Cela. Reaction of diazepam and related benzodiazepines with chlorine. Kinetics, transformation products and in-silico toxicological assessment Water Research 120 (2017) 280-289 with DOI: 10.1016/j.watres.2017.04.063

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

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Abstract

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

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

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

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1. Introduction

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

In the same way as other pharmaceuticals, diazepam and its metabolites can enter wastewater treatment plants (WWTPs) (Calisto and Esteves, 2009; De Almeida et al., 2015; Kosjek et al., 2011; Racamonde et al., 2014), by urinary excretion or direct spills, and then they can reach surface water if not completely removed at WWTPs (Kosjek et al., 2011; Mendoza et al., 2014), which are finally a source of drinking water. A common step in drinking water production is the use of disinfection methods to reduce the level of microorganisms or contaminants in the drinking water, by using e.g. chlorine, ozone, chloramination, chlorine dioxide, H_2O_2, ferrate or UV radiation (Postigo and Richardson, 2014; Sharma, 2008). However, such disinfectants agents can generate a range of transformation products (TPs) which are sometimes more toxic than the precursor compound (Gonzalez-Marino et al., 2016; Postigo and Richardson, 2014). In the case of benzodiazepines, photodegradation (Calisto et al., 2011; Jakimska et al., 2014; Kosjek et al., 2011; West and Rowland, 2012) and hydrolysis (Cabrera et al., 2005; Han et al., 1977) have been previously reported as well as the advanced oxidative treatments (Bautitz and Nogueira, 2010; Bautitz et al., 2012). However, despite the fact that chlorine is the most widely used disinfectant, the study of its reaction with benzodiazepines has not been performed so far.

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

2. Materials and method

2.1. Chemicals and materials

All benzodiazepine standards were purchased from Cerilliant (Round Rock, TX, USA) as 1 mg mL^{-1} solutions in methanol (MeOH) or acetonitrile (ACN). All concentrations cited here refer to the neutral species. Table 1 shows the name of the benzodiazepines considered, their abbreviation and some physico-chemical properties. Mixtures and dilutions were prepared in methanol and kept at -20 ºC.

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

2.2. Real samples

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

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

2.3. Chlorination experiments

Chlorination experiments were carried out with 10 mL of water in 16 mL amber closed vials at room temperature (20±2°C). For preliminary test and the study of factors affecting to the reaction kinetics, ten milliliters of ultrapure water were buffered at pH 6, 7 or 8, with a KH_2PO_4/K_2HPO_4 0.03 M solution, and spiked with the target analytes at 0.1-0.25 µg mL^{-1}. Also, potassium bromide was added at 0.05-0.1 µg mL^{-1} in some experiments. These pH values and bromide concentrations, which is known to react to HBrO with chlorine, were selected as to represent typical surface waters (Gonzalez-Marino et al., 2016). After free chlorine addition at concentrations in the 0.5-10 µg mL^{-1} range, also representing typical dosages during drinking water production (Gonzalez-Marino et al., 2016), the amber vials were manually shaken for 2-3 s. Then, 6-10 aliquots of 1 mL were collected at increasing reaction times, from time 0 s (before chlorine addition) to 120 min-96 h (depending of the reaction rate of the drug). These aliquots were transferred to 2 mL vials containing 10 µL of ascorbic acid (60 mg mL^{-1}) as quenching agent to stop the reaction.

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

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

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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^{-1} 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^{-1}; 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 $[M+H]^+$ 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 (accesible 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 (accesible 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_{50} (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_{50}, 96-hour fish LC_{50} and 96-hour green algae half maximal effective
175 concentration (EC_{50}) 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-

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

3. Results and discussion

3.1. Preliminary tests

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

3.2. Parameters affecting the reaction kinetics

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

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

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

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

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 ±1.6 mDa (equivalent to ± 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).

3.3.2. Nordazepam

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

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

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

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

3.3.3. Diazepam

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

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

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

Finally, as mentioned, DIA-TPV, is an analog to OXA-TP/NOR-TPIV, showing the same sequential loss of benzene, CO and HCN in their spectra (Fig. 2 and S11). Moreover, DIA-TPV structure has an N-methyl group, which is evident from the losses of methane or CH_3 from its spectrum (Fig. S11).

3.4 Reaction in real sample matrices

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

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

3.5. QSAR estimation of ecotoxicological implications

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

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

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

4. Conclusions

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

Acknowledgement

This study has been supported by Xunta de Galicia (EM2014-004 and GRC2013-020) and FEDER/ERDF. I.C. acknowledges her postdoctoral formation grant to Xunta de Galicia government.

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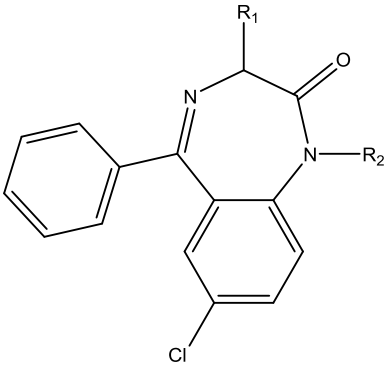
468

469 **Tables**

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

Structure	Compounds (abbreviation)	R_1	R_2	Log K_{ow} [a]	pK_a [a]
	Oxazepam (OXA)	OH	H	2.24	10.9
	Nordazepam (NOR)	H	H	2.93	11.7
	Diazepam (DIA)	H	CH_3	2.82	11
	Temazepam (TEM)	OH	CH_3	2.19	11.6

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

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

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

[a] Statistically significant factors and interactions at the 95% confidence level

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

	t_R (min)	Formula	Calculated m/z $[M+H]^+$	Experimental m/z $[M+H]^+$	Score	Error (mDa)	Error (ppm)	DBE
Oxazepam	12.9	$C_{15}H_{11}ClN_2O_2$	287.0582	287.0589	95.56	-0.52	-1.8	11
OXA-TP [a]	13.4	$C_{14}H_9ClN_2O$	257.0476	257.0478	99.3	-0.2	-0.79	11
Nordazepam	13.7	$C_{15}H_{11}ClN_2O$	271.0633	271.0632	99.76	0.02	0.08	11
NOR-TPI	14.6	$C_{14}H_9ClN_2$	241.0527	241.0522	98	0.61	2.6	11
NOR-TPII	15.1	$C_{14}H_8Cl_2N_2$	275.0137	275.0132	98.68	0.54	2.0	11
NOR-TPIII	14.8	$C_{15}H_{12}ClNO_3$	290.0578	290.0572	92.15	0.62	2.1	10
NOR-TPIV[a]	13.6	$C_{14}H_9ClN_2O$	257.0476	257.0470	98.66	0.51	2.0	11
Diazepam	13.82	$C_{16}H_{13}ClN_2O$	285.0789	285.0799	96.91	-0.87	-3.1	11
DIA-TPI	15.2	$C_{14}H_{12}ClNO$	246.068	246.0685	98.19	-0.44	-1.8	9
DIA-TPII	14.1	$C_{16}H_{14}ClNO_3$	304.0735	304.0752	88.5	-1.54	-5.0	10
DIA-TPIII	14.5	$C_{16}H_{14}ClNO_3$	304.0735	304.0738	98.83	-0.07	-0.23	10
DIA-TPIV	13.9	$C_{16}H_{11}ClN_2O_2$	299.0582	299.0578	96.27	0.12	0.39	12
DIA-TPV	13.8	$C_{15}H_{11}ClN_2O$	271.0633	271.0641	94.21	-0.46	-1.7	11

[a] NOR-TPIV and OXA-TP were the same by-product (see discussion in section 3.3.2).

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

Compound	**Ultrapure water (pH 6.9)**	**Creek (pH 6.7)**	**River (pH 6.1)**
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)

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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^{-1}), toxic (1-10 mg L^{-1}), harmful (10-100 mg L^{-1}) and not harmful (> 100 mg L^{-1}) (United Nations, 2011).

Compound	ECOSAR							TEST	
	Chemical class	Acute toxicity (mg L^{-1})			Chronic toxicity (mg L^{-1})			96h *Fathead Minnow* LC_{50} (mg L^{-1})	Mutagenicity
		Fish (96h LC_{50})	*Daphnid* (48h LC_{50})	Algae (96h EC_{50})	Fish	*Daphnid*	Algae		
OXA	Amide	50.36[b]	47.88[b]	1.70	0.11	3.32	1.83	1.06	Negative
OXA-TP[c]	Neutral organic[a]	95.78	56.34	48.55	9.76	6.06	13.75	2.02	Positive
NOR	Amide	19.22[b]	14.16[b]	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[c]	Neutral organic[a]	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[a]	212.73[b]	121.56[b]	92.97	20.95	12.07	24.67	0.18	Positive
DIA-TPV	Neutral organic[a]	144.47	83.626	67.46	14.45	8.60	18.44	1.53	Positive

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

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Figure 1

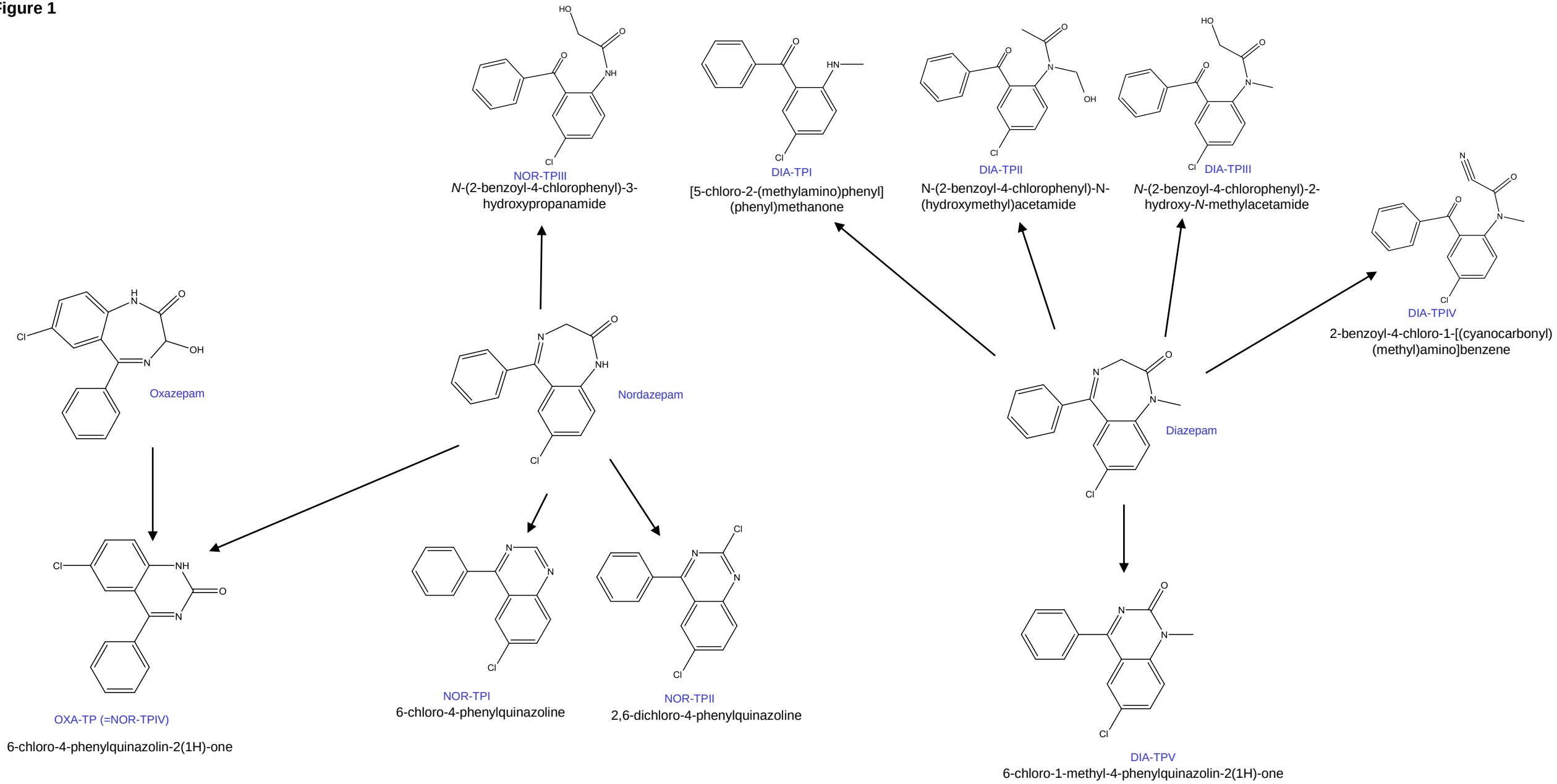


Fig. 1. Proposed reaction pattern of oxazepam, nordazepam and diazepam with chlorine.

Figure 2

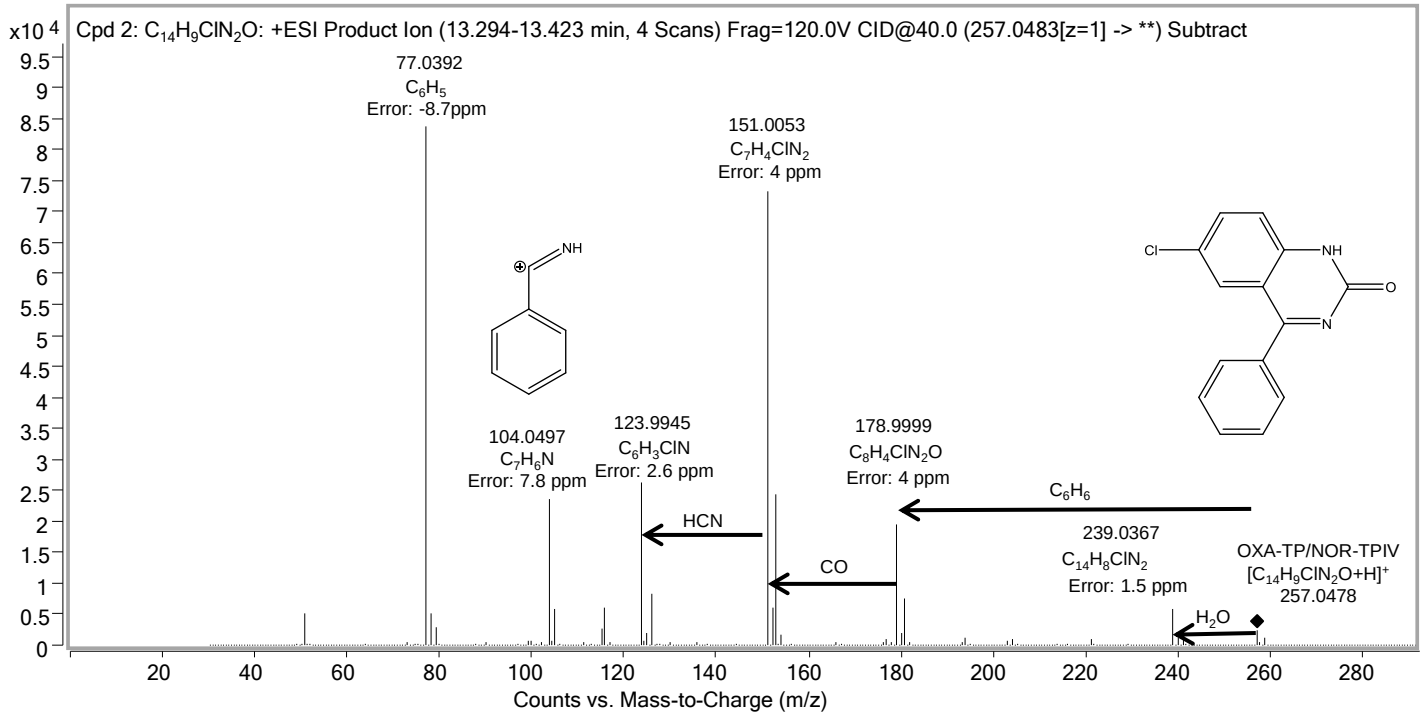


Fig. 2. QTOF product ion scan spectra of oxazepam TP (OXA-TP). The spectrum of oxazepam is presented in the Supplementary Information (Fig. S5)

Figure 3

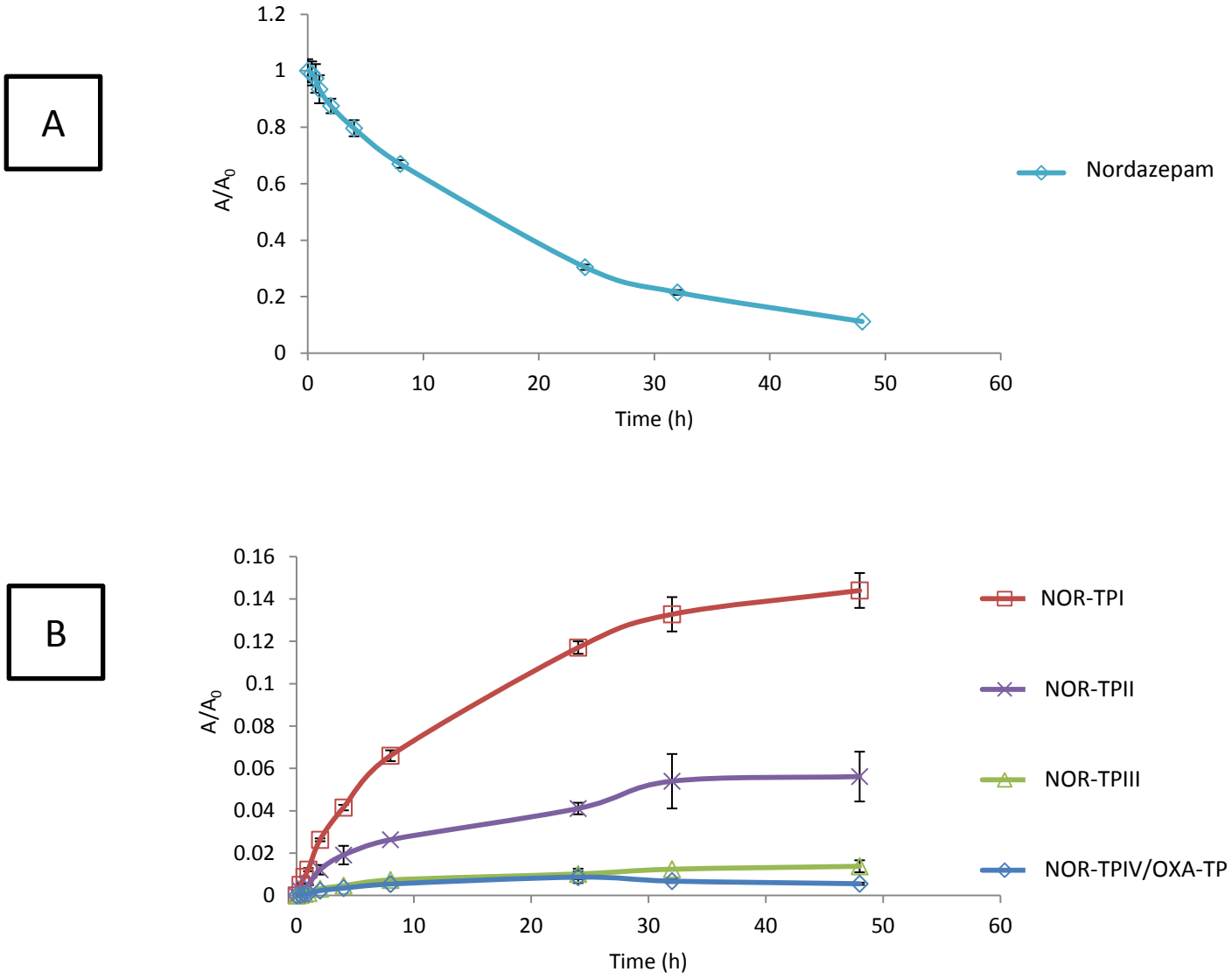


Fig. 3. Reaction time-profile of (A) Nordazepam and (B) its TPs. Results normalized to the signal measured for nordazepam at t=0 s.

Figure 4

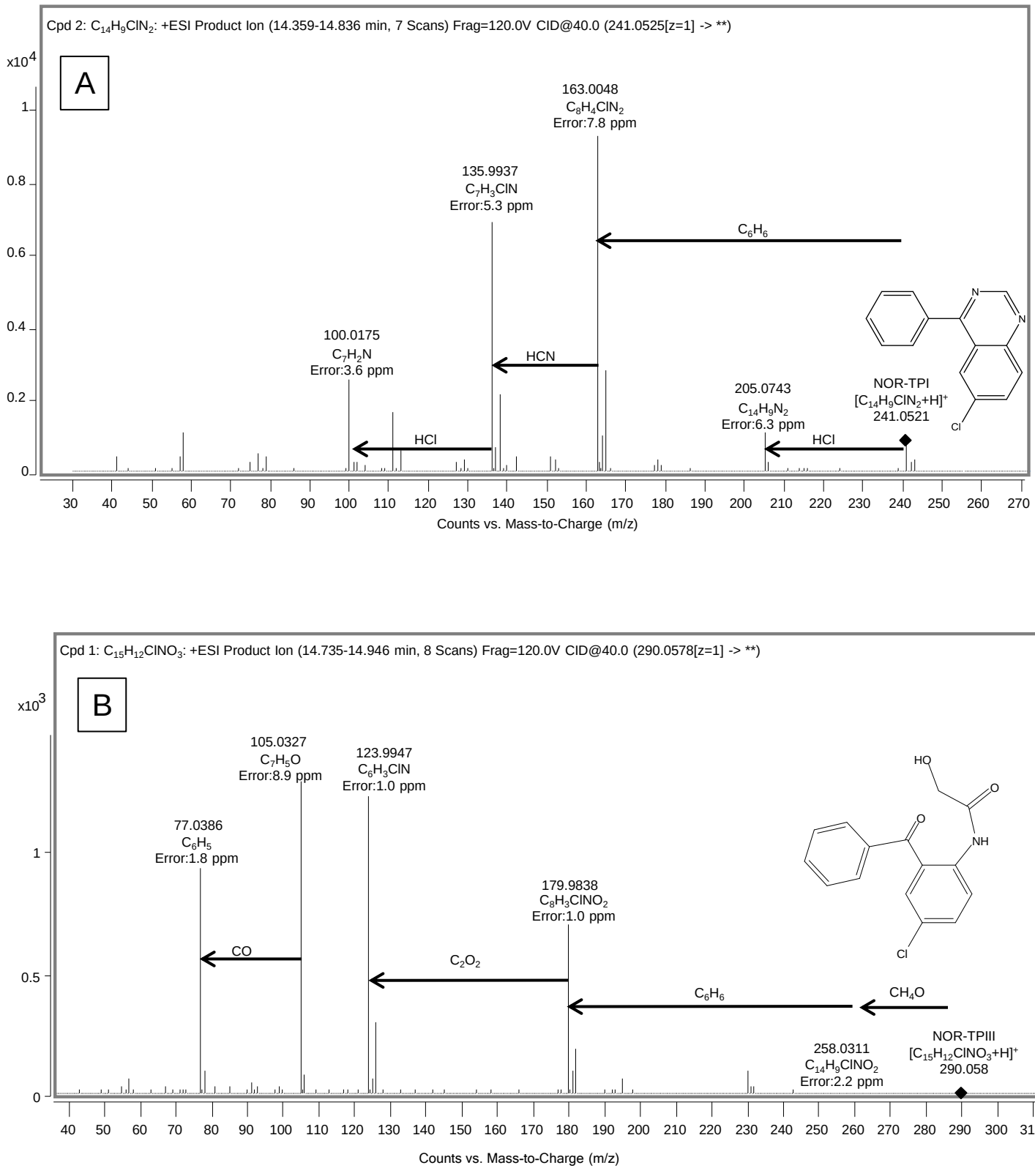


Fig. 4. QTOF product ion spectra of some nordazepam TPs: (A) NOR-TPI and (B) NOR-TPIII. The remaining spectra are presented in the Supplementary Information (Fig. S6-S7).

Figure 5

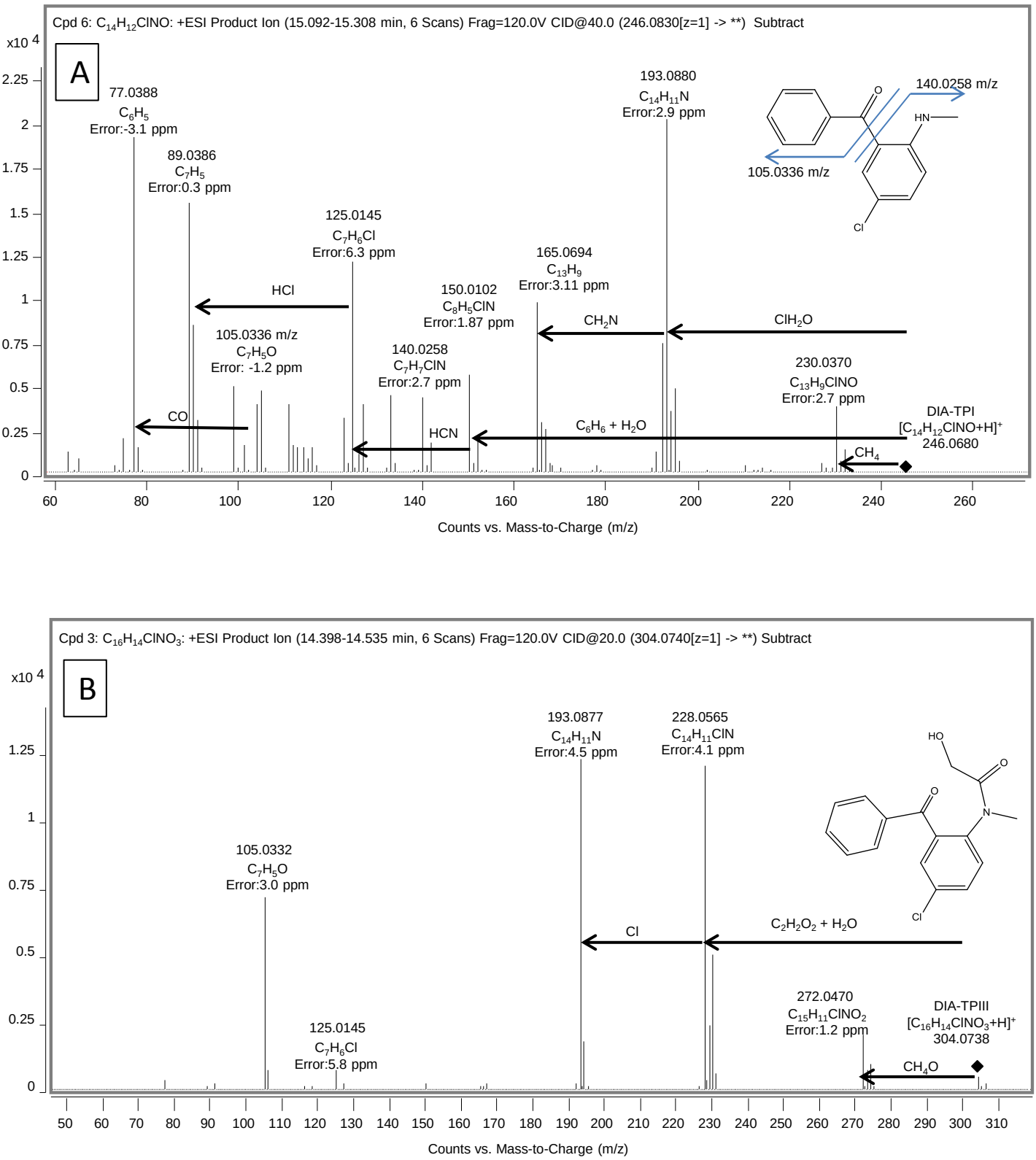
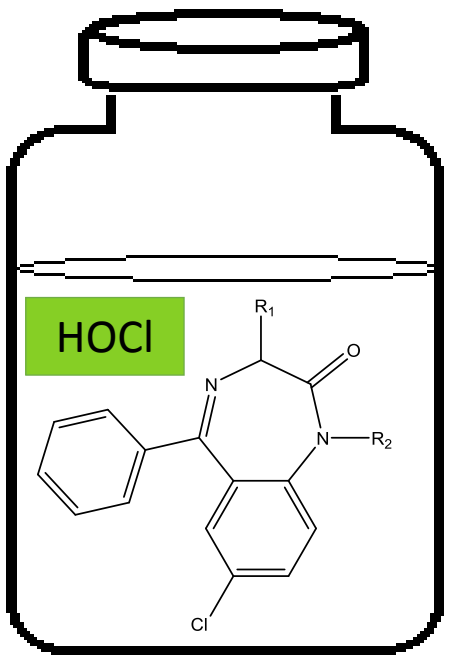
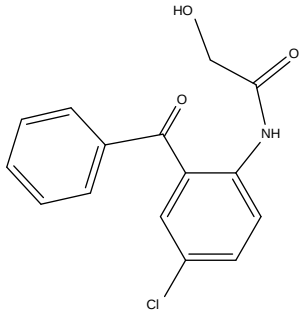


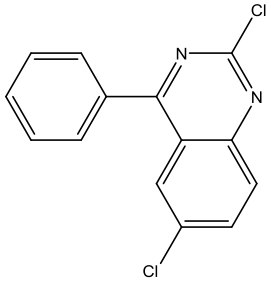
Fig. 5. QTOF product ion spectra of some diazepam TPs: (A) DIA-TPI and (B) DIA-TPIII. The remaining spectra are presented in the Supplementary Information (Fig. S8-S11).

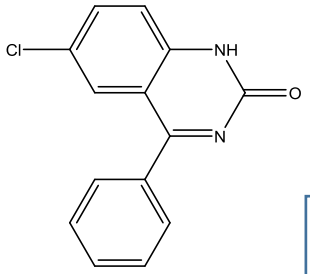
Graphical Abstract



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 (μg mL^{-1})	Bromide (ng mL^{-1})	$t_{1/2}$ (min)	k (M^{-1} s^{-1})	$t_{1/2}$ (h)	k (M^{-1} s^{-1})	$t_{1/2}$ (h)	k (M^{-1} 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

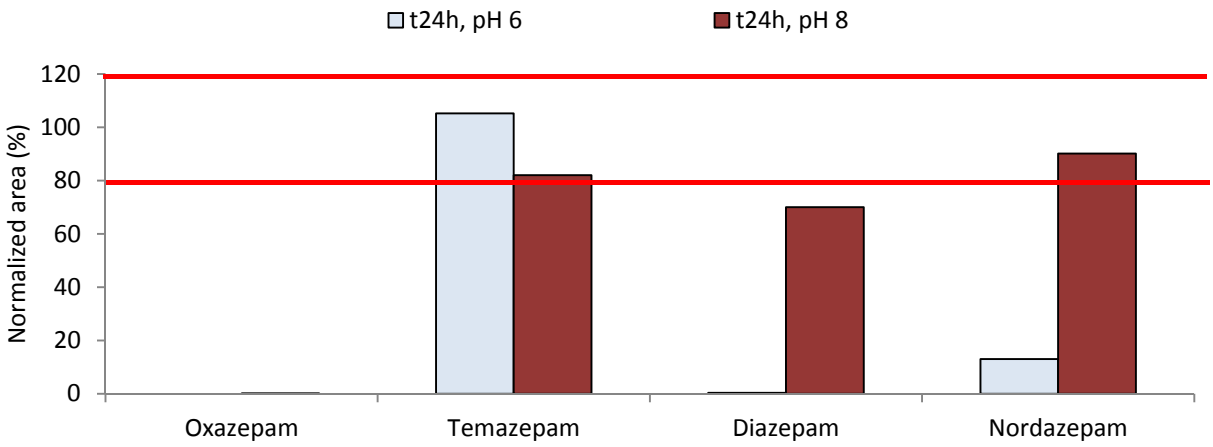


Fig. S1. Stability of benzodiazepines in chlorinated water for 24 h at pH 6 and pH 8 (n=2). Results normalized to the signal measured at t = 0 s. Red lines indicate 80% and 120% of benzodiazepine area against the area measured at time 0 s.

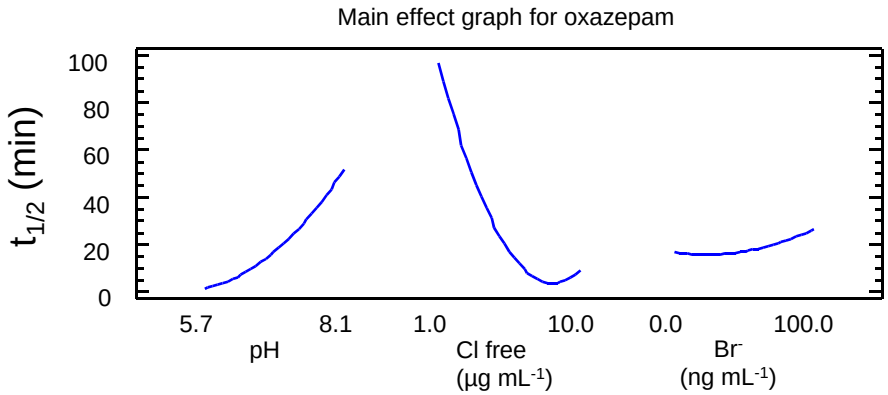


Fig. S2. Main effects graph for oxazepam.

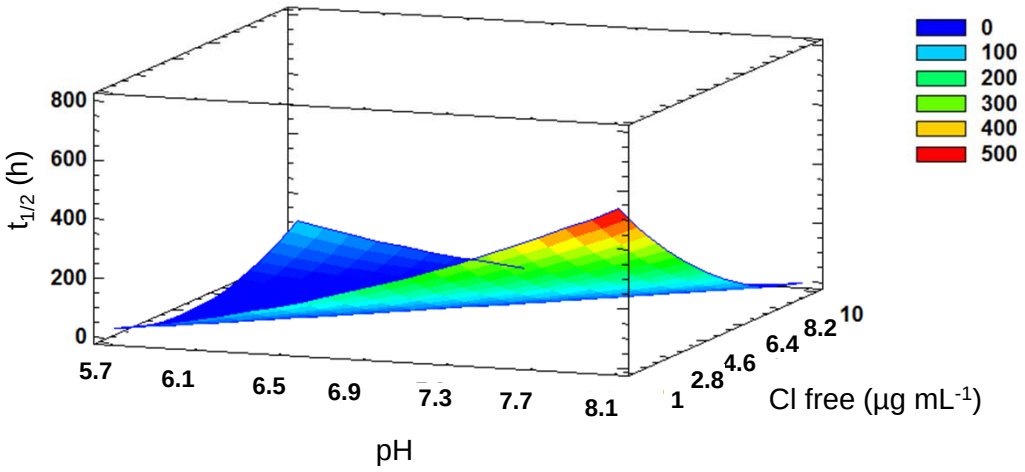


Fig. S3. Response surface plot for diazepam. t1/2 as a function of chlorine concentration and pH. Bromide level at 50 ng mL^{-1}.

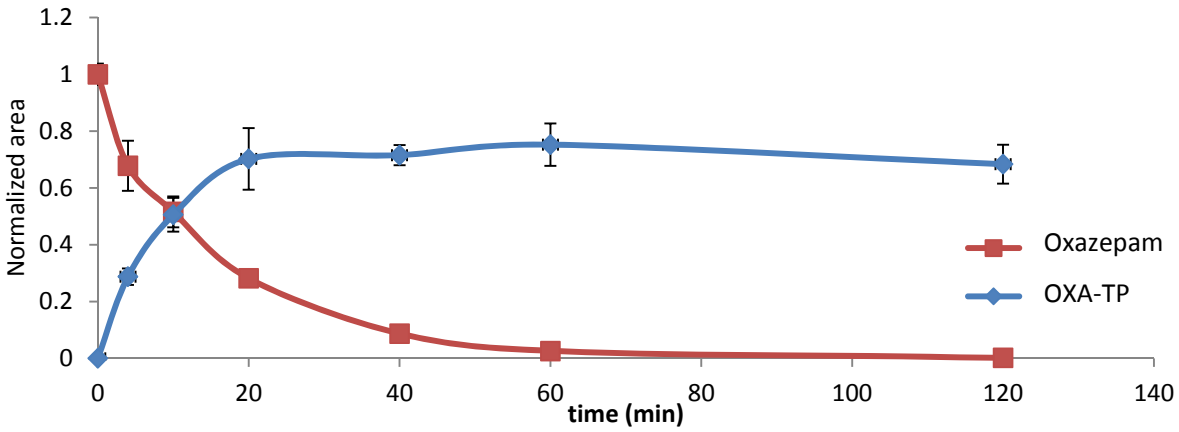


Fig. S4. Reaction time-profile of oxazepam and formation of its transformation product. Area normalized to the response of oxazepam at time=0.

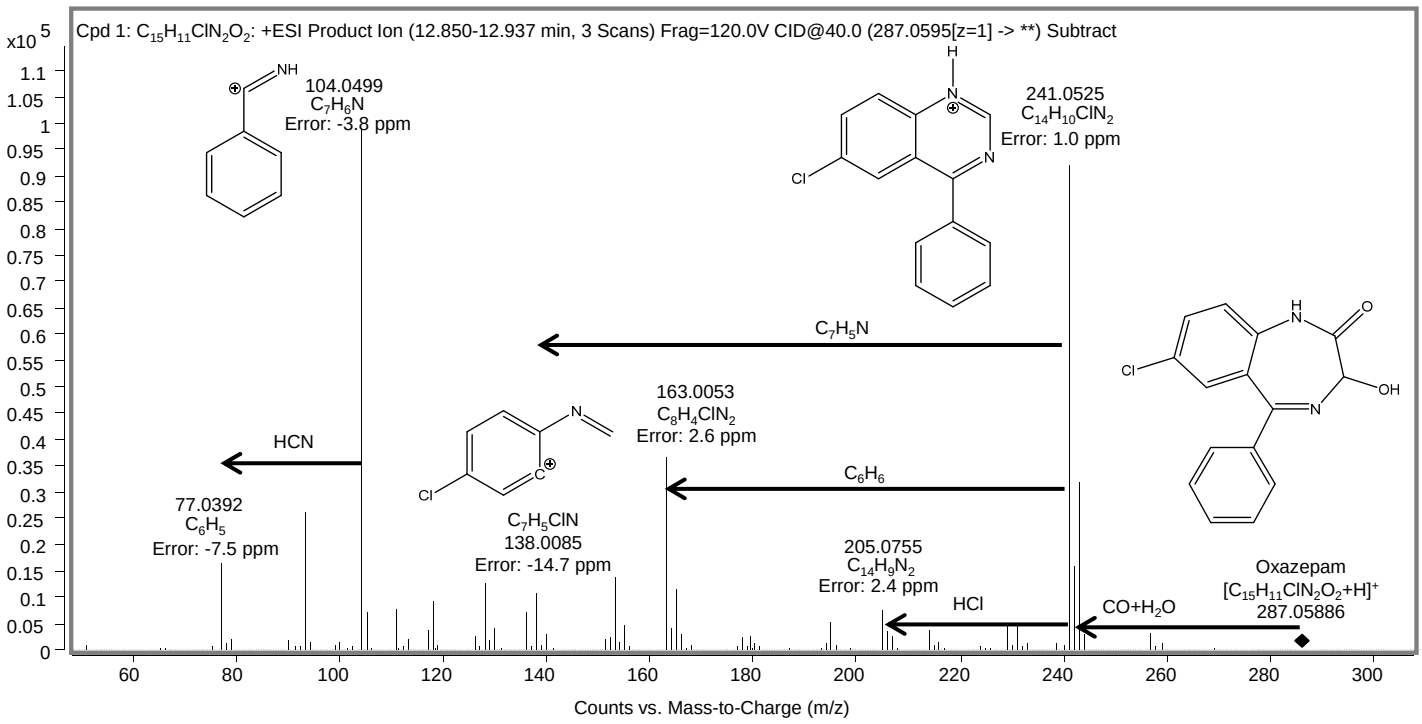


Fig. S5. QTOF product ion spectrum for oxazepam. More details for interpretation of oxazepam MS/MS spectrum are given by Niessen et al. Mass Spectrometry Reviews 30 (2011) 626-663

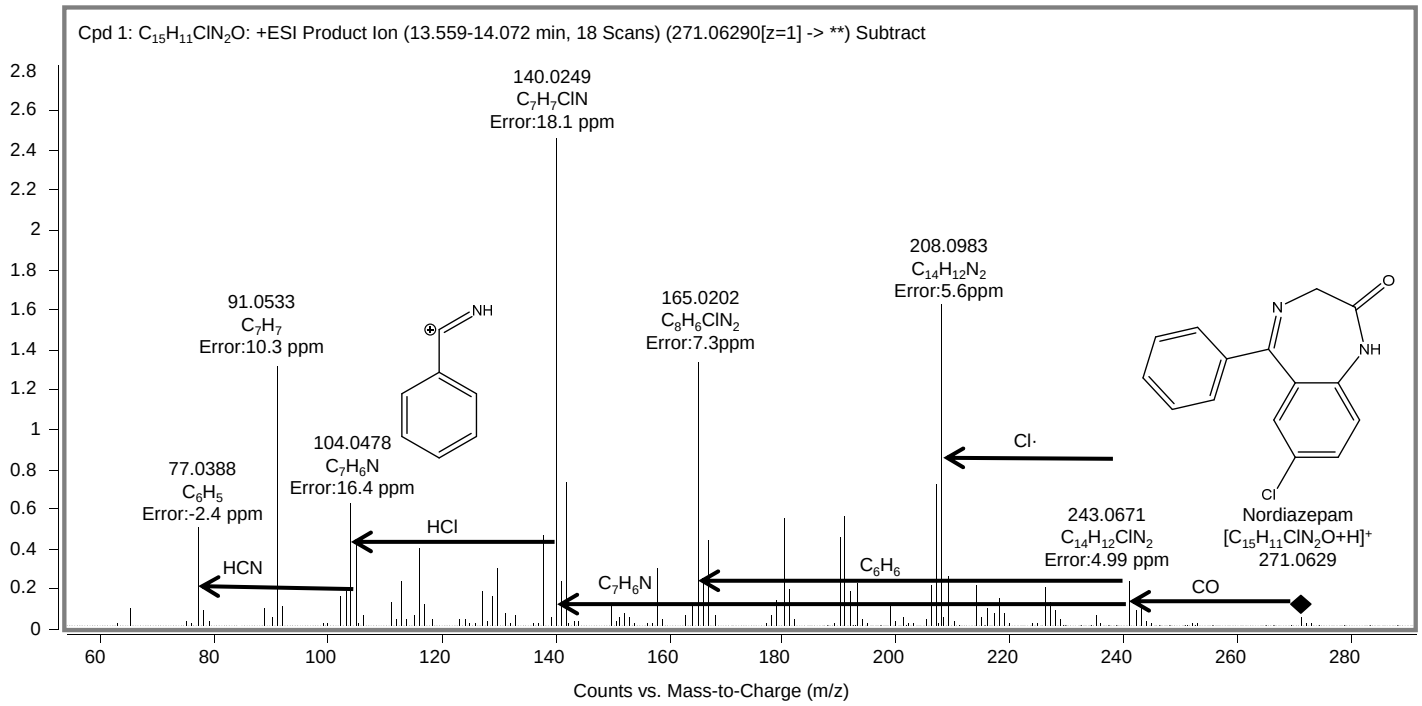


Fig. S6. QTOF product ion spectrum for nordazepam. More details for interpretation of MS/MS spectrum are given by Niessen et al. Mass Spectrometry Reviews 30 (2011) 626-663

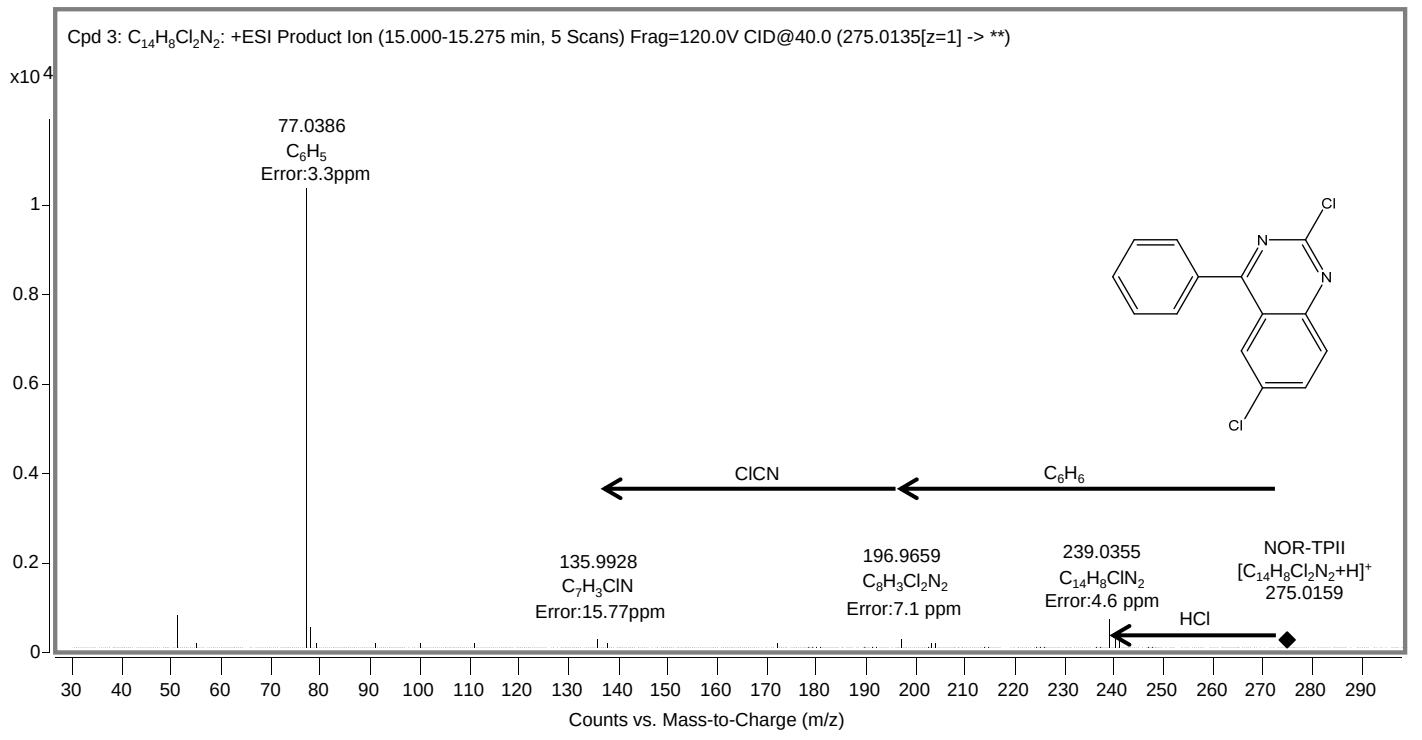


Fig. S7. QTOF product Ion spectrum for NOR-TPII

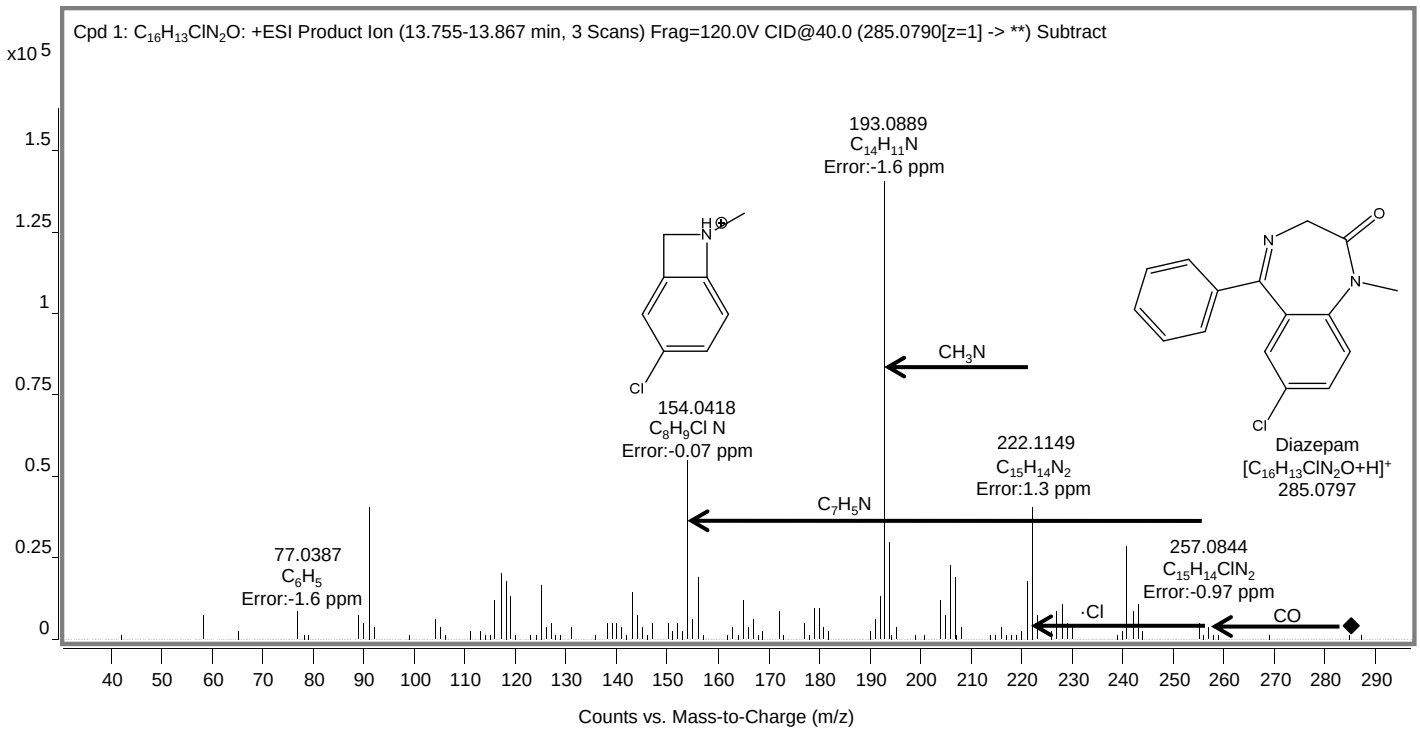


Fig. S8. QTOF product ion MS/MS spectrum for diazepam. More details for interpretation of MS/MS spectrum are given by Niessen et al. Mass Spectrometry Reviews 30 (2011) 626-663

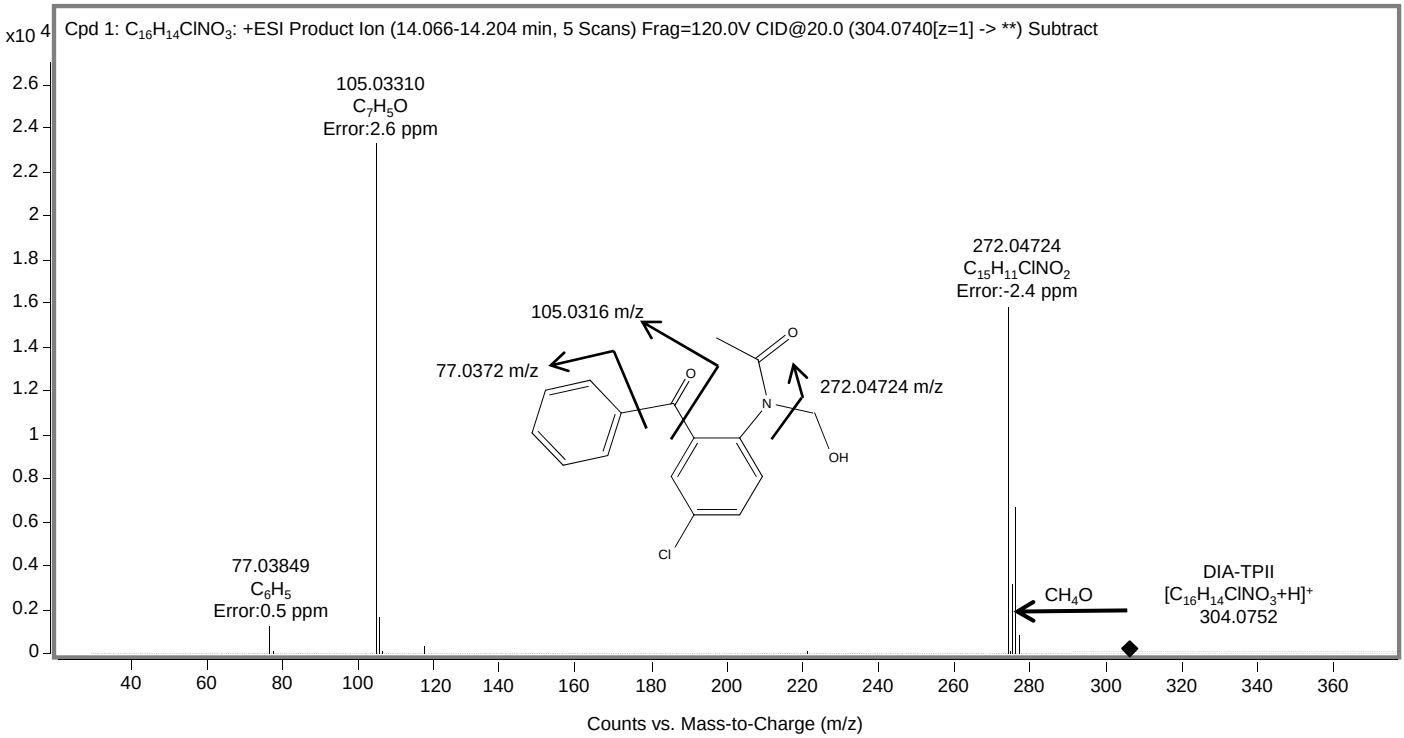


Fig. S9. QTOF Product Ion spectrum for DIA-TPII

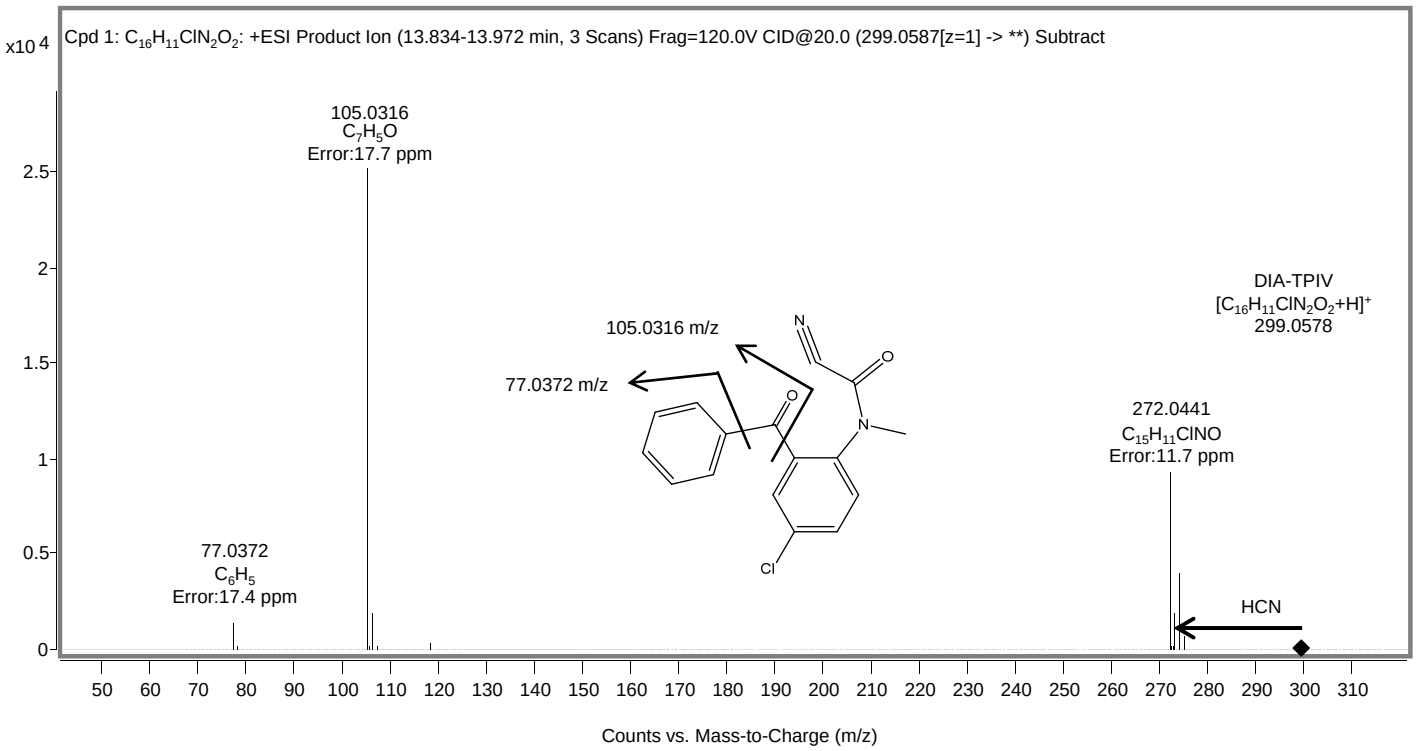


Fig. S10. QTOF Product Ion spectrum for DIA-TPIV

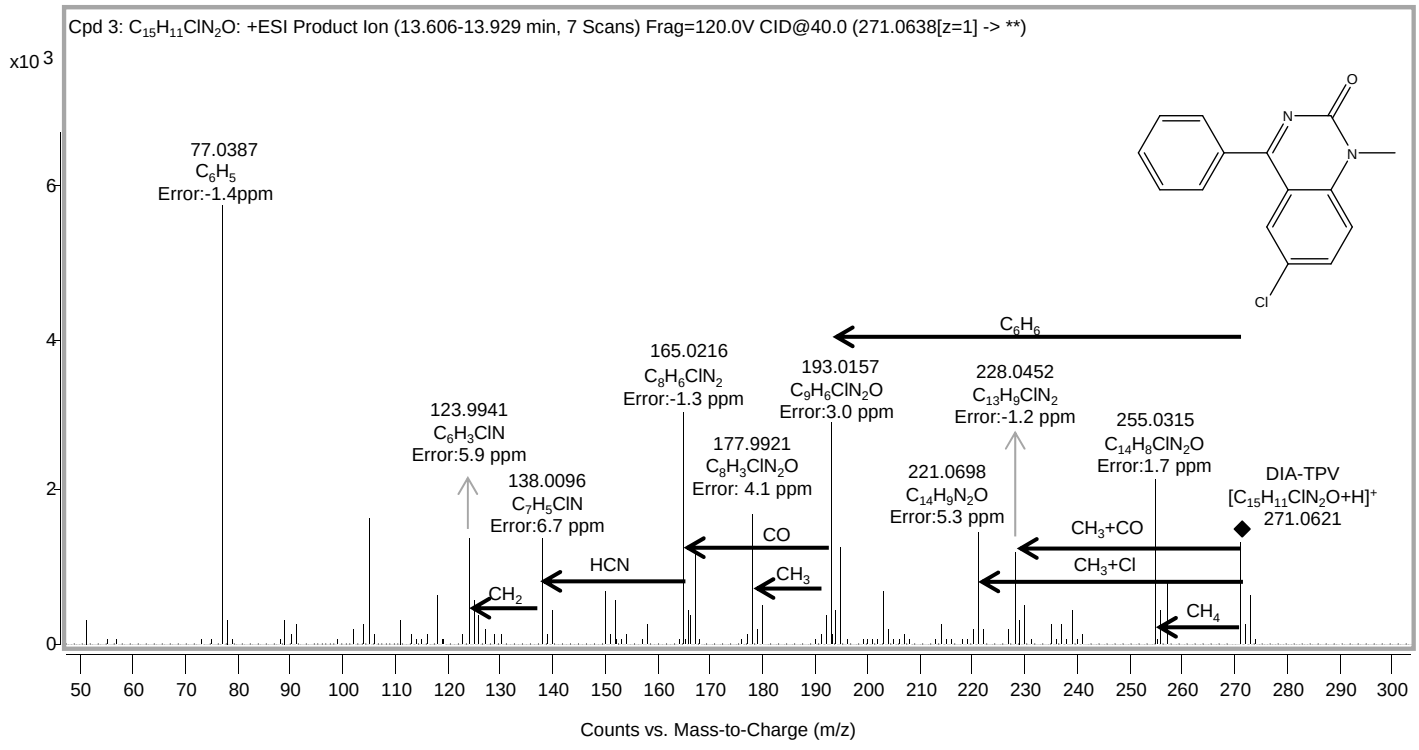


Fig. S11. QTOF Product Ion spectrum for DIA-TPV

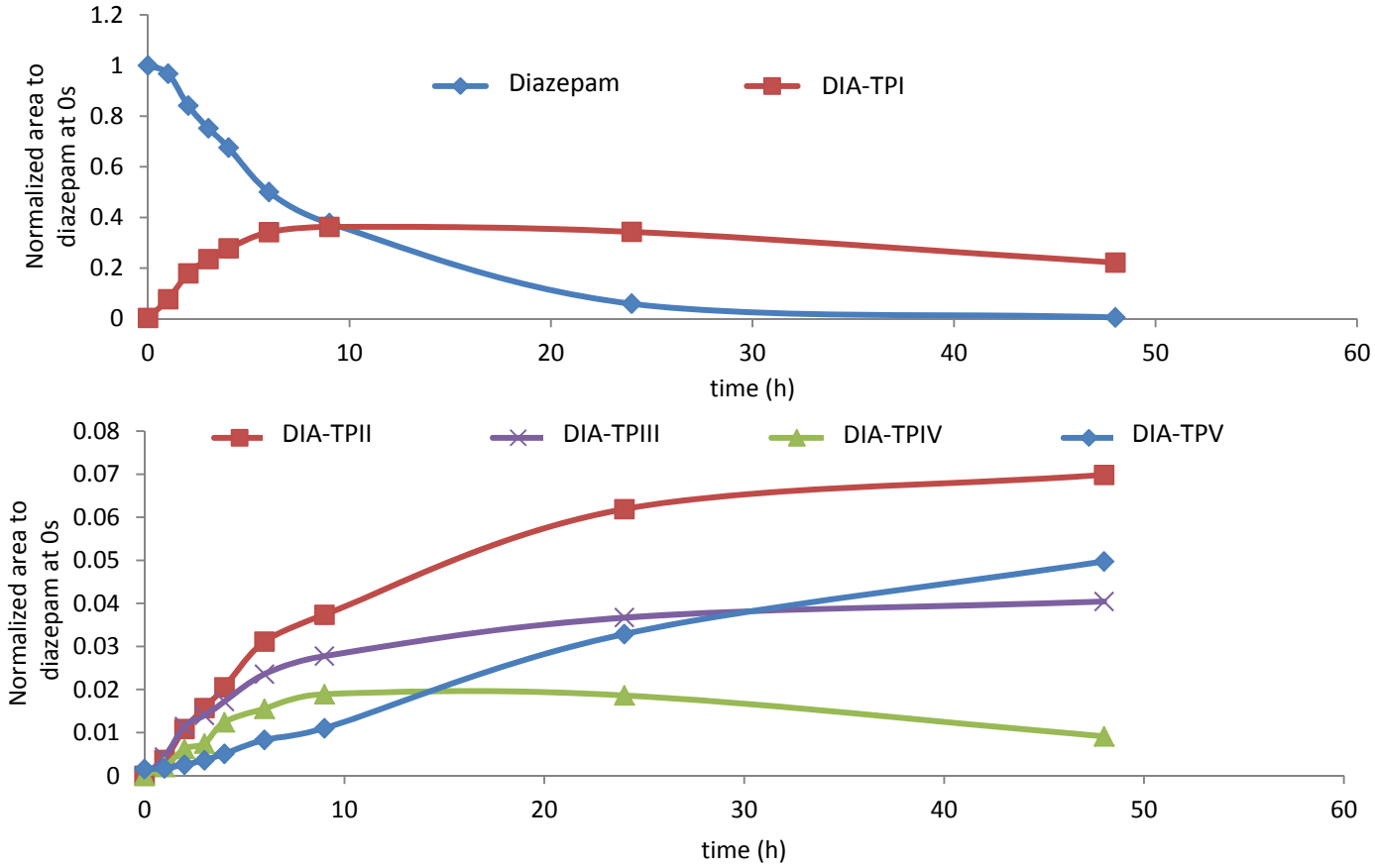


Fig. S12. Time-profile for diazepam reaction and formation of TPs