  
177 (<https://www.epa.gov/tsca-screening-tools/ecological-structure-activity-relationships-ecosar->

178 predictive-model (accessible 04/07/2015),” n.d.; Melnikov et al., 2016). In TEST, the toxicity values can  
179 be estimated with different QSAR methodologies and a large number of molecular descriptors such  
180 as structural or electronic parameters. The 48-hour *Daphnia magna* LC<sub>50</sub>, 96-hour fathead minnow  
181 LC<sub>50</sub>, *Tetrahymena pyriformis* 50% growth inhibition concentration (IGC<sub>50</sub>) and Ames mutagenicity  
182 (induction of revertant colony growth of *Salmonella thyphimurium*) endpoints were estimated by  
183 consensus method, which uses an average value of the calculated toxicities by five different  
184 developed QSAR methodologies and gives the most accurate predictions (Gramatica, 2004).  
185 However, since the predictions for 48h *Daphnia magna* LC<sub>50</sub> were only obtained for some compounds  
186 and *Tetrahymena pyriformis* IGC<sub>50</sub> were not produced for the parent pharmaceuticals, they were not  
187 further considered.

188

### 189 **3. Results and discussion**

#### 190 **3.1. Preliminary tests**

191 Preliminary studies were carried out in order to evaluate the stability of diazepam, nordazepam,  
192 oxazepam and temazepam in water in the presence of chlorine (10 µg mL<sup>-1</sup>) for 24h at pH values 6  
193 and 8. A control experiment was also conducted in parallel without chlorine. All studied  
194 benzodiazepines were stable in ultrapure water but, in presence of chlorine, diazepam, nordazepam  
195 and oxazepam significantly reacted while temazepam signal decrease was lower than 20% (Fig. S1).  
196 Therefore, the kinetics of nordazepam, diazepam and oxazepam, were studied in detail, whereas  
197 temazepam was deemed stable, since in real samples other constituents will in practice deplete  
198 chlorine, making temazepam reaction negligible.

#### 199 **3.2. Parameters affecting the reaction kinetics**

200 The influence of parameters such as pH, chlorine and bromide concentration in the degradation  
201 kinetics was studied simultaneously using an Box-Behnken experimental design (Quintana et al.,  
202 2012a, 2012b), which has three levels for each factor considered: pH 6, 7 and 8; free chlorine at 1, 5.5  
203 and 10 µg mL<sup>-1</sup> levels; and bromide levels at 0, 50 and 100 ng mL<sup>-1</sup>. A total of 15 experiments (Table  
204 S1), including three central points, were performed by taking at least 9 aliquots per experiment at  
205 different reaction times from 0 s to a maximum of 8.5 h for oxazepam, 96 h for diazepam and 72 h for

206 nordazepam. The natural logarithm of the ratio of the area obtained at certain time divided by those  
207 obtained from time 0 s, against the reaction time were fitted to a linear model, where the slope is the  
208 pseudo-first order velocity constant ( $k'$ ) (in excess of oxidant). Then, half-lives were calculated as  $t_{1/2} =$   
209  $\ln 0.5/k'$  for each experiment ranging between 1.9 and 146 h for oxazepam, 1.8 and 87 h for  
210 nordazepam and 2.5-637 h for diazepam (Table S1). These magnitudes were used as experimental  
211 response, which was statistically analyzed (Statgraphics Centurion XVI software, Statpoint  
212 Technologies, Warrenton, VA, USA) in order to evaluate the significance of the main effects  
213 associated to each factor and their two-factor interactions. Alternatively, second order rate constants  
214 ( $k$ ) could be also calculated as the product of  $k'$  and the initial chlorine concentration (Table S1). As it  
215 can be observed, oxazepam reaction was faster ( $1.8\text{-}43 \text{ M}^{-1} \text{ s}^{-1}$ ) in comparison with nordazepam ( $0.13\text{-}$   
216  $1.16 \text{ M}^{-1} \text{ s}^{-1}$ ) and diazepam ( $0.02\text{-}2.0 \text{ M}^{-1} \text{ s}^{-1}$ ).

217 Table 2 shows the standardized values for main factor effects and their two-factor interactions.  
218 Bromide was not statistically significant in any case, whereas conversely, the free chlorine dose was  
219 the most important factor, the only one statistically significant for the three benzodiazepines, with a  
220 negative effect on the half-lives, so, as expected, the higher the concentration of chlorine the faster the  
221 kinetics. In the case of oxazepam, the quadratic term (BB in Table 2) was also statistically significant,  
222 meaning that the relationship is not linear, i.e. the reaction velocity increased with chlorine levels until  
223 high levels where the velocity remain constant (Fig. S2). The fact that bromide is not statistically  
224 significant can be attributed to the fact that halogenation is not a very relevant route in the reaction,  
225 with only one minor TP being observed from chlorine introduction (see section 3.3.)

226 For diazepam and oxazepam, the pH was also statically significant playing a positive effect in the  $t_{1/2}$ ,  
227 that is, reaction was slower at higher pH values. An explanation is that the  $\text{ClO}^-$  is less oxidant than  
228  $\text{HClO}$  as it has well described in the literature (Nam et al., 2014; Sharma et al., 2014), since the three  
229 benzodiazepines are neutral along the whole pH range studied ( $\text{pK}_a\text{s} = 11\text{-}12$ , as predicted by  
230 [www.chemaxon.com](http://www.chemaxon.com)). Moreover, the chlorine-pH interaction was statically significant for diazepam. As  
231 observed in Fig. S3, the pH effect was only appreciated at low chlorine doses.

232

### 233 3.3. Identification of transformation products

234 Table 3 compiles the retention time, formulae, exact and calculated  $m/z$ , error and double bound  
235 equivalents (DBE) of the identified TPs, which are numbered according to its precursor pharmaceutical  
236 with a roman number, in intensity order (e.g. TPI is more intense than TPII and so on). Mass errors  
237 regarding to the proposed formulae were lower than  $\pm 1.6$  mDa (equivalent to  $\pm 5$  ppm). Also, the  
238 normalized score was higher than 88%. After empirical formulae selection, their structure was  
239 tentatively elucidated by interpretation of MS/MS spectra as described below for each benzodiazepine.  
240 Fig. 1 presents the proposed reaction pattern and the structure of identified TPs. No brominated TP  
241 was observed in any case, consistently with the lack of effect of bromide in the kinetics described  
242 above and the fact that only a minor chlorination TP was observed (NOR-TPII, see below).

243

### 244 3.3.1. Oxazepam

245 Only one TP, 6-chloro-3,4-dihydro-4-phenyl-2-quinazolinone (labelled as OXA-TP), was identified for  
246 oxazepam (Fig. 1). OXA-TP was generated during first 20 min by apparent elimination of  
247 formaldehyde from oxazepam, remaining stable for the rest of the experiment (Fig. S4). Also, since the  
248 normalized intensity of OXA-TP is about 80% of the precursor benzodiazepine (Fig. S4), it is assumed  
249 that transformation to this TP is (almost) quantitative. As the number of double bounds equivalents  
250 (DBEs) did not change, a 6-membered ring was formed resulting in a quinazolinone (Fig. 1), as  
251 supported by the MS/MS interpretation.

252 The MS/MS spectra of OXA-TP (Fig. 2) and oxazepam (Fig. S5) show both the loss of  $H_2O$  and  $CO$ . In  
253 the case of oxazepam, these are consecutive, but not for OXA-TP, where they occur by two different  
254 alternative routes. Thus, the loss of  $H_2O$  in OXA-TP spectrum could be explained from the enol  
255 tautomer to yield the ion  $C_{14}H_8ClN_2^+$  (239.0367  $m/z$ ) and the  $CO$  loss to form ion 151.00563  $m/z$  from  
256 178.9999  $m/z$  confirming that the ketone group remain in the structure. In addition, both spectra show  
257 the loss of benzene and the presence of benzonitrile ion ( $C_7H_6N^+$ , 104.0497  $m/z$ ) and the presence of  
258 a *p*-chloroaniline ion ( $C_7H_4ClN_2^+$ , 138.0085 and  $C_6H_3ClN^+$ , 123.9945) proving, therefore, that the  
259 phenyl rings remained unchanged.

260 Actually, OXA-TP has already been described in the literature as one of the photoproducts of  
261 oxazepam (Calisto et al., 2011; West and Rowland, 2012).

262

### 263 3.3.2. Nordazepam

264 Four compounds were identified as nordazepam TPs (Table 3 and Fig. 1). The time-profile plot  
265 showing the dissipation of nordazepam and the formation of TPs is presented in Fig. 3, where the  
266 least intense product, labeled as NOR-TPIV, is in fact the same as OXA-TP. Besides this minor  
267 product NOR-TPIV (OXA-TP), two further phenylquinazoline products (NOR-TPI and NOR-TPII) were  
268 the major TPs, with DBEs being identical to nordazepam (Table 3). These are generated by  
269 elimination of formaldehyde (NOR-TPI) and then further chlorination (NOR-TPII) or oxidation (NOR-  
270 TPIV). The last one, also minor TP, NOR-TPIII, has a DBE one unit lower than nordazepam, which  
271 suggested a ring opening via hydrolysis of the 4,5-azomethine bond (Archontaki et al., 1998) to form a  
272 benzophenone. Further detail on the identification of these TPs, on the basis of MS/MS spectra (Fig. 4  
273 and Fig S7), are given below.

274 Unlike nordazepam (Fig. S6), the CO loss was not observed in NOR-TPI nor NOR-TPII MS/MS  
275 spectra, in agreement with the empirical formulae (Table 3), thus confirming the elimination of the  
276 carbonyl group. Besides, nordazepam and these two major TPs showed the loss of benzene in their  
277 spectra to produce the ions  $C_8H_6ClN_2^+$  165.0202 m/z (Nordazepam),  $C_8H_4ClN_2^+$  163.0048 m/z (NOR-  
278 TPI) and  $C_8H_3Cl_2N_2^+$  196.9659 m/z (NOR-TPII), proving that the benzene ring is not altered and  
279 suggesting the condensation of the 7-membered ring to the 6-membered ring (as explained for OXA-  
280 TP/NOR-TPIV). Further fragmentation of  $C_8H_4ClN_2^+$  (163.0048 m/z, NOR-TPI) involved also the loss  
281 of HCN (Fig. 4A). However, ClCN is lost from  $C_8H_3Cl_2N_2^+$  (196.9659 m/z) instead of HCN in the case of  
282 NOR-TPII (Fig. S7), which points to the introduction of a chlorine atom in between the two nitrogens.

283 As concerns NOR-TPIII (Fig. 4B), the main MS/MS fragmentation route starts with elimination of  
284 methanol from the terminal hydroxyl group, followed by benzene (to produce 179.9838 m/z),  
285 confirming that the benzene ring remained unaltered. Then, this fragment also yields 123.9947 m/z by  
286 eliminating twice carbon monoxide, which confirms the presence of two carbonyl groups in the  
287 structure. Finally, the ion corresponding to 105.0327 m/z ( $C_7H_5O$ ) indicates that one carbonyl moiety is  
288 attached to the benzene ring, thus suggesting the aperture of the benzodiazepine ring and further  
289 oxidation to the benzoquinone.

290 Besides OXA-TP/NOR-TPIV (mentioned in 3.3.1), NOR-TPI has already been identified as diazepam  
291 photoproduct in the literature (West and Rowland, 2012). NOR-TPII and NOR-TPIII have not been  
292 reported in the literature as TPs as far as we know. However, NOR-TPII has been used as a precursor  
293 in the synthesis of alprazolam (“Alprazolam,” 2007).

294

### 295 3.3.3. Diazepam

296 The reaction of diazepam with chlorine leads to 5 TPs (Table 3 and Fig. 1). Four of them (DIA-TPI-IV)  
297 are produced by opening of the benzodiazepinic ring, while the fifth (less intense) one (DIA-TPV) is  
298 analog to OXA-TP/NOR-TPIV, but with an N-methyl group. The only TP described so far in the  
299 literature is actually the most intense one, DIA-TPI, which has been reported as a phototransformation  
300 product (Jakimska et al., 2014; West and Rowland, 2012) and also formed by N-bromosuccinimide  
301 oxidation (Nanda et al., 2014).

302 The MS/MS spectra (Fig. 5 and Fig S9-S10) from DIA-TPI to DIA-TPIV show that the four product ion  
303 spectra contain an ion assigned to  $C_7H_5O^+$  (105.0327 m/z), analogously to NOR-TPIII (see 3.3.2, Fig  
304 4B), indicating that these three TPs are again benzophenones. DIA-TPI spectrum exhibits also a  
305 product ion with 140.0258 m/z ( $C_7H_7ClN$ ) which correspond to the other part of the molecule and  
306 leaves basically the assigned structure as the only feasible (Fig. 5A). The major ion at 193.0880 m/z,  
307 corresponding to the loss of Cl and water (which is a typical loss from OH moieties) could be explained  
308 from the enol form of the ketone. DIA-TPII and DIA-TPIII have the same empirical formula and show in  
309 both cases the loss of methanol in the MS/MS spectra (Fig. 5B and Fig S9), which could indicate the  
310 presence of a terminal alcohol, so they are expected to have a very similar structure. DIA-TPII does  
311 not have any further intense peak (besides the phenylic cation, 77.03849 m/z), whereas that of DIA-  
312 TPIII shows the same peak to DIA-TPI at 193.0880 m/z and its chlorinated analog (228.0565 m/z),  
313 which indicates that the structure of DIA-TPIII is more similar to DIA-TPI, than in the case of DIA-TPII.  
314 Thus, we propose DIA-TPII being hydroxylated at the N-methyl group and DIA-TPIII hydroxyl attached  
315 at the acetamide group. In the case of DIA-TPIV, the spectra (see Fig. S10) only points to the  
316 benzophenone structure (ions 105.0316 and 77.0372 m/z), also showing an additional loss of HCN.  
317 On the basis of this spectrum, DBE values and the structures of the other TPs, we propose the

318 structure presented in Fig. 1, as an intermediate before evolution to further TPs, whose intensity  
319 actually decreases over time (Fig. S12).

320 Finally, as mentioned, DIA-TPV, is an analog to OXA-TP/NOR-TPIV, showing the same sequential  
321 loss of benzene, CO and HCN in their spectra (Fig. 2 and S11). Moreover, DIA-TPV structure has an  
322 N-methyl group, which is evident from the losses of methane or CH<sub>3</sub> from its spectrum (Fig. S11).

323

### 324 **3.4 Reaction in real sample matrices**

325 Finally, the reaction was studied with two real sample matrices, viz. a river impacted by a WWTP  
326 effluent discharge and a pristine creek water. Table 4 reports the half-lives obtained from ultrapure and  
327 these two samples, being significantly higher in the real samples. Diazepam and nordazepam reaction  
328 did only take place with the less complex matrix (creek water) and the apparent half-lives were close  
329 to 1 day. In the case of oxazepam, the reaction took place in both matrices with half-lives of about 1 h.  
330 Actually, the half-life in the river sample was lower than in creek sample, despite of its apparent higher  
331 complexity. A possible explanation is the lower pH in the river, accordingly with what was mentioned in  
332 section 3.2.

333 Regarding the TPs detected in these samples, all TPs except NOR-TPIII were detected when  
334 nordazepam chlorination was carried out in the creek sample. Non detection of NOR-TPIII may be  
335 explained because its formation did not occur or matrix effects in ESI increasing the limit of detection  
336 for this chemical. For diazepam, all TPs were detected. And, for oxazepam, the reaction is basically a  
337 transformation to OXA-TP in the two studied matrices. Therefore, reaction may take place, depending  
338 on sample complexity, being particularly relevant for oxazepam.

339

### 340 **3.5. QSAR estimation of ecotoxicological implications**

341 A preliminary evaluation of the toxicity of the TPs was carried out with TEST and ECOSAR software  
342 as described in the section 2.4. The results obtained are compiled in Table 5. The observed  
343 differences for predicted accurate toxicity in fish using both software have already been reported in the  
344 bibliography (Barron et al., 2012; Melnikov et al., 2016). Besides the differences in the parameters and  
345 algorithm used, *Fathead Minnow* is the only fish considered when TEST software is applied; while

346 several genera and families are considered in ECOSAR. For oxazepam, in all cases, its only TP, OXA-  
347 TP, showed a lower predicted toxicity (higher acute and chronic concentration values) than the  
348 precursor drug itself. In the case of Nordiazepam and according to ECOSAR data, NOR-TPI and  
349 NOR-TPII would present higher acute toxicity using fish and *daphnid* as end points (lower LC<sub>50</sub>) and  
350 higher chronic toxicity for *daphnid* endpoint (lower chronic toxicity concentration); and, the estimated  
351 toxicity for NOR-TPIII were higher than nordazepam in all cases (lower acute and chronic  
352 concentration values). However, considering TEST data, only the predicted acute toxicity of NOR-TPII  
353 for *Fathead Minnow* is higher than parent drugs (lower LC<sub>50</sub>). According to ECOSAR results, DIA-TPI  
354 and DIA-TPIII would exhibit higher toxicity (lower acute and chronic toxicity concentration) than parent  
355 drugs, while the TEST results showed that DIA-TPIV would be more toxic than diazepam.

356 Regarding to mutagenicity estimated by TEST, diazepam, nordazepam and oxazepam would show no  
357 mutagenicity, while the TPs (except DIA-TPI, DIA-TPII and DIA-TPIII) may be mutagens. Therefore,  
358 further research is needed to evaluate the real toxicity of these compounds, particularly given the  
359 contradictory results obtained by both QSARs.

360

#### 361 **4. Conclusions**

362 Diazepam, nordazepam and oxazepam significantly react with free chlorine. In excess of chlorine, the  
363 transformations kinetics followed first-order pseudo-kinetics with  $t_{1/2}$  affected positively by pH and  
364 negatively by chlorine dosage, without being influenced by bromide. The TPs detected were mainly  
365 generated by pyrimidine or benzophenone formation. Moreover, in real samples these TPs can also  
366 be formed, when the organic load of the samples is not too high. Finally, their toxicity was evaluated  
367 using the EPA TEST and ECOSAR software showing that some TPs could be more toxic than the  
368 pharmaceuticals parent chemicals and all, except three, would show a positive result in the  
369 mutagenicity test. However, differences between acute toxicity values predicted with the two software  
370 tools for fish were observed, pointing to a need for a real toxicity estimation.

371

#### 372 **Acknowledgement**

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- 468

Table 1. Structure and some physico-chemical properties of the target benzodiazepines.

Structure	Compounds (abbreviation)	R <sub>1</sub>	R <sub>2</sub>	Log K <sub>ow</sub> <sup>a</sup>	pK <sub>a</sub> <sup>a</sup>
	Oxazepam (OXA)	OH	H	2.24	10.9
	Nordazepam (NOR)	H	H	2.93	11.7
	Diazepam (DIA)	H	CH <sub>3</sub>	2.82	11
	Temazepam (TEM)	OH	CH <sub>3</sub>	2.19	11.6

<sup>a</sup> Values obtained using the Advanced Chemistry Development (ACD/Labs) software V11.02 © 1994-2011 ACD/Labs

Table 2. Standardized values for main effects and the two factors interaction.

	Oxazepam	Nordazepam	Diazepam
A:pH	6.32 <sup>a</sup>	0.48	2.73 <sup>a</sup>
B:Free chlorine level	-11.41 <sup>a</sup>	-3.19 <sup>a</sup>	-3.04 <sup>a</sup>
C:Bromide level	1.26	-0.56	-0.37
AA	1.61	-2.11	1.21
AB	-0.48	0.24	-3.02 <sup>a</sup>
AC	-0.51	-0.03	0.00
BB	6.74 <sup>a</sup>	1.63	1.78
BC	-2.50	0.82	0.52
CC	0.78	0.97	-0.69

<sup>a</sup> Statistically significant factors and interactions at the 95% confidence level

Table 3. Retention time ( $t_R$ ), formula, calculated and experimental  $m/z$ , error and double bond equivalents (DBE) for identified compounds.

	$t_R$ (min)	Formula	Calculated $m/z$ [M+H] <sup>+</sup>	Experimental $m/z$ [M+H] <sup>+</sup>	Score	Error (mDa)	Error (ppm)	DBE
Oxazepam	12.9	C <sub>15</sub> H <sub>11</sub> ClN <sub>2</sub> O <sub>2</sub>	287.0582	287.0589	95.56	-0.52	-1.8	11
OXA-TP <sup>a</sup>	13.4	C <sub>14</sub> H <sub>9</sub> ClN <sub>2</sub> O	257.0476	257.0478	99.3	-0.2	-0.79	11
Nordazepam	13.7	C <sub>15</sub> H <sub>11</sub> ClN <sub>2</sub> O	271.0633	271.0632	99.76	0.02	0.08	11
NOR-TPI	14.6	C <sub>14</sub> H <sub>9</sub> ClN <sub>2</sub>	241.0527	241.0522	98	0.61	2.6	11
NOR-TPII	15.1	C <sub>14</sub> H <sub>8</sub> Cl <sub>2</sub> N <sub>2</sub>	275.0137	275.0132	98.68	0.54	2.0	11
NOR-TPIII	14.8	C <sub>15</sub> H <sub>12</sub> ClNO <sub>3</sub>	290.0578	290.0572	92.15	0.62	2.1	10
NOR-TPIV <sup>a</sup>	13.6	C <sub>14</sub> H <sub>9</sub> ClN <sub>2</sub> O	257.0476	257.0470	98.66	0.51	2.0	11
Diazepam	13.82	C <sub>16</sub> H <sub>13</sub> ClN <sub>2</sub> O	285.0789	285.0799	96.91	-0.87	-3.1	11
DIA-TPI	15.2	C <sub>14</sub> H <sub>12</sub> ClNO	246.068	246.0685	98.19	-0.44	-1.8	9
DIA-TPII	14.1	C <sub>16</sub> H <sub>14</sub> ClNO <sub>3</sub>	304.0735	304.0752	88.5	-1.54	-5.0	10
DIA-TPIII	14.5	C <sub>16</sub> H <sub>14</sub> ClNO <sub>3</sub>	304.0735	304.0738	98.83	-0.07	-0.23	10
DIA-TPIV	13.9	C <sub>16</sub> H <sub>11</sub> ClN <sub>2</sub> O <sub>2</sub>	299.0582	299.0578	96.27	0.12	0.39	12
DIA-TPV	13.8	C <sub>15</sub> H <sub>11</sub> ClN <sub>2</sub> O	271.0633	271.0641	94.21	-0.46	-1.7	11

<sup>a</sup> NOR-TPIV and OXA-TP were the same by-product (see discussion in section 3.3.2).

475

Table 4. Half-lives of the benzodiazepines in ultrapure water and real samples spiked with  $10 \mu\text{g mL}^{-1}$  of free chlorine and  $1 \mu\text{g mL}^{-1}$  of the benzodiazepine.

<b>Compound</b>	<b>Ultrapure water (pH 6.9)</b>	<b>Creek (pH 6.7)</b>	<b>River (pH 6.1)</b>
Oxazepam	9.3 min	75 min	52 min
Nordazepam	7.5 h	23 h	n/a
Diazepam	3.9 h	20 h	n/a

n/a: not applicable (no reaction)

476

Table 5. Ecotoxicological data of oxazepam, nordazepam, diazepam and their degradation products (with elucidated structure) predicted by US EPA TEST and ECOSAR software. Results can be classified as very toxic (< 1 mg L<sup>-1</sup>), toxic (1-10 mg L<sup>-1</sup>), harmful (10-100 mg L<sup>-1</sup>) and not harmful (> 100 mg L<sup>-1</sup>) (United Nations, 2011).

Compound	ECOSAR						TEST		
	Chemical class	Acute toxicity (mg L <sup>-1</sup> )			Chronic toxicity (mg L <sup>-1</sup> )			96h <i>Fathead</i> <i>Minnow</i> LC <sub>50</sub> (mg L <sup>-1</sup> )	Mutagenicity
		Fish (96h LC <sub>50</sub> )	<i>Daphnid</i> (48h LC <sub>50</sub> )	Algae (96h EC <sub>50</sub> )	Fish	<i>Daphnid</i>	Algae		
OXA	Amide	50.36 <sup>b</sup>	47.88 <sup>b</sup>	1.70	0.11	3.32	1.83	1.06	Negative
OXA-TP <sup>c</sup>	Neutral organic <sup>a</sup>	95.78	56.34	48.55	9.76	6.06	13.75	2.02	Positive
NOR	Amide	19.22 <sup>b</sup>	14.16 <sup>b</sup>	0.73	0.06	1.41	0.99	0.64	Negative
NOR-TPI	Neutral organic	8.45	5.52	7.36	0.96	0.80	2.64	0.95	Positive
NOR-TPII	Neutral organic	2.55	1.77	3.01	0.32	0.30	1.23	0.21	Positive
NOR-TPIII	Amide	14.32	9.53	0.57	0.05	1.1	0.85	1.28	Positive
NOR-TPIV <sup>c</sup>	Neutral organic <sup>a</sup>	95.78	56.34	48.55	9.76	6.06	13.75	2.02	Positive
DIA	Amide	26.89	21.44	0.98	0.07	1.91	1.24	0.48	Negative
DIA-TPI	Neutral organic	1.99	1.39	2.42	0.25	0.24	1.01	2.72	Negative
DIA-TPII	Amide	34.74	29.24	1.24	0.08	2.41	1.49	2.15	Negative
DIA-TPIII	Amide	7.89	4.39	0.34	0.03	0.65	0.60	1.57	Negative
DIA-TPIV	Neutral organic <sup>a</sup>	212.73 <sup>b</sup>	121.56 <sup>b</sup>	92.97	20.95	12.07	24.67	0.18	Positive
DIA-TPV	Neutral organic <sup>a</sup>	144.47	83.626	67.46	14.45	8.60	18.44	1.53	Positive

<sup>a</sup> Not related to existent chemical class. <sup>b</sup> The solubility was not enough to measure the predicted effect. <sup>c</sup> OXA-TP and NOR-TPIV are be the same compound.

Figure 1

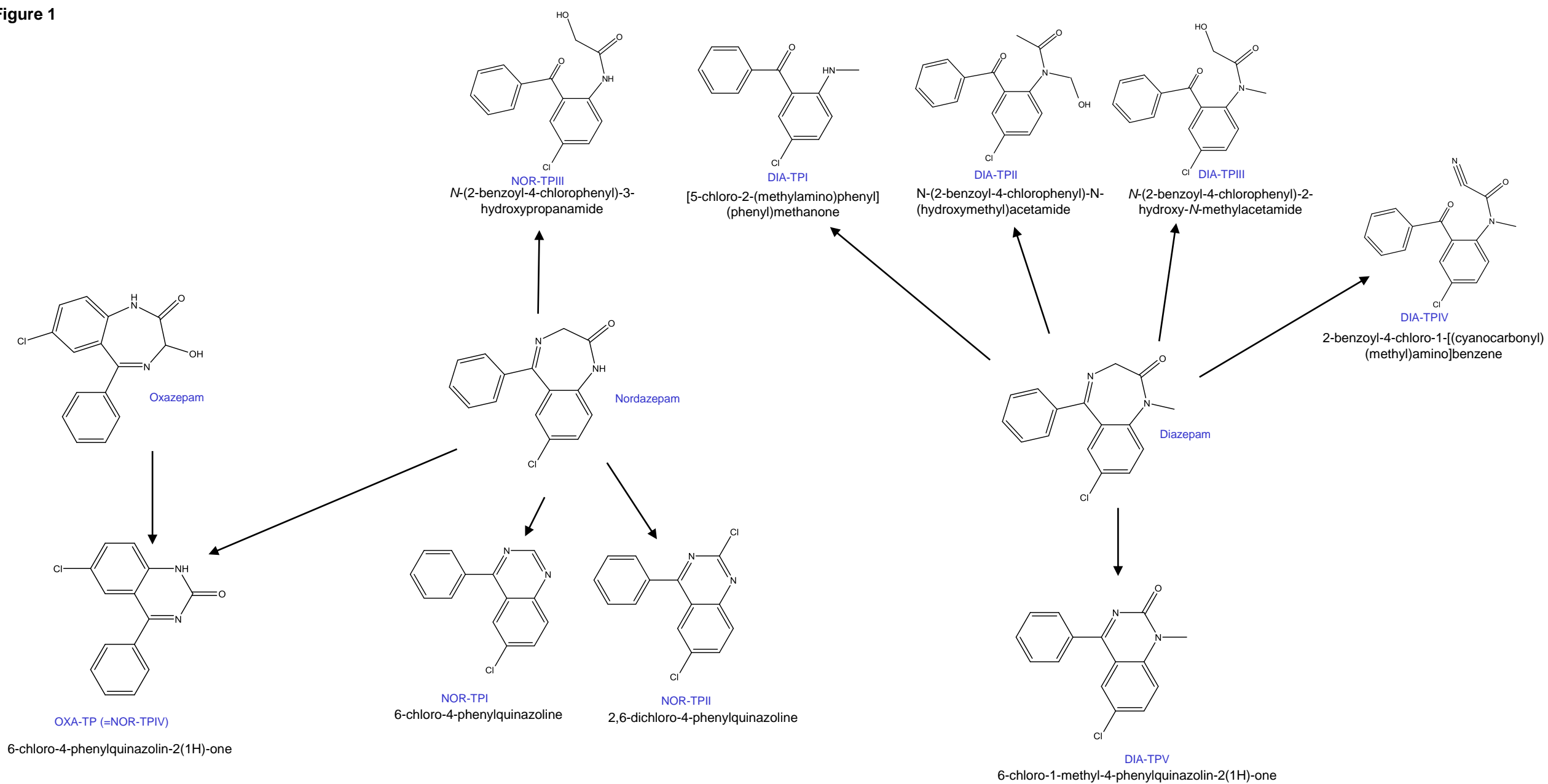


Fig. 1. Proposed reaction pattern of oxazepam, nordazepam and diazepam with chlorine.

Figure 2

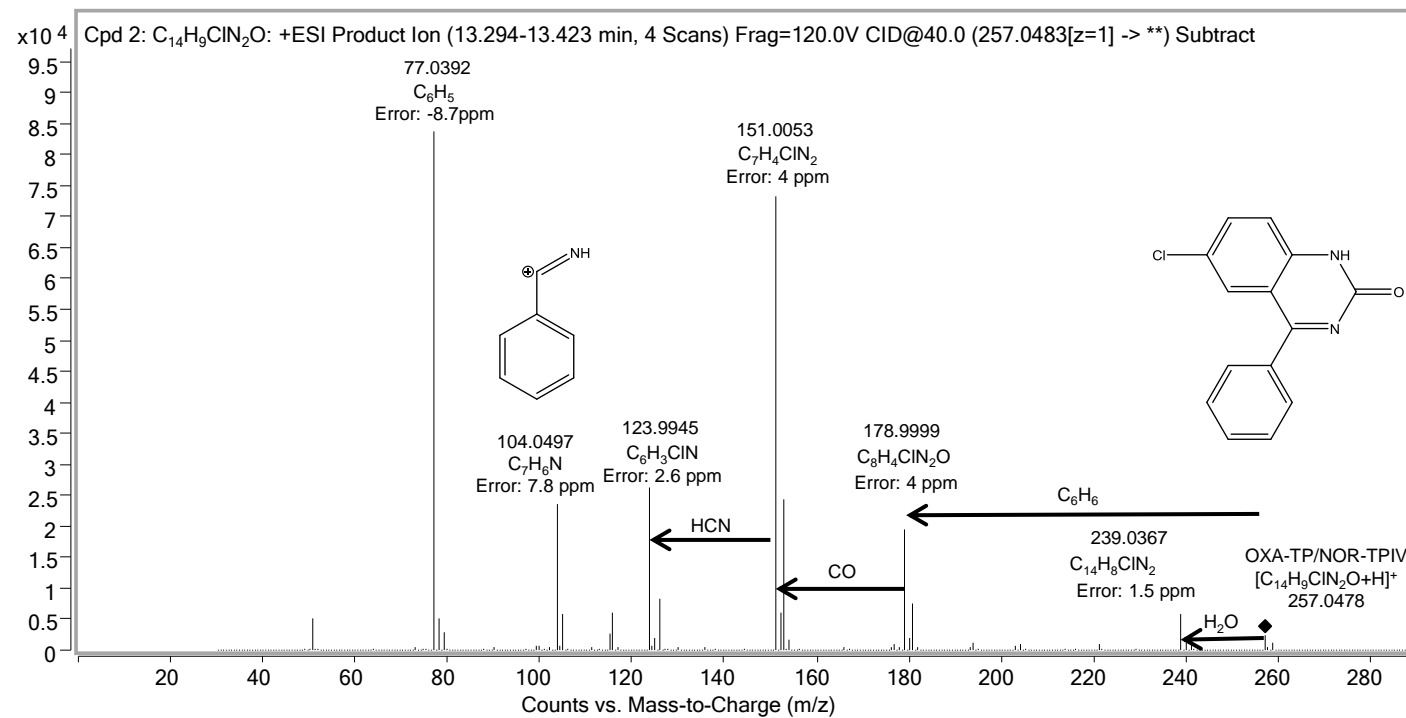
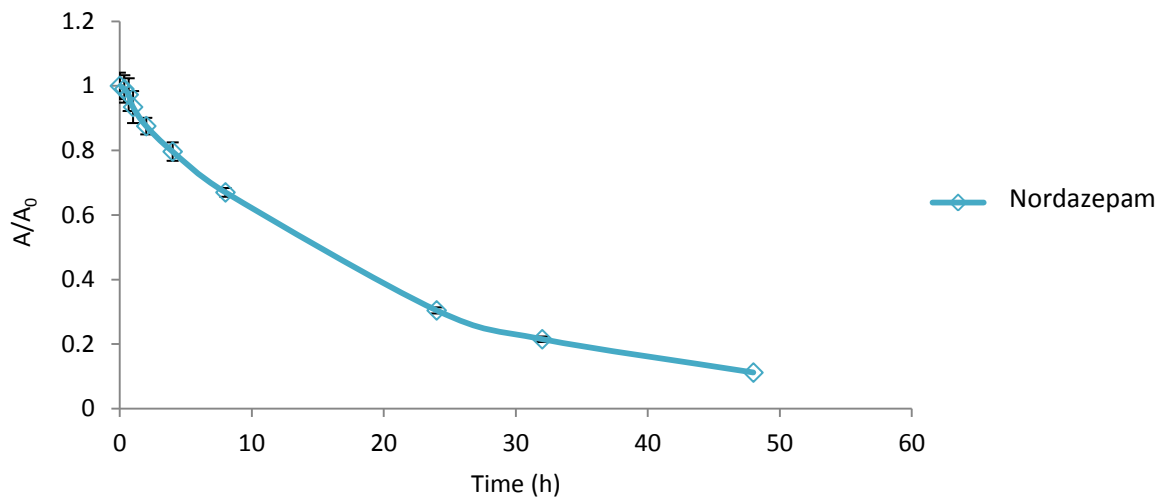


Fig. 2. QTOF product ion scan spectra of oxazepam TP (OXA-TP). The spectrum of oxazepam is presented in the Supplementary Information (Fig. S5)

Figure 3

A



B

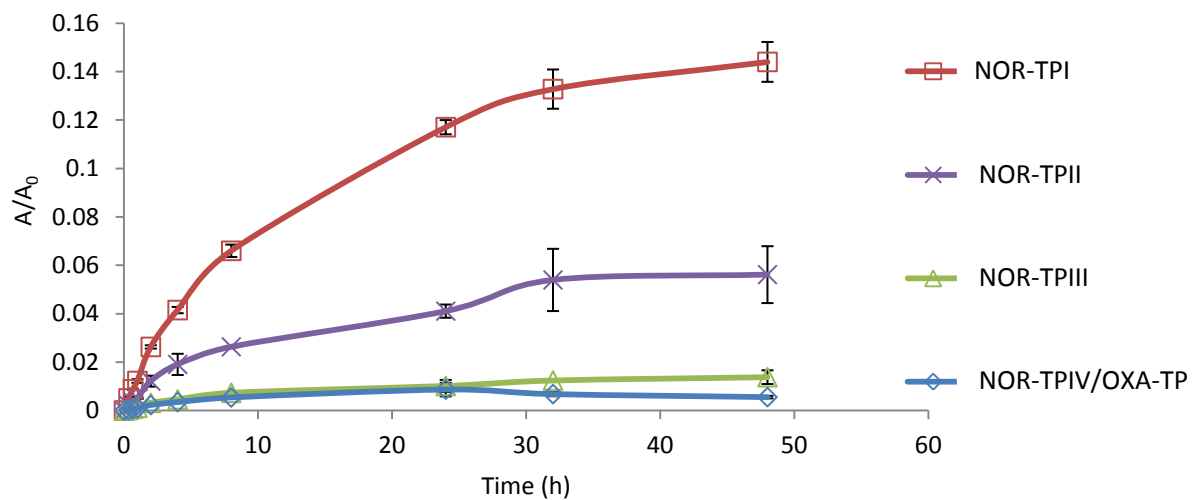


Fig. 3. Reaction time-profile of (A) Nordazepam and (B) its TPs. Results normalized to the signal measured for nordazepam at  $t=0$  s.

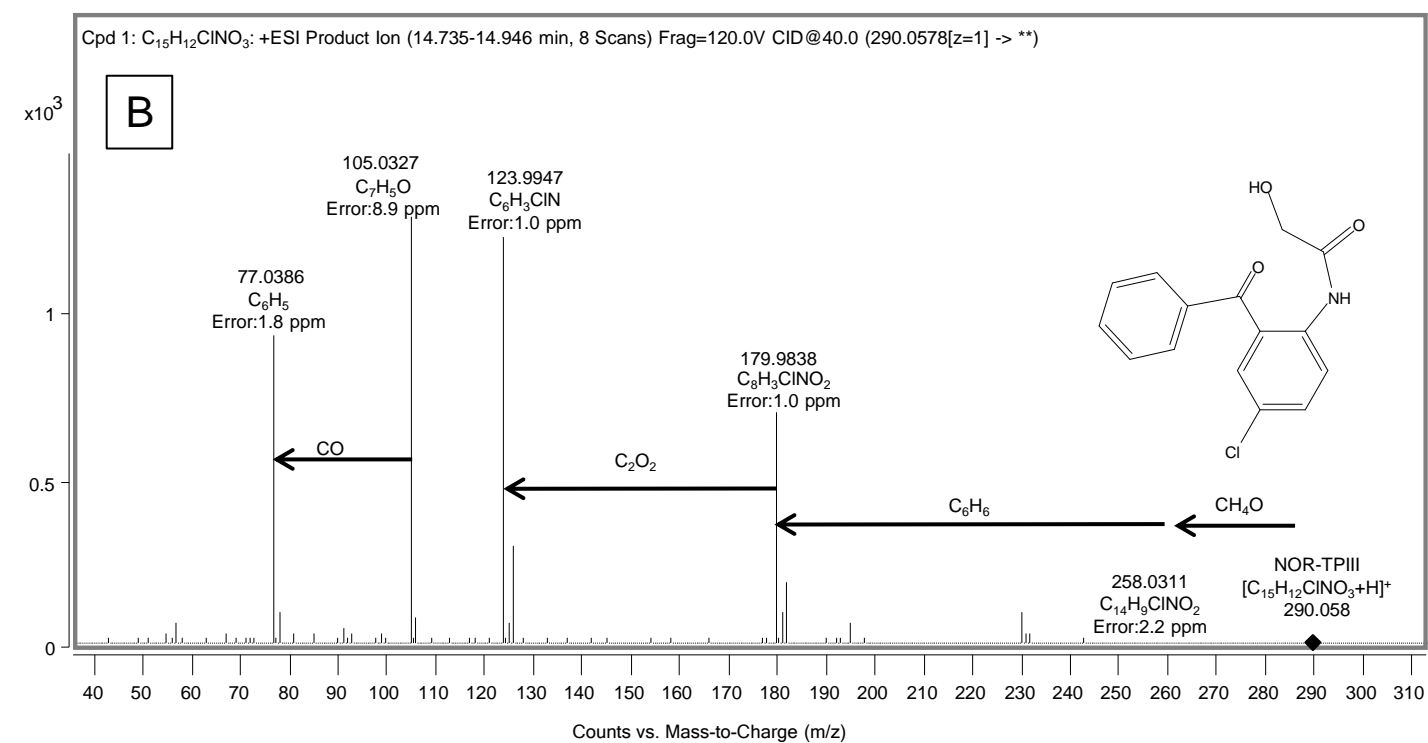
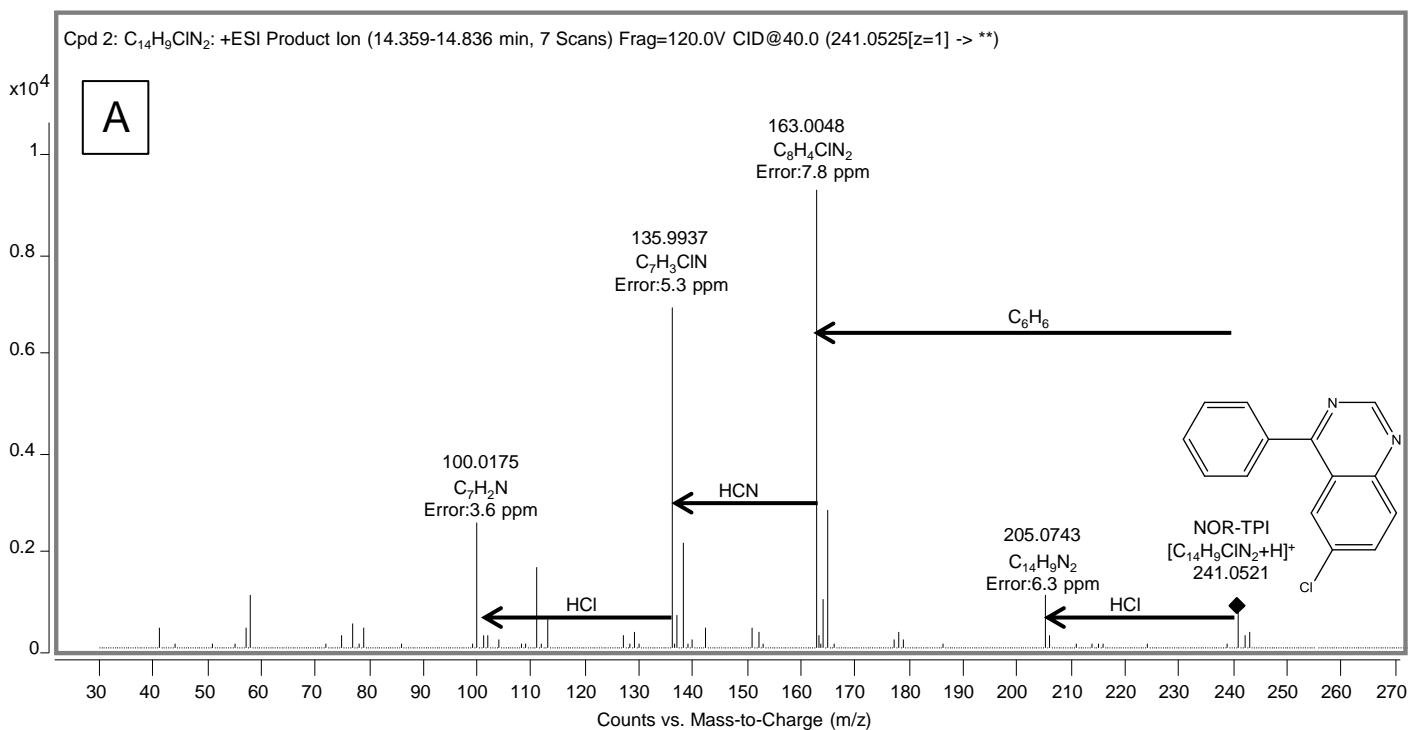
**Figure 4**

Fig. 4. QTOF product ion spectra of some nordazepam TPs: (A) NOR-TPI and (B) NOR-TPIII. The remaining spectra are presented in the Supplementary Information (Fig. S6-S7).

**Figure 5**

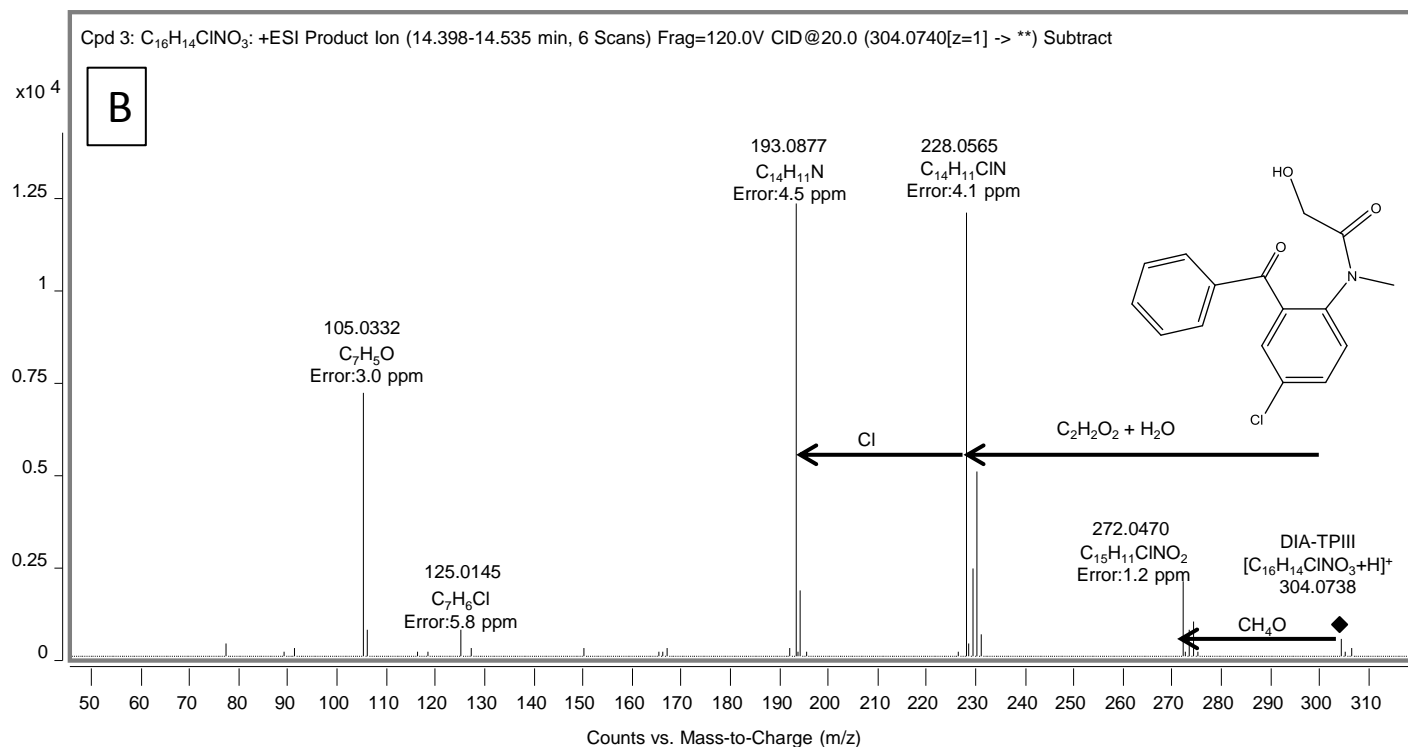
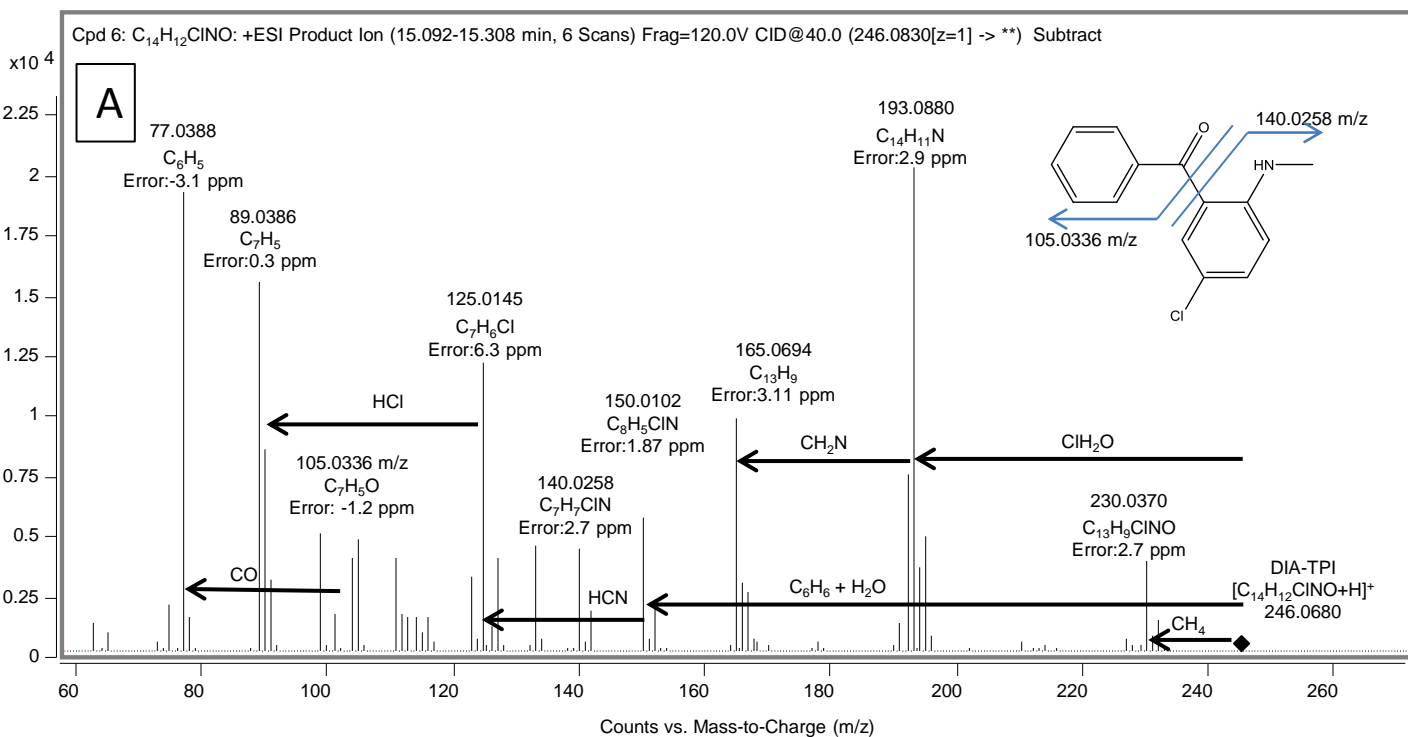
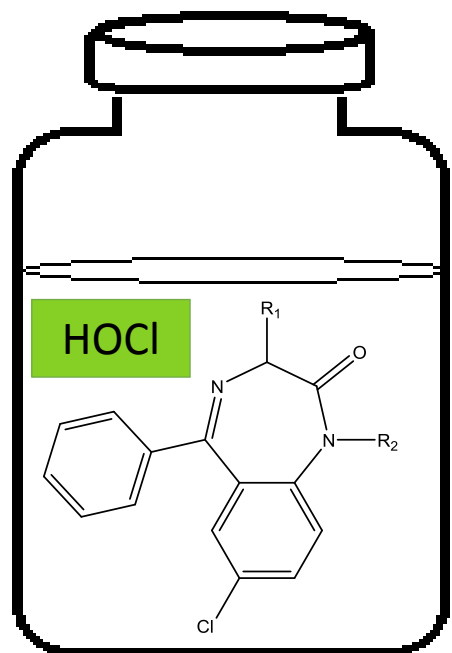
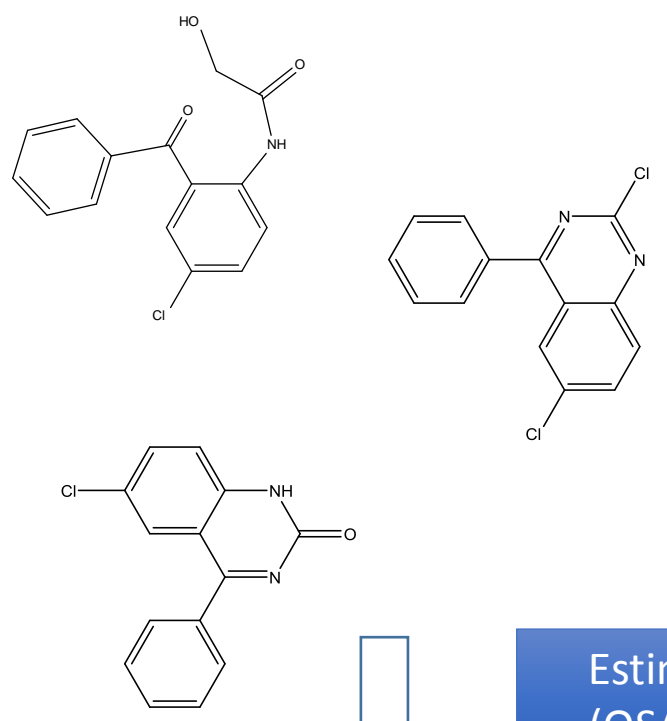


Fig. 5. QTOF product ion spectra of some diazepam TPs: (A) DIA-TPI and (B) DIA-TPIII. The remaining spectra are presented in the Supplementary Information (Fig. S8-S11).



Identification of transformation products by QTOF-MS



Estimation ecotoxicity (QSAR software tools)



**Electronic Supplementary Material**

**Reaction of diazepam and related benzodiazepines with chlorine. Kinetics, transformation products and *in-silico* toxicological assessment**

I. Carpinteiro, R. Rodil, J.B. Quintana, R.Cela

Table S1. Box-Behnken design plan, obtained half-life time ( $t_{1/2}$ ) and apparent second-order constant (k).

Exp.	Factor			Oxazepam		Nordazepam		Diazepam	
	pH	Free chlorine ( $\mu\text{g mL}^{-1}$ )	Bromide ( $\text{ng mL}^{-1}$ )	$t_{1/2}$ (min)	k ( $\text{M}^{-1} \text{s}^{-1}$ )	$t_{1/2}$ (h)	k ( $\text{M}^{-1} \text{s}^{-1}$ )	$t_{1/2}$ (h)	k ( $\text{M}^{-1} \text{s}^{-1}$ )
1	6.9	5.5	50	17.2	8.55	16.2	0.15	7.6	0.32
2	5.7	1	50	87.6	9.22	18.7	0.72	6.6	2.04
3	8.1	1	50	141.4	5.72	18.6	0.72	637.6	0.02
4	5.7	10	50	1.9	42.48	1.8	0.74	2.5	0.55
5	8.1	10	50	44.5	1.82	10.2	0.13	21.3	0.06
6	5.7	5.5	0	4.2	34.55	2.1	1.16	2.8	0.88
7	8.1	5.5	0	63.0	2.33	10.2	0.24	70.5	0.03
8	6.9	5.5	50	16.9	8.70	17.9	0.14	9.8	0.25
9	5.7	5.5	100	5.3	27.85	3.0	0.82	2.8	0.88
10	8.1	5.5	100	52.5	2.80	10.1	0.24	70.2	0.04
11	6.9	1	0	92.4	8.75	86.7	0.16	182.6	0.07
12	6.9	10	0	9.3	8.73	7.3	0.19	3.9	0.35
13	6.9	1	100	146.1	5.48	58.6	0.23	76.6	0.18
14	6.9	10	100	8.3	9.77	7.5	0.18	3.7	0.36
15	6.9	5.5	50	17.6	8.36	15.9	0.15	8.9	0.28

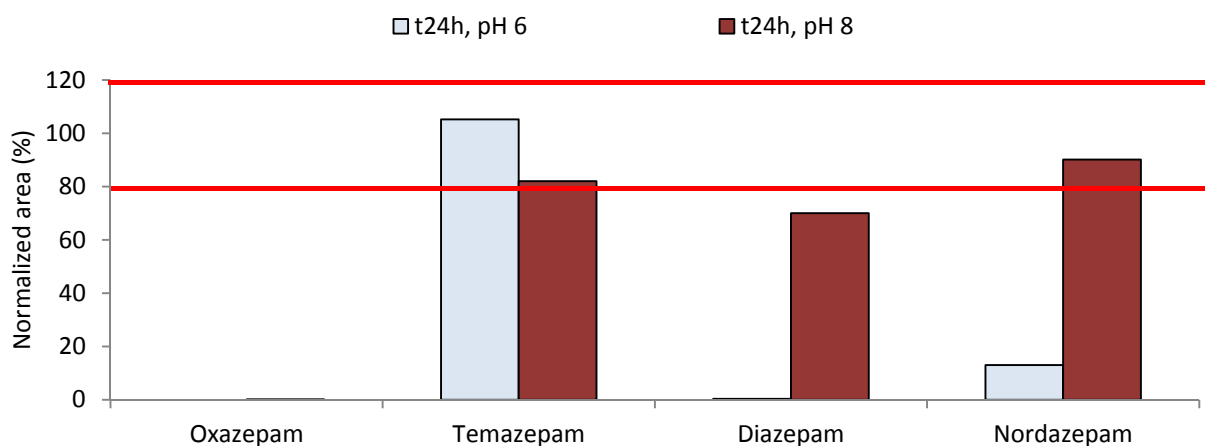


Fig. S1. Stability of benzodiazepines in chlorinated water for 24 h at pH 6 and pH 8 (n=2). Results normalized to the signal measured at t = 0 s. Red lines indicate 80% and 120% of benzodiazepine area against the area measured at time 0 s.

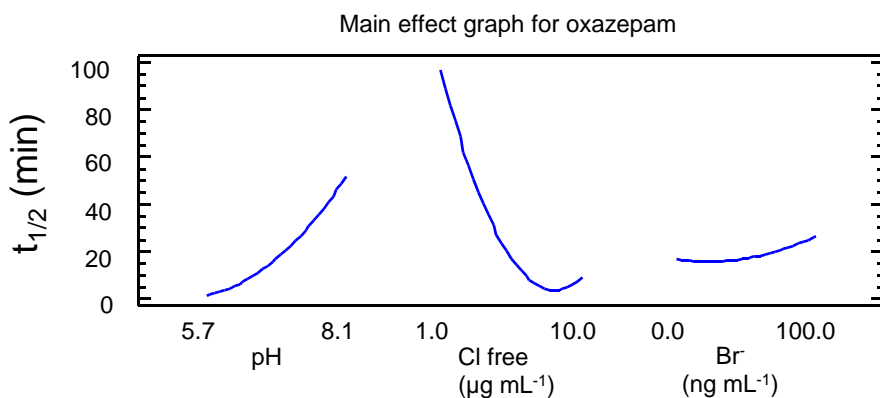


Fig. S2. Main effects graph for oxazepam.

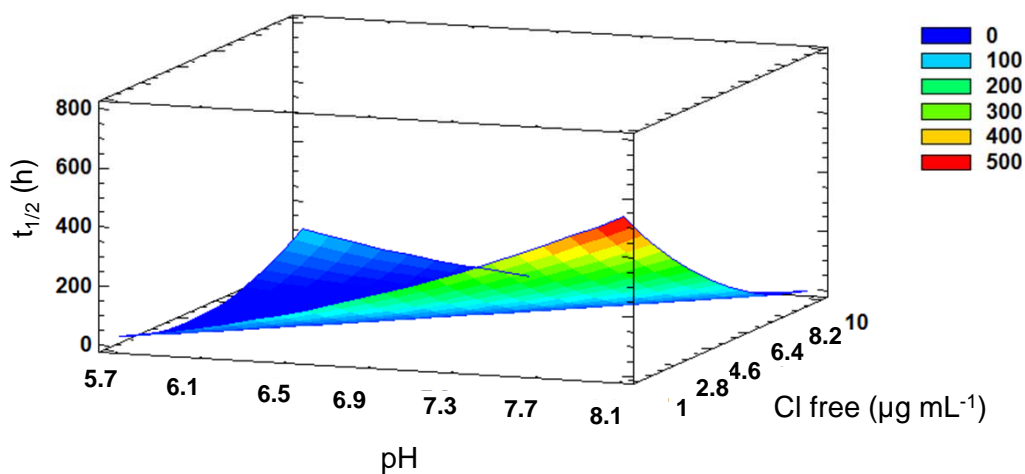


Fig. S3. Response surface plot for diazepam.  $t_{1/2}$  as a function of chlorine concentration and pH. Bromide level at 50 ng mL<sup>-1</sup>.

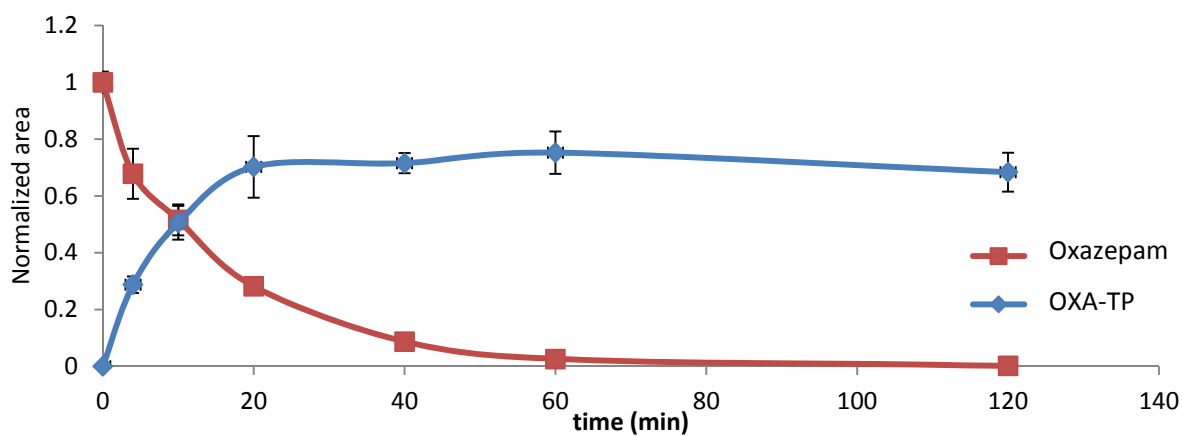


Fig. S4. Reaction time-profile of oxazepam and formation of its transformation product. Area normalized to the response of oxazepam at time=0.

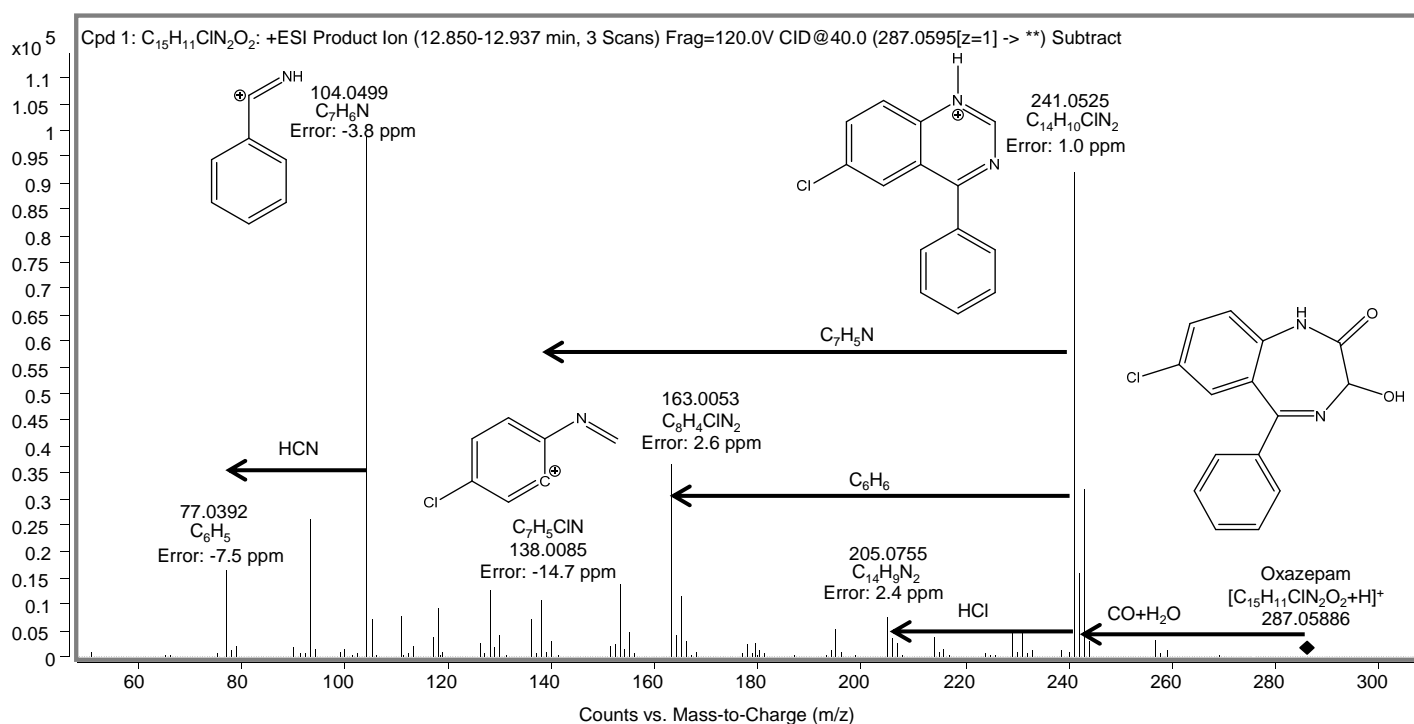


Fig. S5. QTOF product ion spectrum for oxazepam. More details for interpretation of oxazepam MS/MS spectrum are given by Niessen et al. Mass Spectrometry Reviews 30 (2011) 626-663

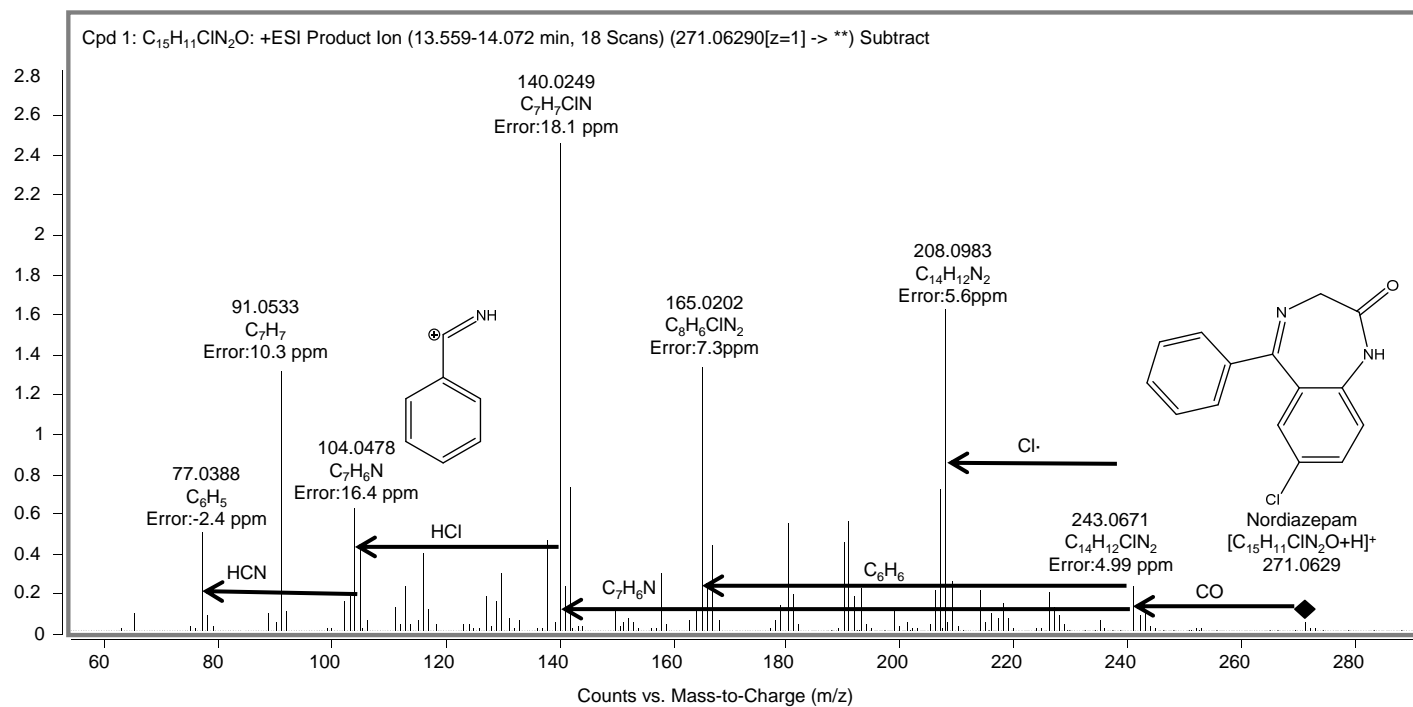


Fig. S6. QTOF product ion spectrum for nordiazepam. More details for interpretation of MS/MS spectrum are given by Niessen et al. Mass Spectrometry Reviews 30 (2011) 626-663

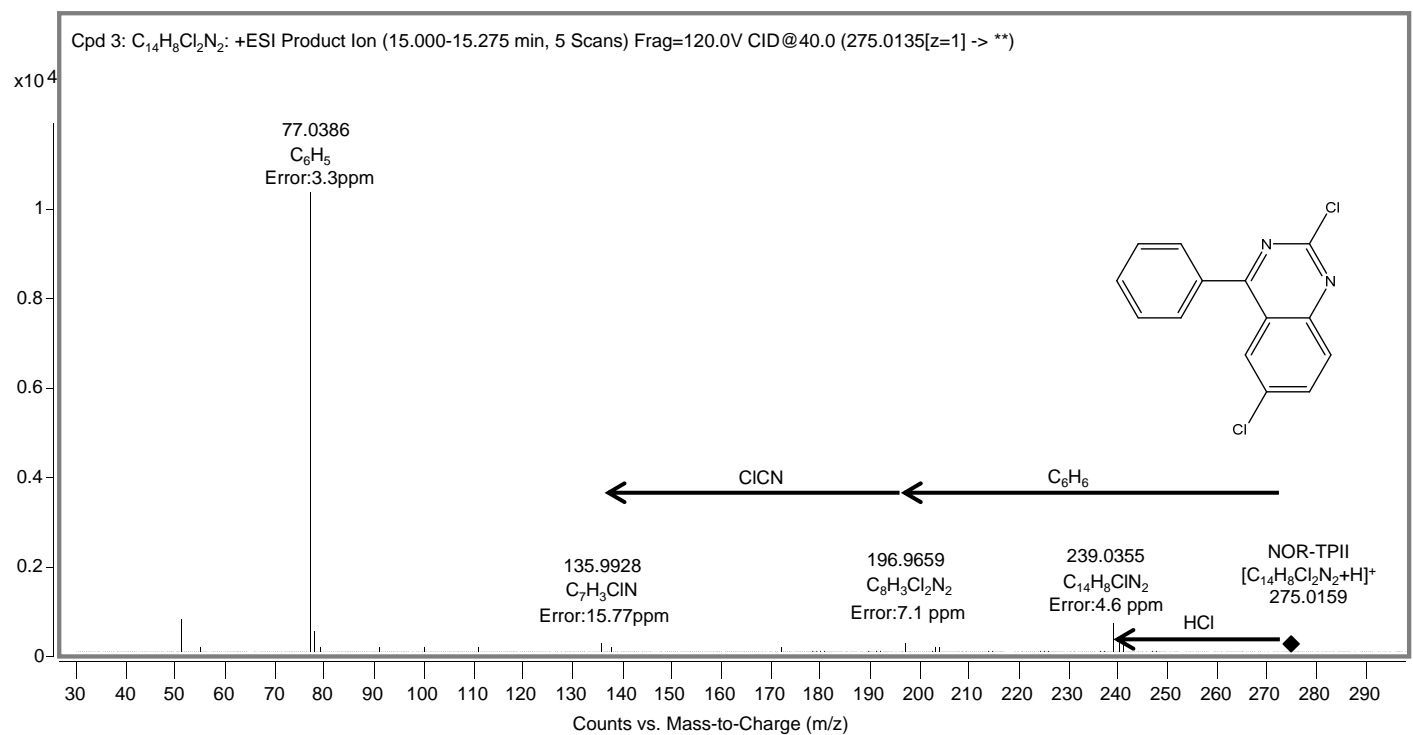


Fig. S7. QTOF product ion spectrum for NOR-TPII

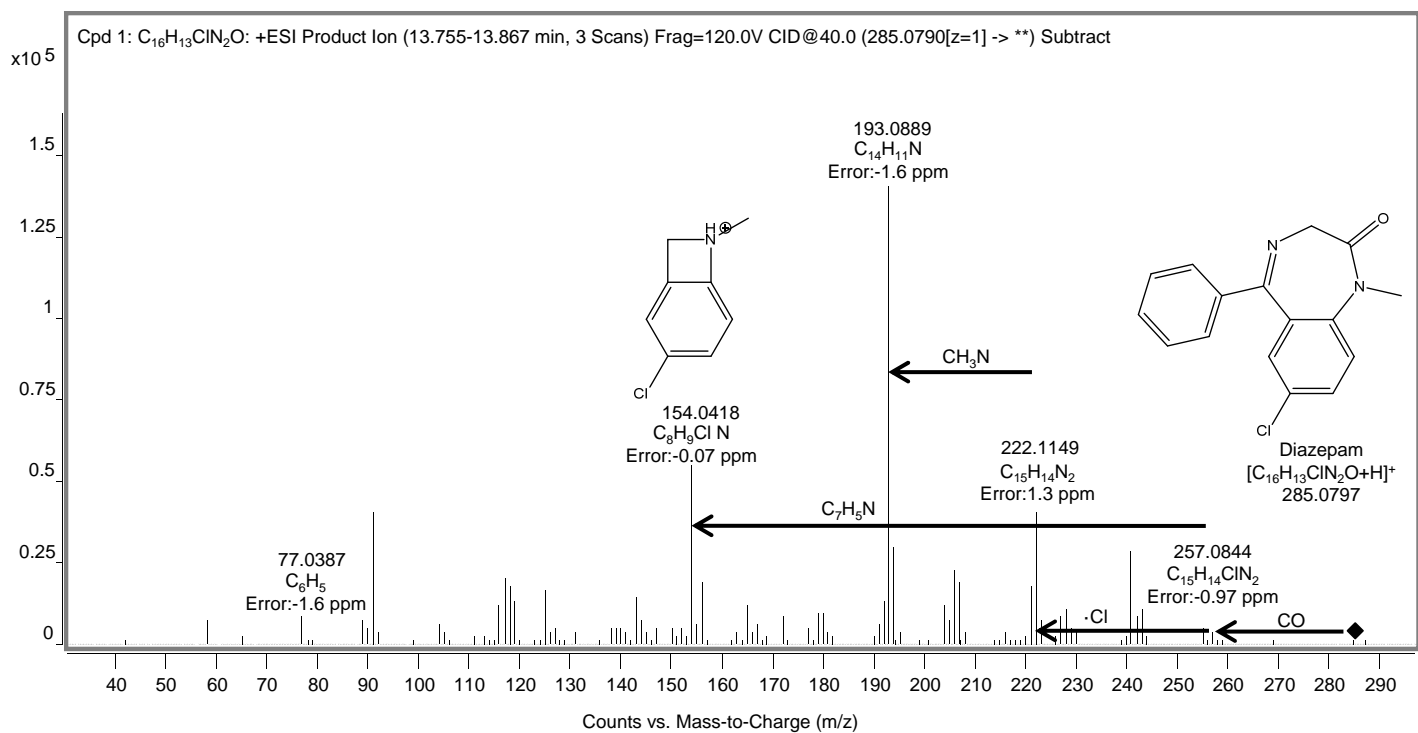


Fig. S8. QTOF product ion MS/MS spectrum for diazepam. More details for interpretation of MS/MS spectrum are given by Niessen et al. Mass Spectrometry Reviews 30 (2011) 626-663

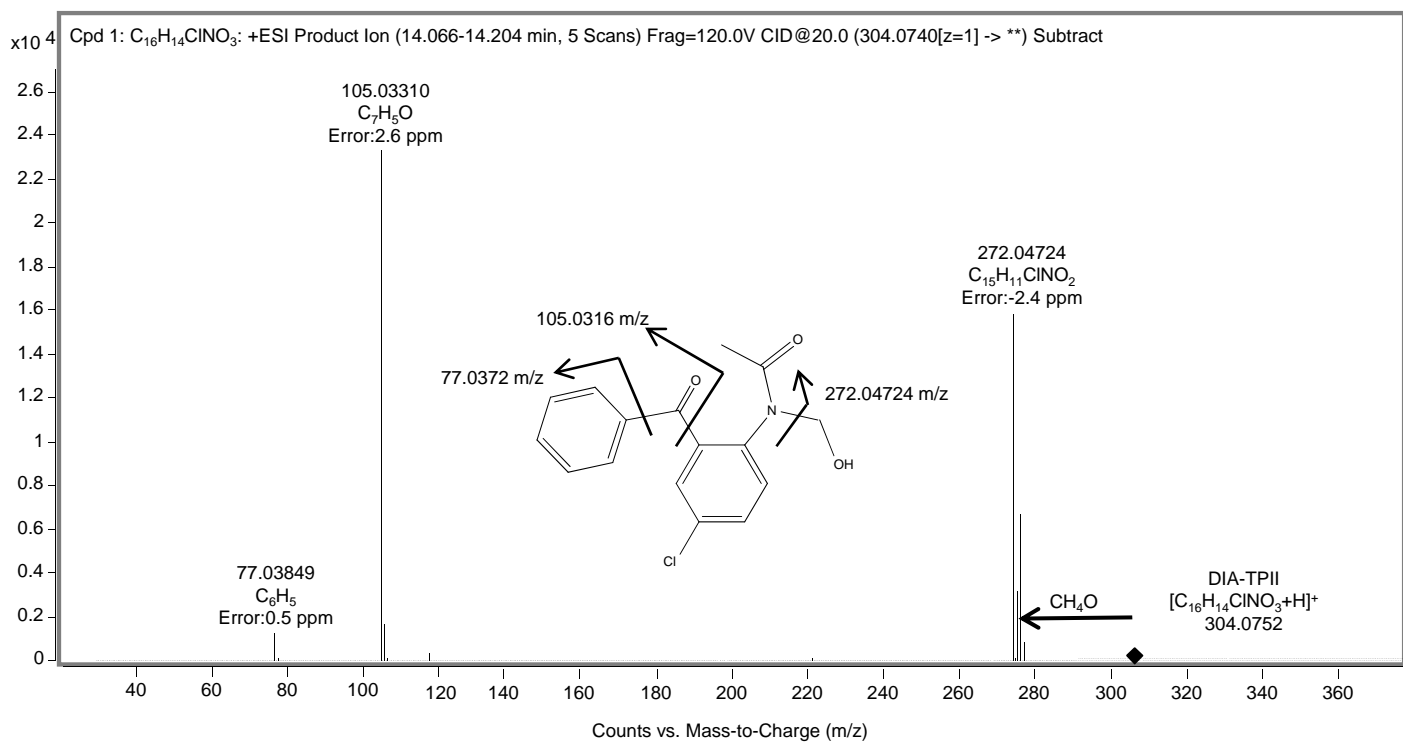


Fig. S9. QTOF Product Ion spectrum for DIA-TPII

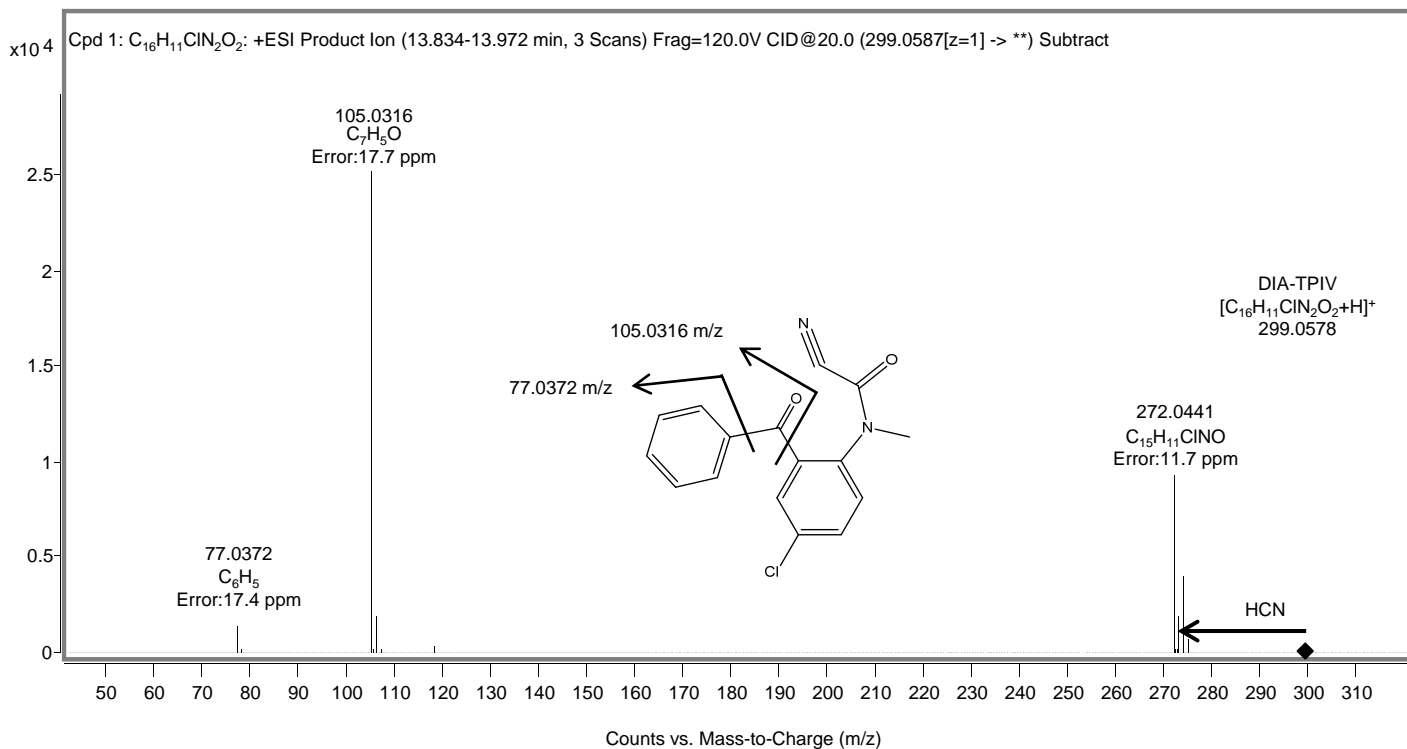


Fig. S10. QTOF Product Ion spectrum for DIA-TPIV

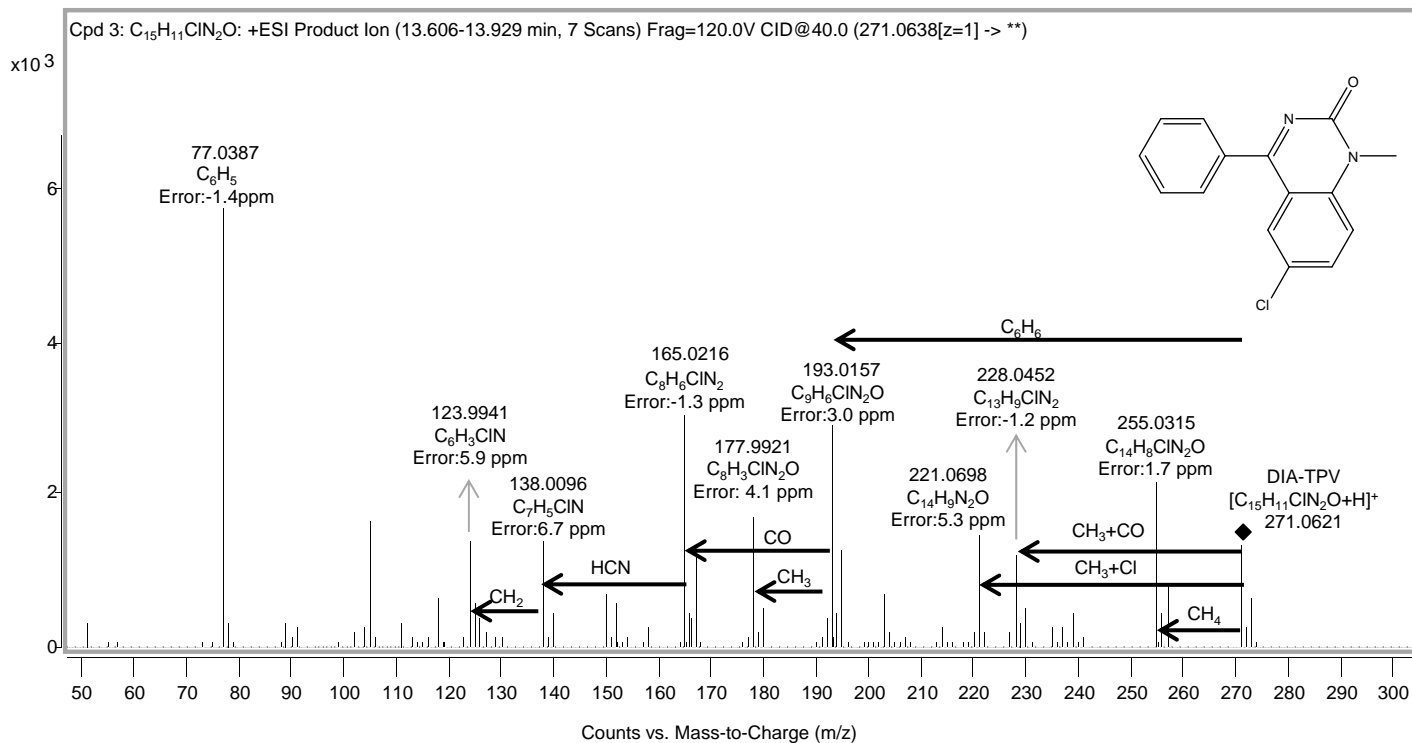


Fig. S11. QTOF Product Ion spectrum for DIA-TPV

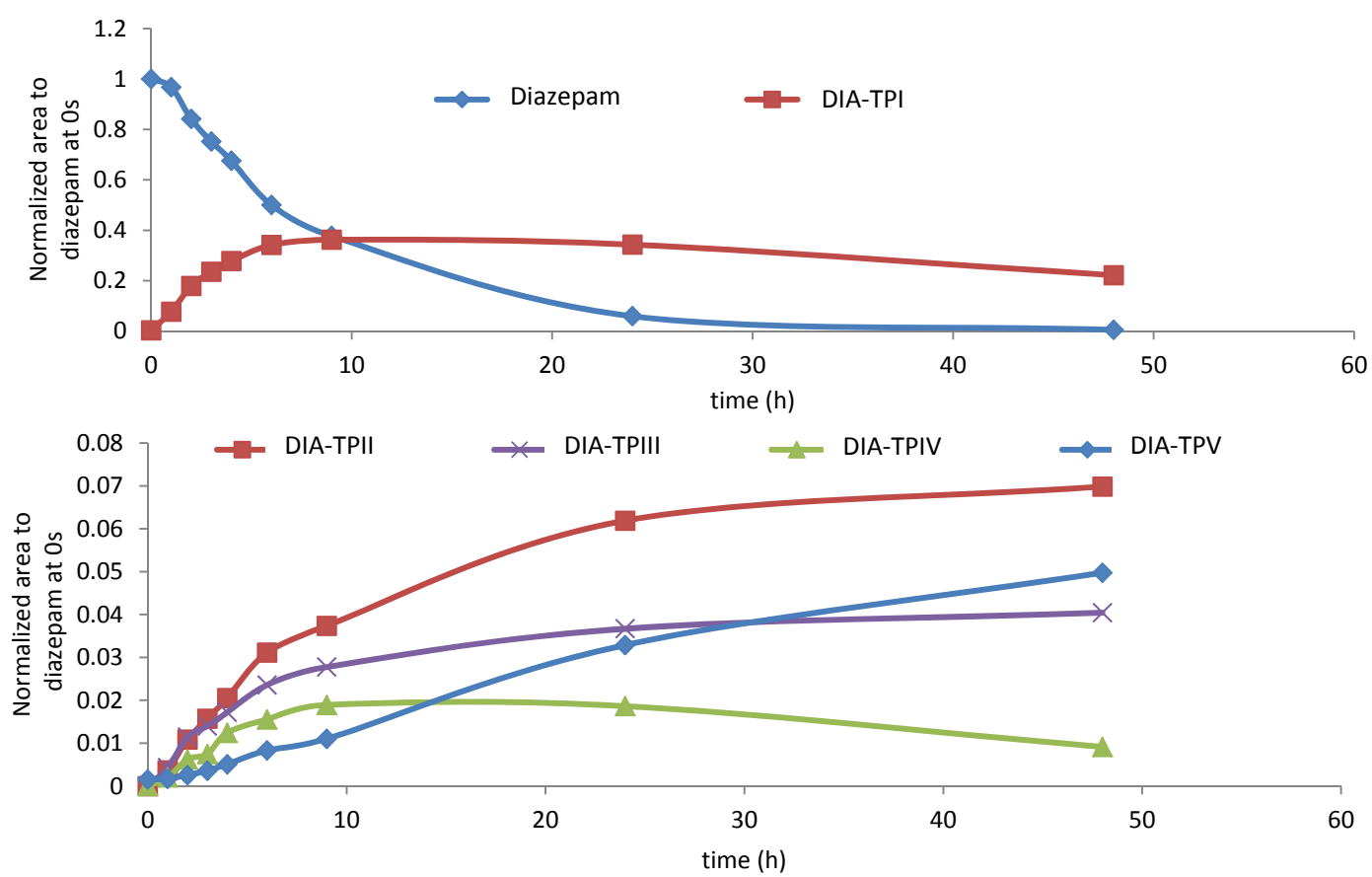


Fig. S12. Time-profile for diazepam reaction and formation of TPs