

1 **Reduction of temazepam to diazepam and lorazepam to delorazepam during enzymatic**
2 **hydrolysis**

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22

1 **Abstract**

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3 It has been previously reported that treatment of urinary oxazepam by commercial β -
4 glucuronidase enzyme preparations, from *Escherichia coli*, *Helix pomatia*, and *Patella*
5 *vulgata*, results in production of nordiazepam (desmethyldiazepam) artefact. In this study, we
6 report that this unusual reductive transformation also occurs in other benzodiazepines with a
7 hydroxyl group at the C3 position such as temazepam and lorazepam. As determined by LC-
8 MS analysis, all three enzyme preparations were found capable of converting urinary
9 temazepam into diazepam following enzymatic incubation and subsequent liquid-liquid
10 extraction procedures. For example, when *H. pomatia* enzymes were used with incubation
11 conditions of 18 h and 50 °C, the percentage conversion, although small, was significant –
12 approximately 1 % (0.59% - 1.54%) in both patient and spiked blank urines. Similarly, using
13 *H. pomatia* enzyme under these incubation conditions, a reductive transformation of urinary
14 lorazepam into delorazepam (chlordesmethyldiazepam) occurred. These findings have both
15 clinical and forensic implications. Detection of diazepam or delorazepam in biological
16 samples following enzyme treatment should be interpreted with care.

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20 **Key Words** β -Glucuronidase · temazepam · diazepam · lorazepam · delorazepam · mass
21 spectrometry

22

1 **Introduction**

2

3 Benzodiazepines are widely prescribed for their sedative, hypnotic, muscle relaxant and
4 anticonvulsant properties. They are also associated with misuse, abuse and are implicated in
5 crimes such as drug-facilitated sexual assault [1-4]. As such, benzodiazepines are among the
6 most frequently encountered substances in clinical and forensic toxicological analyses. There
7 are several factors which contribute to the challenging nature of benzodiazepine analysis and
8 result interpretation. Firstly, there are over 50 different benzodiazepines commercially
9 available for clinical use [5,6]. Secondly, many benzodiazepines form multiple metabolites
10 with varying biological activity, and many metabolites are drug substances in their own right
11 [7]. These can have distinct pharmacological profiles, and are used for specific clinical
12 indications. For example, diazepam, marketed as Valium[®], is one of the most widely used
13 benzodiazepines in the world for the treatment of anxiety, seizure and for preoperative
14 anaesthesia induction. Part of its excretion from the body is metabolism by hepatic enzymes to
15 nordiazepam and temazepam, which are both further metabolised to oxazepam. Consequently,
16 detection of temazepam is often accompanied by the presence of nordiazepam and oxazepam
17 from diazepam ingestion. Temazepam could also be a metabolite of other drugs such as
18 clorazepate, prazepam, and medazepam [8]. Diazepam, on the other hand, could be a
19 metabolite of tetrazepam [9-11]. Finally, benzodiazepines are prone to degradation and
20 chemical conversion. It has been reported that diazepam can be a thermal degradation product
21 of ketazolam [12]. Some of these benzodiazepine transformations are summarised in Scheme
22 I. Acid-catalysed hydrolysis procedures used to de-conjugate benzodiazepine glucuronides
23 during urine drug testing are known to decompose some benzodiazepines into common
24 benzophenones [13-15]. Recently, we have reported that the commonly believed “mild”
25 hydrolysis method employing β -glucuronidase is also a source of artefact production. We

1 found that either oxazepam glucuronide present in patient urine, or oxazepam added to blank
2 urine, could be reduced to nordiazepam (desmethyldiazepam) during treatment of urine
3 specimens by commercial β -glucuronidase enzyme preparations from *Escherichia coli*, *Helix*
4 *pomatia*, and *Patella vulgata*. Formation of the nordiazepam artefact was positively correlated
5 with incubation temperature, incubation time, oxazepam concentration and enzyme
6 concentration [16]. In this study, we report the reductive transformation of temazepam and
7 lorazepam, two benzodiazepines sharing structural similarities with oxazepam, observed
8 during treatment of urine specimens with commercial β -glucuronidase enzyme preparations.

9

10 **Material and methods**

11

12 *Chemicals and urine specimens*

13 Drug standards temazepam, diazepam and lorazepam were purchased from Alltech-Applied
14 Science Labs (State College, PA) and were prepared as 1 mg/mL individual standard solutions
15 in methanol. Internal standard α -hydroxyalprazolam- d_5 (HO-Alp- d_5 , at 0.1 mg/mL in
16 methanol) was supplied from Cerilliant (Austin, TX). *H. pomatia* β -glucuronidase preparation
17 (Type H-3, 99,000 units/mL), *P. vulgata* β -glucuronidase preparation (Type L-II, lyophilized
18 powder, 2,262,000 units/g solid) and *E. coli* β -glucuronidase preparation (Type VII-A,
19 lyophilized powder, 12,495,000 units/g protein) were purchased from Sigma-Aldrich (Castle
20 Hill, NSW, Australia). The enzymes supplied in powder form were prepared at a concentration
21 of 100,000 units/mL by dissolving in 1 M ammonium acetate buffer pH 4.5 for *P. vulgata*
22 enzyme and 1 M sodium phosphate buffer pH 6.8 for *E. coli* enzyme. All enzymes were stored
23 in a refrigerator no longer than 6 months from time of purchase to completion of experiments.
24 Enzyme activities were based on manufacturer's specification and were not further tested.

1 Water was purified by a Milli-Q system obtained from Millipore (Sydney, Australia). All
2 solvents and other chemicals were analytical grade or better.

3
4 Urine samples containing temazepam and lorazepam were obtained from the Toxicology Unit,
5 Pacific Laboratory Medicine Services, Macquarie Hospital, North Ryde, NSW, Australia.
6 These samples were residual specimens from patients prescribed temazepam or lorazepam and
7 were supplied after de-identification. All patient samples had been kept refrigerated by the
8 testing laboratory for 1 to 2 weeks or frozen for 1 to 6 months before use in this study. None of
9 the urine specimens were pooled; they were analysed directly without any treatment e.g.
10 centrifugation after warming to room temperature. Donor information including sex, age, and
11 dose were not available. Bacterial growth in the samples during storage was not checked.
12 Blank urine specimens were obtained from healthy donors. During spiking sample preparation
13 for positive temazepam or lorazepam, 30 μ L of the 1 mg/mL stock standard solution
14 (temazepam or lorazepam) in methanol was added to 1 mL of the blank urine. The sample thus
15 prepared had a concentration of temazepam or lorazepam at 30,000 ng/mL and contained 3%
16 (v/v) methanol.

17
18 ***Enzymatic hydrolysis***

19 Into 1 mL of urine specimens in a 10-mL screw-cap test tube were added 1500 units of β -
20 glucuronidase preparation (15 μ L of the stock enzyme solutions) and 1 mL of 1M ammonium
21 acetate buffer pH 4.5 for *H. pomatia* enzyme and *P. vulgata* enzyme or 1 mL of 1 M sodium
22 phosphate buffer pH 6.8 for *E. coli* enzyme. For quantitative LC-MS analysis, 250 ng of
23 internal standard (50 μ L of 5 μ g/mL HO-Alp-d₅ solution) was added into the specimens. They
24 were then incubated at 50°C for 18 h unless otherwise specified.

25

1 ***Liquid-liquid extraction***

2 A previously published extraction method for benzodiazepines was adapted in the study [16].
3 Briefly, into the above enzyme-treated samples were added 0.5 mL 2 M sodium hydroxide
4 solution and 5 mL dichloromethane/isopropanol (9:1) as extracting solvents. The samples
5 were mixed on a blood roller mixer for 20 min and centrifuged at 3000 rpm for 5 min. After
6 aspirating the top aqueous layers into waste, the bottom organic layers were mixed with
7 approximately 200 mg anhydrous sodium sulphate and then transferred into clean tubes. After
8 evaporation under a gentle stream of nitrogen at 30°C, the dried extracts were reconstituted in
9 100 µL methanol for LC-MS analysis.

10

11 ***LC-MS***

12 LC-MS analyses were carried out on an Agilent 1200 series LC system coupled to an Agilent
13 6460 triple quadrupole MS system with an electrospray ionisation (ESI) source (Agilent
14 Technologies, Forest Hills, VIC, Australia). The system was operated by the MassHunter
15 program. A Gemini C18 column (150 mm x 3.0 -mm i.d., 5-µm particle size, Phenomenex,
16 Lane Cove, NSW, Australia) was maintained at 25 °C. Chromatography was performed by
17 employing an isocratic elution of a mobile phase consisting of 30% water containing 5 mM
18 ammonium acetate and 70% methanol at a flow rate of 0.3 mL/min. The injection volume was
19 1 µL.

20

21 The MS was run in positive electrospray ionisation mode. Nitrogen was used as the drying
22 gas, nebulising gas, and sheath gas. A flow rate of 10 L/min was set for both the drying gas at
23 300 °C and the sheath gas at 350 °C. Nebulizer pressure was at 35 psi. The electrospray ion
24 source capillary voltage was 3500 V and the nozzle voltage was 1000 V.

25

1 For qualitative analysis of lorazepam and its reductive product, the MS was operated in both
2 full scan mode and product ion scan mode with a mass scan range of 100-350 amu. In scan
3 mode, the fragmentor energy used was 150 V. In product ion scan mode, the fragmentor
4 energy used was 120 V for lorazepam and 150 V for the reduction product. For each
5 compound, two collision energy settings were applied (10 V and 25 V).

6
7 For quantitative analysis of diazepam and temazepam, an LC-MS method run in selected
8 reaction monitoring (SRM) mode was developed. The SRM transitions monitored are listed in
9 Table 1. The dwell time was 100 msec for each transition. The retention times for HO-Alp-d₅,
10 temazepam and diazepam were at 4.74 min, 5.84 min and 7.65 min, respectively. A
11 representative SRM chromatogram for temazepam and diazepam (both at 1 ng/mL) is shown
12 in Fig. 1. For positive identification of analytes, the retention time and ion ratios must be
13 within $\pm 2\%$ and $\pm 20\%$, respectively, when compared to those of the calibrating standards.
14 These criteria were not applicable to the identification of delorazepam due to lack of a
15 reference standard.

16
17 ***Quantitative LC-MS method validation***

18 The liquid-liquid extraction method employed in the study yielded recoveries of 79% for
19 temazepam, 86% for diazepam and 92% for HO-Alp-d₅. Calibration of temazepam and
20 diazepam was performed separately by spiking blank urine samples (1 mL) with reference
21 standards. Two calibration ranges were investigated for temazepam. The low concentration
22 calibrators ranged from 1 – 1,000 ng/mL (1, 10, 50, 100, 300, and 500, 1,000 ng/mL). The
23 high concentration calibrators covered a range of 1,000 – 30,000 ng/mL (1,000, 2,000, 5,000,
24 10,000, 20,000, 30,000 ng/mL). Due to the expected low concentrations of diazepam, only a
25 low calibration range (1– 1,000 ng/mL) was deemed necessary. After adding 250 ng HO-Alp-

1 d₅ (internal standard) and 1500 units of β-glucuronidase into each sample, enzymatic
2 hydrolysis and extraction procedures described above were followed. The extract was
3 analysed by the quantitative LC-MS method. Calibration curves were linear for all
4 concentration ranges investigated and for all hydrolysis conditions employed. The correlation
5 coefficient values were greater than 0.99 for all calibration conditions for both temazepam and
6 diazepam. The limit of quantitation (LOQ) was found at 1 ng/mL for both analytes (see the
7 electronic supplementary material for LOQ standards). The LOQ was determined following
8 conventional definitions, that is, acceptable RT (± 2%), ion ratios (± 20%), and quantitative
9 values (± 20% of the nominal values). The limit of detection (LOD) was not determined and
10 the LOQ was used as the LOD in this study.

11
12 To investigate the matrix effect, blank urine (1 mL) was mixed with 1500 units of *H. pomatia*
13 enzyme preparation and incubated at 50 °C for 18 h. The sample was extracted using the
14 liquid-liquid extraction procedures described above and the extract was dissolved in 100 μL of
15 methanol. Reference standards of temazepam, diazepam and HO-Alp-d₅, each at 50 ng, were
16 then added to the 100 μL methanol extract of the urine blank (urine) or to 100 μL methanol
17 (solvent). Analysis of the two samples revealed that there was minimal difference in absolute
18 peak areas of the SRM signals for each compound between the two samples. The same results
19 were obtained on 3 repeat experiments. Average peak areas for urine samples were calculated
20 at 98% for HO-Alp-d₅, 92% for temazepam and 99% for diazepam relative to those of the
21 solvent alone. The data suggested an 8% ion suppression for temazepam and negligible matrix
22 effects for HO-Alp-d₅ and diazepam.

23

24 **Results**

25

1 ***Formation of diazepam during enzyme hydrolysis of urines from patients prescribed***
2 ***temazepam***

3 Five urine samples from patients prescribed temazepam were analysed by the quantitative LC-
4 MS method. These samples were incubated for 18 h at 50 °C with or without the presence of
5 *H. pomatia* enzyme. The results are summarized in Table 2. When enzyme was not present
6 during incubation, there was no detectable diazepam in any of the samples. However, in all the
7 enzyme treated specimens, there were small but measurable amounts of diazepam detected,
8 ranging from 0.59 % to 1.54 % of the total temazepam present. The amount of free temazepam
9 detected in samples without enzyme treatment accounted for less than 5.5% (3.5% - 5.4%) of
10 the total temazepam released after enzyme hydrolysis. Oxazepam and nordiazepam were also
11 measured using the previously published method [16]. The oxazepam concentrations in the
12 enzyme treated samples accounted for 10-22% of temazepam concentrations measured (data
13 not shown). Nordiazepam concentrations accounted for up to 2% of total oxazepam present.
14 The trace amounts of nordiazepam were highly likely a result of reductive transformation of
15 oxazepam during enzyme treatment of urine samples as reported previously [16].

16
17 ***Formation of diazepam from incubation of pure reference temazepam standard with***
18 ***enzymes***

19 To investigate whether or not the above observed reductive transformation of temazepam to
20 diazepam occurs with temazepam as the pure drug substance, a commercial temazepam
21 standard was used for the study. Temazepam at a concentration of 30,000 ng/mL was added to
22 blank urine and incubated with *H. pomatia* enzyme (1500 units/mL) at 50 °C for 0, 2, 5, and
23 18 h following addition of internal standard. Results from LC-MS analyses of the samples are
24 shown in Fig. 2. There was a positive correlation between incubation time and diazepam
25 artefact formation. After incubation at 50 °C for 18 h, diazepam was produced at a

1 concentration of 213 (± 41) ng/mL ($n=3$). There were trace amounts of diazepam (at
2 approximately 25 ng/mL) detectable in the corresponding control experiments in which
3 temazepam (30,000 ng/mL) was incubated under the same conditions without the presence of
4 enzyme. A similar amount of diazepam (21 ± 9 ng/mL) was also noticeable in the control
5 experiment in which temazepam (30,000 ng/mL) was analysed directly without the sample
6 preparation procedures. It was concluded that the temazepam standard contained a trace
7 amount of diazepam as an impurity ($<0.1\%$). However, reductive conversion of temazepam to
8 diazepam during enzyme incubation was evident from the significantly elevated level of
9 diazepam in the enzyme treated samples. The percentage of temazepam converted to diazepam
10 from 18 h incubation at 50 °C was approximately 0.60% after taking into consideration the
11 trace amount of diazepam impurity in the temazepam starting material. When temazepam
12 (30,000 ng/mL) added in blank urine was incubated with β -glucuronidase preparations from *P.*
13 *vulgata* or *E. coli* (1500 units/mL) at 50°C for 18 h ($n=3$), diazepam was detected at a
14 concentration of 174 (± 36) or 51 (± 11) ng/mL, respectively. These results are summarised in
15 Table 3.

16

17 ***Reductive transformation of lorazepam in urine during enzyme treatment***

18 Lorazepam is another benzodiazepine with a hydroxy group at the C3 position in its structure,
19 a feature shared by both oxazepam and temazepam. It is therefore anticipated that lorazepam
20 will undergo the same reductive transformation process during enzyme treatment to yield
21 delorazepam (chlordesmethyldiazepam). Since delorazepam reference standard was not
22 commercially available, its formation in the system was not quantitatively determined.

23

24 Lorazepam (30,000 ng/mL) was added to blank urine and incubated with *H. pomatia* enzyme
25 (1500 units/mL) for 18 h at 50 °C. The extracts were analysed by LC-MS in scan mode. As

1 shown in Fig. 3, the starting material lorazepam (labelled as 1) eluted at 5.40 min (Fig. 3a) and
2 its MS spectrum showed the $[M+H]^+$ ions at m/z 321 for $C_{15}H_{11}N_2O_2Cl_2$ ($^{35}Cl/^{35}Cl$), m/z 323
3 for $C_{15}H_{11}N_2O_2Cl_2$ ($^{35}Cl/^{37}Cl$), and m/z 325 for $C_{15}H_{11}N_2O_2Cl_2$ ($^{37}Cl/^{37}Cl$) (Fig. 3d). The
4 presence of the 2 chlorine atoms in lorazepam was evident from the isotopic profile (9:6:1) of
5 the protonated molecules. The small total ion chromatogram (TIC) peak (labelled as 2) eluting
6 at 6.31 min (Fig. 3a) was identified as the reduction product of lorazepam. MS of the product
7 showed the $[M+H]^+$ ions at m/z 305, m/z 307 and m/z 309, 16 atomic mass units lower than
8 the corresponding protonated molecular ions of lorazepam (Fig. 3e). The characteristic
9 isotopic profile (9:6:1) of the $[M+H]^+$ ions suggests that the product retains the 2 chlorine
10 atoms in its structure. This product could not be found in the other two TIC traces from control
11 specimens in which lorazepam was either incubated without enzyme for 18 h (Fig. 3b) or
12 incubated with enzyme for 0 h (Fig 3c). The substance was not found in the lorazepam
13 reference material as an impurity. The product was tentatively identified as delorazepam
14 (chlordesmethyldiazepam, 7-chloro-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-
15 one) based on careful study of the MS/MS spectra acquired in product ion scan mode.

16
17 The delorazepam-containing extract was re-analysed using LC-MS operated in the product ion
18 scan mode. To verify ion assignment, both the $^{35}Cl/^{35}Cl$ -containing ion at m/z 305 and the
19 $^{35}Cl/^{37}Cl$ -containing ion at m/z 307 were used as the precursor ions. The product ion spectra
20 for delorazepam after applying collision energy of 25 V are shown in Fig. 4a-b. The
21 fragmented ions resulted from the collision induced dissociation (CID) process resembled
22 those from nordiazepam (Fig. 4c-d). The fragmentation pathway depicted in Scheme II could
23 account for the major fragment ions observed in the spectra. Protonated delorazepam
24 dissociates in the gas phase by initially eliminating CO (pathway I) which is a feature shared
25 by all benzodiazepines [17-20]. Subsequent loss of the chlorophenyl group (C_6H_5Cl , pathway

1 III) and the chlorobenzonitrile group ($C_6H_4(CN)Cl$, pathway V) gives fragment ions at m/z
2 165/167 and m/z 140/142, respectively, which are identical to those from nordiazepam. Loss
3 of a chlorine radical via pathway II results in the formation of ions at m/z 242/244. This
4 fragmentation pathway is supported by the observed analogous fragment ion at m/z 208 in the
5 mass spectrum of nordiazepam. A loss of a chlorine radical from the C2' position instead of
6 the C7 position via pathway VI may also be contributing to the formation of the ions at m/z
7 242/244. A neutral loss of HCl via pathway VII leads to the formation of fragment ions at m/z
8 241/243. There is strong evidence to suggest the chlorine lost in this pathway is from the C2'-
9 Cl instead of the C7-Cl. Firstly, an analogous neutral loss of HF (20 amu) was observed in
10 CID of N-desalkylflurazepam in which a fluorine is attached to the C2' position [17].
11 Secondly, if a neutral loss of HCl was also possible from the C7-Cl, a corresponding fragment
12 ion at m/z 207 would have been observed in CID of nordiazepam. As shown in Fig. 4c-d, CID
13 of nordiazepam did not produce a fragment ion at m/z 207 (loss of HCl) but a fragment ion at
14 m/z 208 (loss of Cl) only.

15
16 When the collision energy was lowered to 10 V, the product ion scan of the $[M+H]^+$ ion at m/z
17 305 for delorazepam did not yield any fragments. Under the same experimental conditions, the
18 spectrum of its parent compound lorazepam showed two distinct fragments at m/z 303 and m/z
19 275 (Fig. 5). Their corresponding isotope ions were also noted in the full scan spectrum of
20 lorazepam (Fig. 3d). These ions are formed from the common neutral losses: that of water (18
21 amu) to give m/z 303/305/307, followed by CO (28 amu) to give m/z 275/277/279 (Scheme
22 III). This fragmentation pathway is a feature shared by all OH-bearing benzodiazepines such
23 as temazepam and oxazepam in which a hydroxyl group (-OH) is present at C3 [20]. The data
24 strongly suggests that the C3-OH group in lorazepam is replaced by a C3-H group in
25 delorazepam.

1
2 Since delorazepam is not available in Australia, quantitative determination of its formation in
3 the reaction system was not possible due to the lack of a reference standard. Attempts were
4 also made to determine if delorazepam could be formed from enzyme treatment of patient
5 urine following lorazepam administration. To investigate this possibility two lorazepam-
6 positive patient urine samples were analysed for the presence of delorazepam but neither
7 showed any measurable amounts of delorazepam. The two samples analysed had lorazepam
8 concentrations below 3,000 ng/mL (data not shown). Another four lorazepam positive urine
9 specimens sourced for the study were not further investigated since their lorazepam
10 concentrations were all below 1,000 ng/mL. Failure to detect delorazepam in the two samples
11 discussed above could possibly be explained by the low concentrations of lorazepam present
12 in the urine specimens of patients. It is noted that the recommended therapeutic dose of
13 lorazepam is much lower than that of temazepam and oxazepam and hence urinary
14 concentrations of lorazepam and its metabolites in patients treated with lorazepam are lower
15 than those treated with temazepam and oxazepam [21].

16

17 **Discussion**

18

19 We previously reported that treatment of urinary oxazepam by commercial β -glucuronidase
20 enzyme preparations from *E. coli*, *H. pomatia*, and *P. vulgata* yielded nordiazepam
21 (desmethyldiazepam) [16]. We reported that production of nordiazepam from reduction of
22 oxazepam was highest when *H. pomatia* enzyme was used and when incubation was for 18 h
23 at 50 °C. Those same hydrolysis conditions were employed in the current study. We found that
24 the observed reductive transformation reaction was not limited to oxazepam, but also occurred
25 in other benzodiazepines such as temazepam and lorazepam which have a hydroxy group at

1 the C3 position. A general reaction pathway, as depicted in Scheme IV, summarises the
2 observed unusual reductive transformation reaction associated with enzyme treatment of
3 oxazepam, temazepam and lorazepam. This reductive transformation was not observed with
4 the internal standard HO-Alp-d₅ during β -glucuronidase treatment.

5
6 In the experiment to investigate the occurrence of diazepam in urine specimens from patients
7 prescribed only temazepam, a total of five patient specimens were investigated. As shown in
8 Table 2, free temazepam was found to account for less than 5 % of the total temazepam
9 present in the urine samples after enzymatic de-conjugation. This result is in agreement with
10 current knowledge that temazepam is extensively conjugated in urine. Although the conjugate
11 was not directly monitored in the study, it is likely to be temazepam glucuronide based on the
12 literature [21]. Our results also showed that diazepam was only detectable in the enzyme-
13 treated samples (68 - 319 ng/mL) and not in control samples in which enzyme was absent.
14 Since diazepam does not form a glucuronide conjugate [7], the detection of diazepam in urine
15 samples does not require β -glucuronidase treatment. The data strongly suggests that the
16 diazepam detected in the hydrolysed samples was from reductive transformation of
17 temazepam catalysed by commercial enzyme preparations rather than from any other routes or
18 factors including bacterial growth in stored urine specimens. This was further confirmed by
19 investigating the fate of temazepam after addition to blank urine at 30,000 ng/mL followed by
20 incubation with β -glucuronidase. Although the temazepam standard used for the study was
21 found to contain a trace amount of diazepam (<0.1%) as an impurity, the significantly elevated
22 level of diazepam in enzyme-treated samples (213 ng/mL) relative to the samples without
23 enzyme treatment (33 ng/mL) is a clear indication that temazepam can be reduced to form
24 diazepam as an artefact under these conditions. Our study also revealed different yields of
25 diazepam artefact from the three β -glucuronidase enzyme preparations investigated. When the

1 same units of enzymes were used, *E. coli* enzyme produced the least amount of diazepam,
2 while *H. pomatia* enzyme yielded the most products. To understand the reaction mechanisms,
3 further studies are needed to identify the responsible component(s) in the various β -
4 glucuronidase enzyme preparations.

5
6 When lorazepam, another benzodiazepine containing a hydroxyl group was incubated with the
7 same enzymes, a similar reductive transformation pathway was observed whereby lorazepam
8 was reduced to form delorazepam. Structural elucidation of the artefact was based on both the
9 scan MS spectra and the product ion scan MS/MS spectra from LC-MS analysis. When the
10 methods were applied to two urine samples positive for lorazepam, no delorazepam was
11 found. This may be partly explained by the low concentration of lorazepam present in the
12 samples (less than 3000 ng/mL in both cases).

13
14 Urinary drug testing for benzodiazepines in many laboratories in Australia is performed to
15 Australian/New Zealand Standard AS/NZS 4308 [22]. According to the standard, the
16 confirmatory test cut-off level for reporting positive nordiazepam and diazepam in urine is 200
17 ng/mL. It is clear from our study that enzymatic hydrolysis is a potential source of producing
18 false positive results for diazepam. Depending on the enzymatic hydrolysis conditions applied,
19 diazepam artefact formation could have significant implications for benzodiazepine reporting.
20 This is particularly true when enzyme incubation needs to be conducted at a high temperature
21 e.g. 60 °C and for a longer period of time (16-24 h) to accommodate for the concurrent
22 analysis of other drugs such as codeine in the same urine specimens [23,24]. For example,
23 when *H. pomatia* enzyme was used with incubation conditions of 18 h and 50 °C in our study,
24 a urine sample containing 20,000 ng/mL of total temazepam would have likely returned a false
25 positive result for diazepam if an average of 1% of diazepam artefact formation had been

1 assumed. Although β -glucuronidase from *E. coli* produced the least amounts of diazepam
2 artefact, its use may not be desirable because of its known low efficiency in de-conjugating
3 some glucuronides such as lorazepam glucuronide [25]. Due to variables in enzyme hydrolysis
4 such as enzyme type, source of supply, incubation temperature and incubation time, it is
5 advisable that laboratories perform their own assessment on the extent of diazepam artefact
6 formation under their specific analytical conditions. Delorazepam artefact, on the other hand,
7 is expected to have less impact on the reporting of urinary benzodiazepine test results. Firstly,
8 delorazepam, either as a therapeutic substance in its own right [26] or as a metabolite of
9 cloxazolam [27], is not a routinely monitored benzodiazepine analyte for many drug testing
10 laboratories. Secondly, the normal therapeutic dose range for lorazepam is 1-10 mg (usually 2-
11 3 mg) daily, which is significantly lower than that for temazepam (10-40 mg daily) or
12 oxazepam (30-120 mg daily) [21]. Consequently, the concentration of lorazepam in the urine
13 of patients is low. Artefact formation of delorazepam from lorazepam may become an issue in
14 cases of lorazepam overdose or abuse, which may be common in everyday forensic case work.

15

16 **Conclusions**

17

18 The findings of our studies show that detection of diazepam or delorazepam in biological
19 samples following enzyme treatment should be interpreted with care.

20

21 **Acknowledgement**

22

23 We are indebted to the contribution of Associate Professor Michael Dawson who sadly passed
24 away in December 2010.

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1 **Table 1** SRM transition parameters used in the LC-MS method for quantitative analysis of
 2 temazepam and diazepam

Compound	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision (V)
HO-Alp-d5 (internal standard)	330	302 ^a	150	25
	330	284	150	25
Temazepam	301	283	120	10
	301	255 ^a	120	25
Diazepam	285	257 ^a	150	25
	285	241	150	25

3
 4 ^a used for quantification

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 6

7 **Table 2** Urinary benzodiazepines detected in patients on temazepam^a

Sample	Without Enzyme		With Enzyme		
	Temazepam	Diazepam	Temazepam	Diazepam	% Diazepam ^b
1	815 (±148)	Not detectable	23,100 (±2010)	319 (±36)	1.38 (±0.15)
2	908 (±97)	Not detectable	18,400 (±1390)	182 (±41)	0.98 (±0.18)
3	511 (±99)	Not detectable	12,200 (±1690)	71 (±9)	0.59 (±0.14)
4	426 (±127)	Not detectable	8,990 (±511)	138 (±21)	1.54 (±0.20)
5	357 (±81)	Not detectable	6,630 (±219)	68 (±10)	1.02 (±0.12)

8
 9 ^a Concentrations were expressed in ng/mL and were mean values from 3 independent
 10 determinations with standard deviation (SD) in brackets.

11
 12 ^b % Diazepam represents the ratio of diazepam to temazepam in hydrolysed urine specimens.

13

1 **Table 3** Summary of results from study of added temazepam (30,000 ng/mL) in blank urines

Experiment	β -Glucuronidase enzyme at 1500 units/mL	Hydrolysis condition at 50 °C	Diazepam detected \pm SD (n=3) in ng/mL
1	<i>H. pomatia</i>	0 h	28 \pm 8
2	<i>H. pomatia</i>	2 h	40 \pm 7
3	<i>H. pomatia</i>	5 h	85 \pm 28
4	<i>H. pomatia</i>	18 h	213 \pm 41
5	<i>P. vulgata</i>	18 h	174 \pm 36
6	<i>E. coli</i>	18 h	51 \pm 11
7	<i>No enzyme added</i>	0 h	23 \pm 7
8	<i>No enzyme added</i>	2 h	25 \pm 5
9	<i>No enzyme added</i>	5 h	35 \pm 10
10	<i>No enzyme added</i>	18 h	33 \pm 15
11 ^a	-	-	21 \pm 9

2

3 ^a Temazepam (30,000 ng/mL equivalent) was analysed directly without the sample preparation
 4 procedures, i.e. there was no addition of enzyme, no hydrolysis incubation and no liquid-liquid
 5 extraction.

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7

1 **Figure/Scheme Captions**

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4 **Fig. 1** SRM chromatograms for temazepam and diazepam at LOQ (1 ng/mL). Transitions 301
5 → 255 (a) and 301 → 283 (b) are for temazepam (Rt 5.78 min). Transitions 285 → 241 (c)
6 and 285 → 257 (d) are for diazepam (Rt 7.65 min). All transitions have a signal to noise ratio
7 of >10:1

8

9 **Fig. 2** Formation of diazepam from incubation of temazepam (30,000 ng/mL) at 50 °C with (+
10 Enzyme) or without (- Enzyme) *H. pomatia* enzyme (1500 units/mL). Values were means of 3
11 measurements with SD represented by the error bars.

12

13

14 **Fig. 3** TIC of extracts from lorazepam added to blank urine after incubation with *H. pomatia*
15 enzyme for 18 h (a); incubation with no enzyme for 18 h (b); and incubation with enzyme for
16 0 h (c). The full scan MS spectrum (200 amu to 350 amu only) was shown in d for lorazepam
17 (labelled as 1) and e for the reduction product (labelled as 2). No ions at m/z 305/307/309
18 were found in the corresponding TIC windows in traces b and c.

19

20

21 **Fig. 4** Product ion scan spectra of delorazepam (a, b) with precursor ions at m/z 305/307
22 (fragmentor energy 150 V, collision energy 25 V); and nordiazepam (c, d) with precursor ions
23 at m/z 271/273 (fragmentor energy 170 V and collision energy 25 V). Nordiazepam standard
24 was introduced into the mass spectrometer without passing through a chromatographic
25 column.

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2
3 **Fig. 5** Product ion scan spectra of lorazepam. The precursor ions used were at m/z 321 (**a**,
4 $^{35}\text{Cl}/^{35}\text{Cl}$ containing $[\text{M}+\text{H}]^+$) and m/z 323 (**b**, $^{35}\text{Cl}/^{37}\text{Cl}$ containing $[\text{M}+\text{H}]^+$). The collision
5 energy was at 10 V. Lorazepam standard was introduced into the mass spectrometer without
6 passing through a chromatographic column.

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9 **Scheme I** Structures and transformations of some benzodiazepines (i – thermal degradation; ii
10 – metabolic pathway; iii – treatment of β -glucuronidase enzyme preparation)

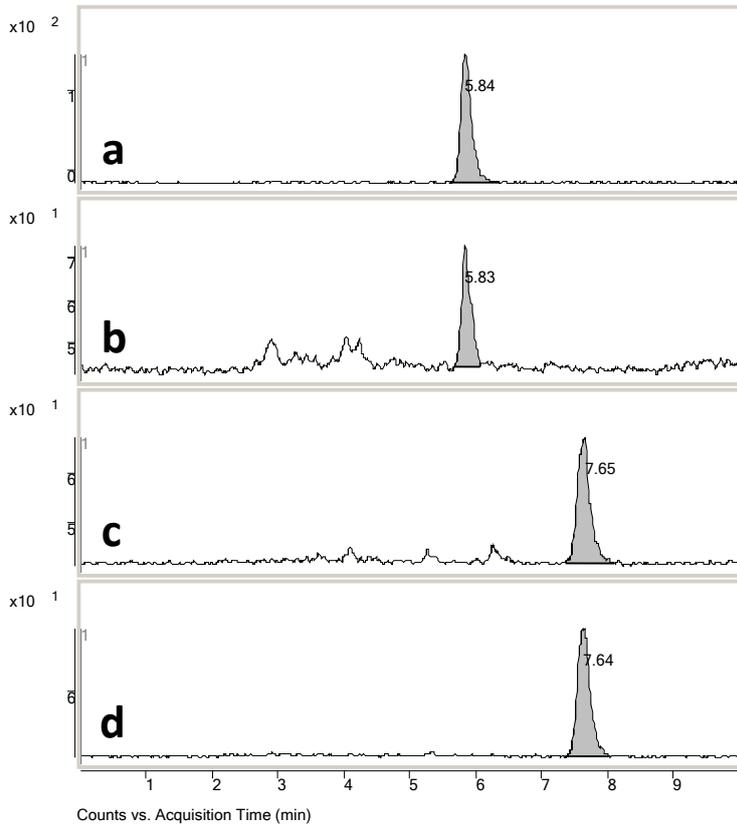
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12 **Scheme II** Proposed fragmentation pathways from CID of delorazepam (DL) and
13 nordiazepam (ND)

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16 **Scheme III** Proposed fragmentation pathways from CID of lorazepam

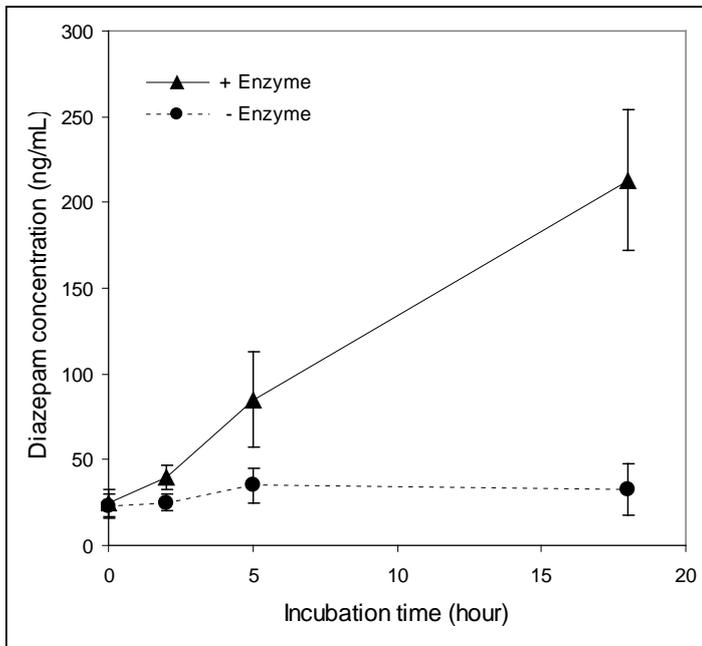
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19 **Scheme IV** Reductive transformation of C3-OH bearing benzodiazepines observed during
20 enzyme hydrolysis employing commercial β -glucuronidase preparations

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1 **Fig. 1**
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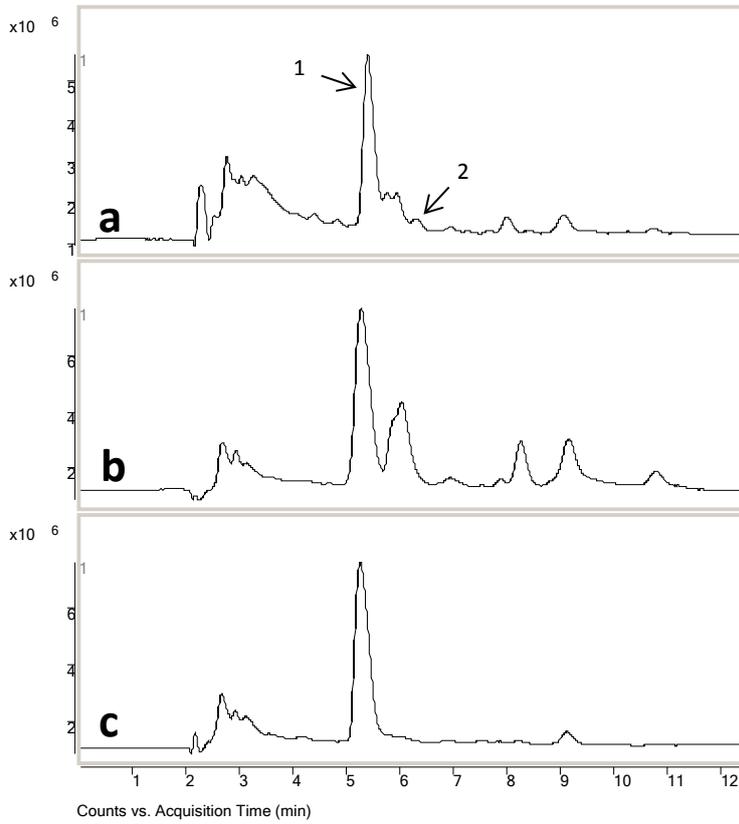


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6 **Fig. 2**
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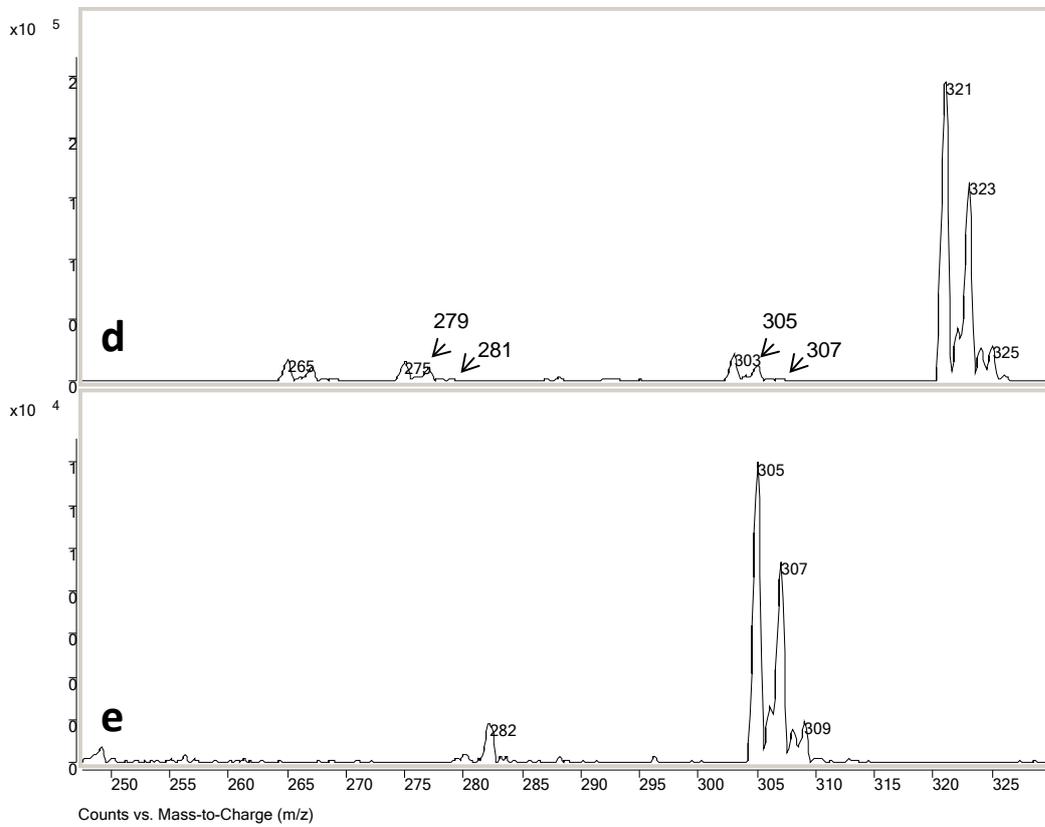


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1 **Fig. 3**
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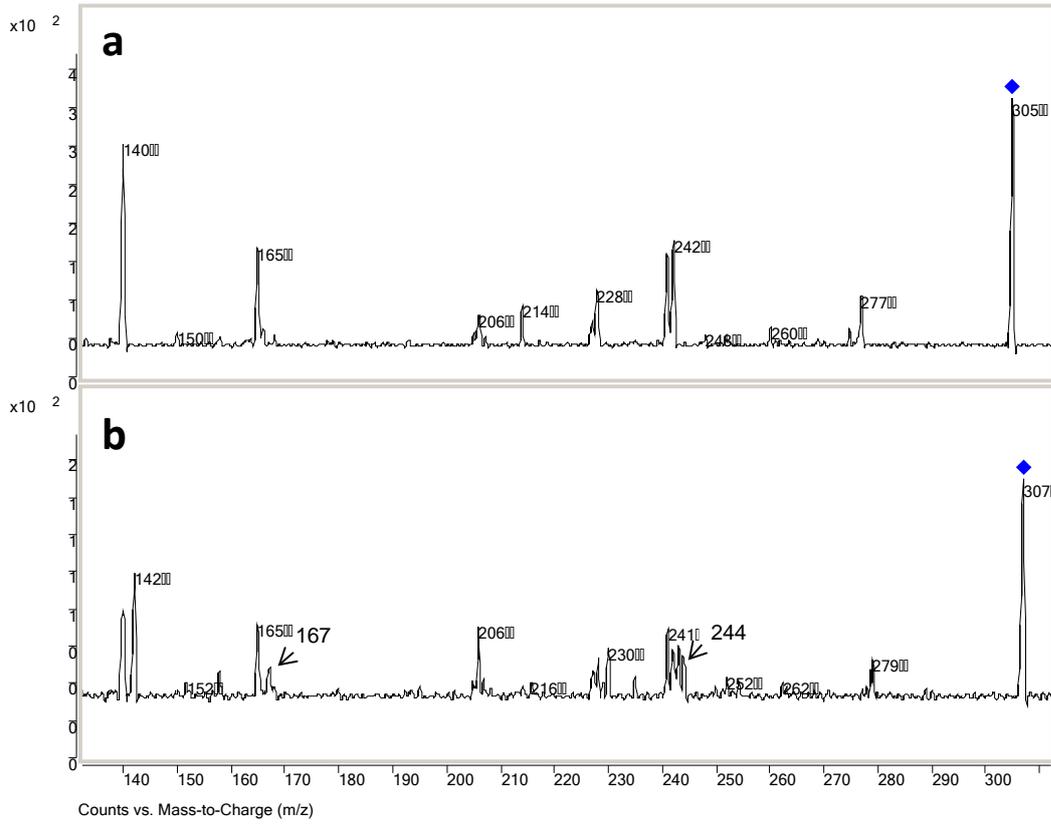


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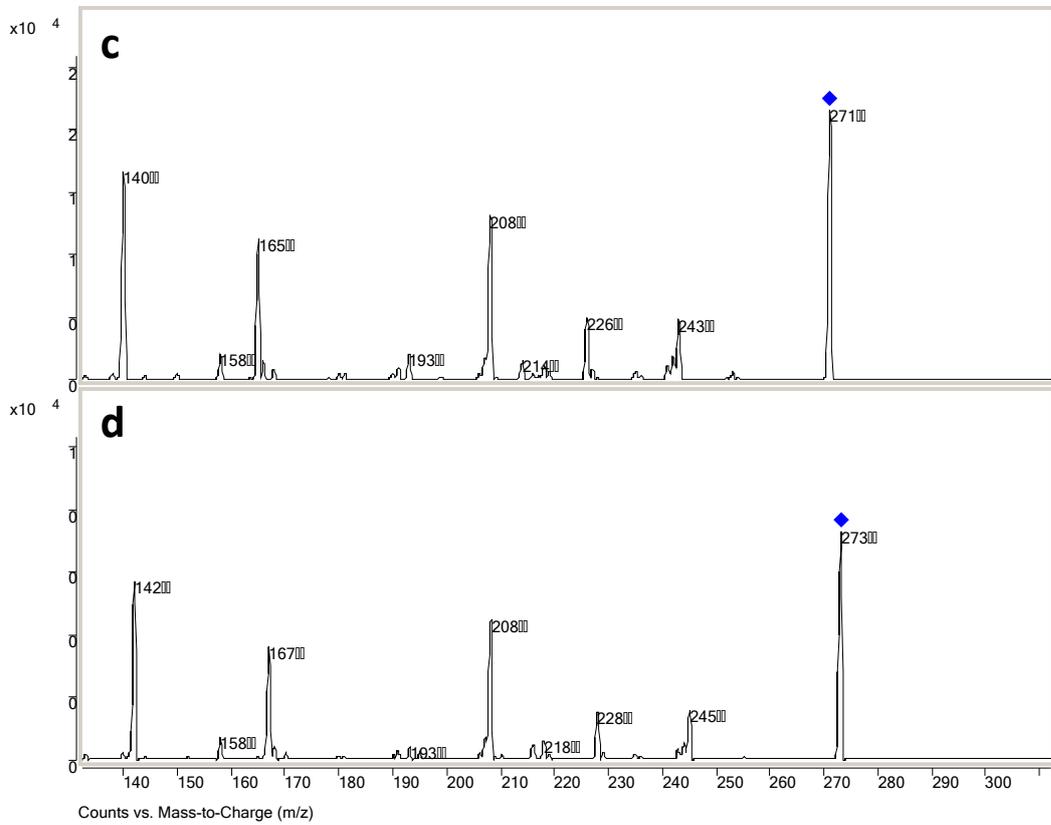


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1 **Fig. 4**
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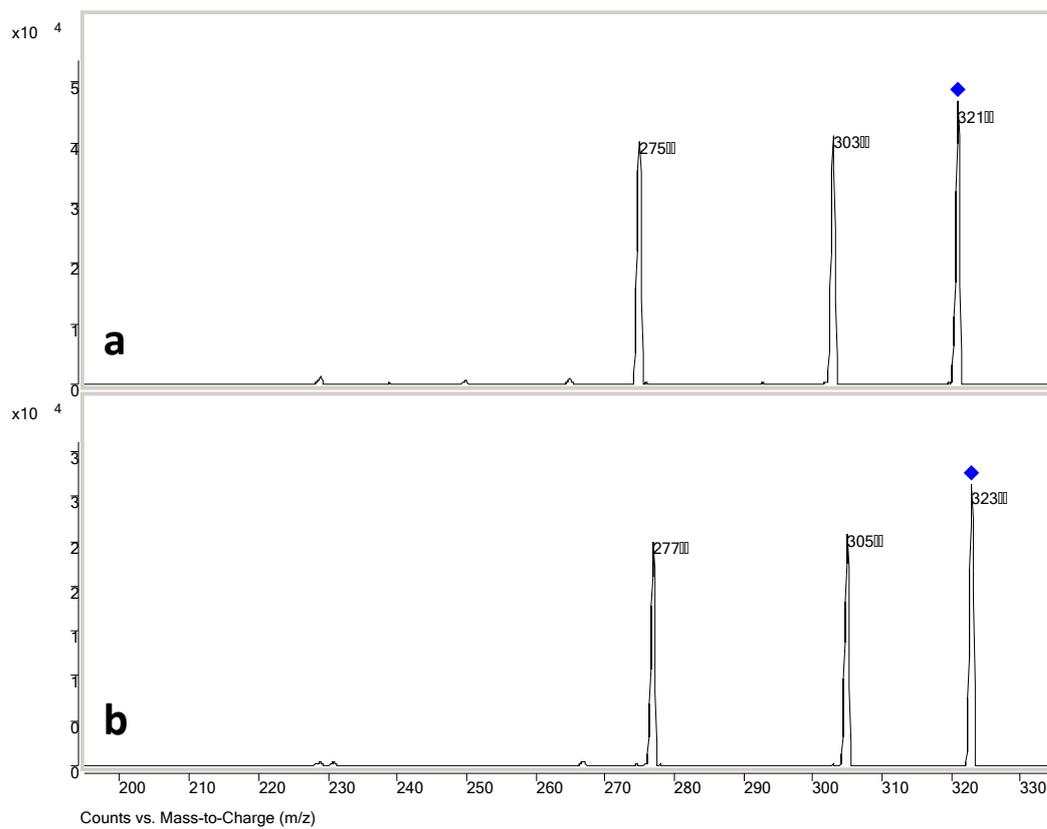


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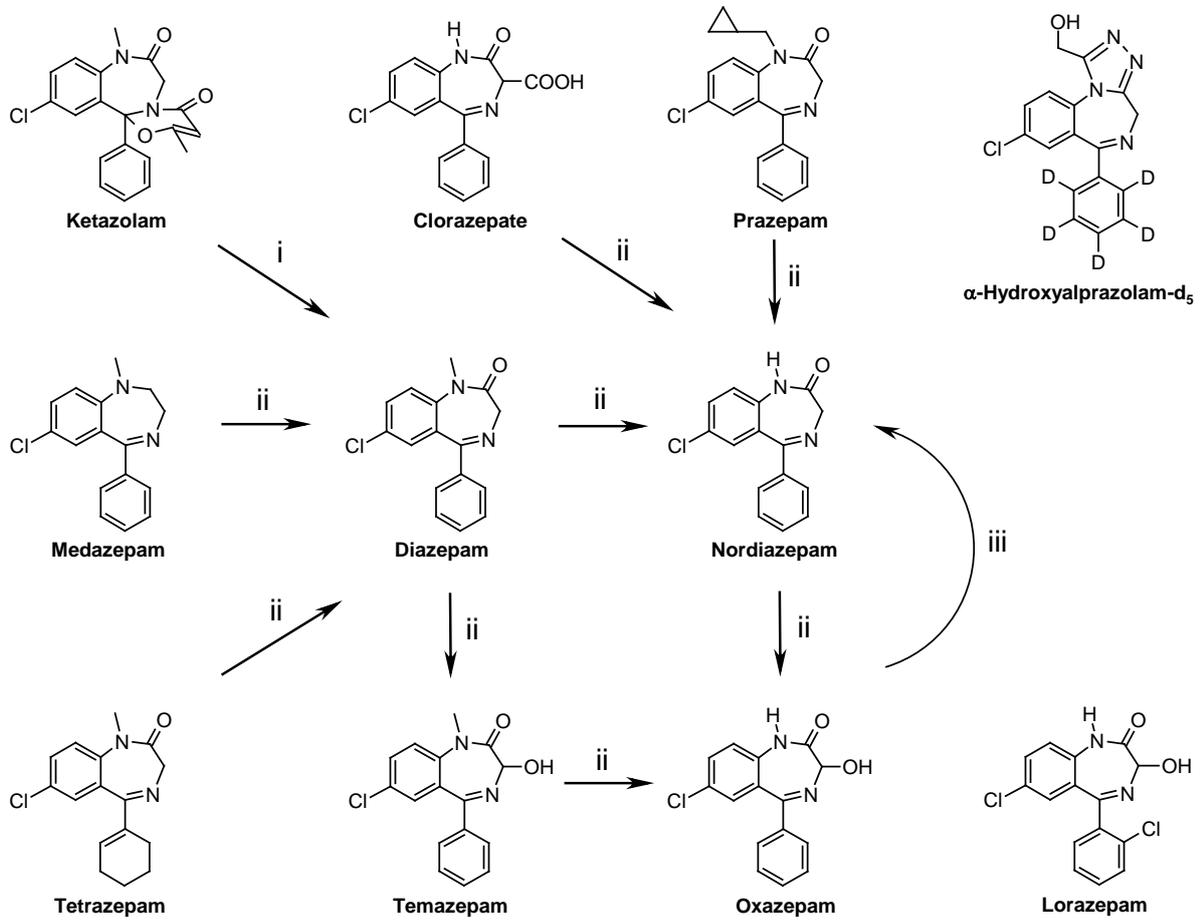
1 **Fig. 5**
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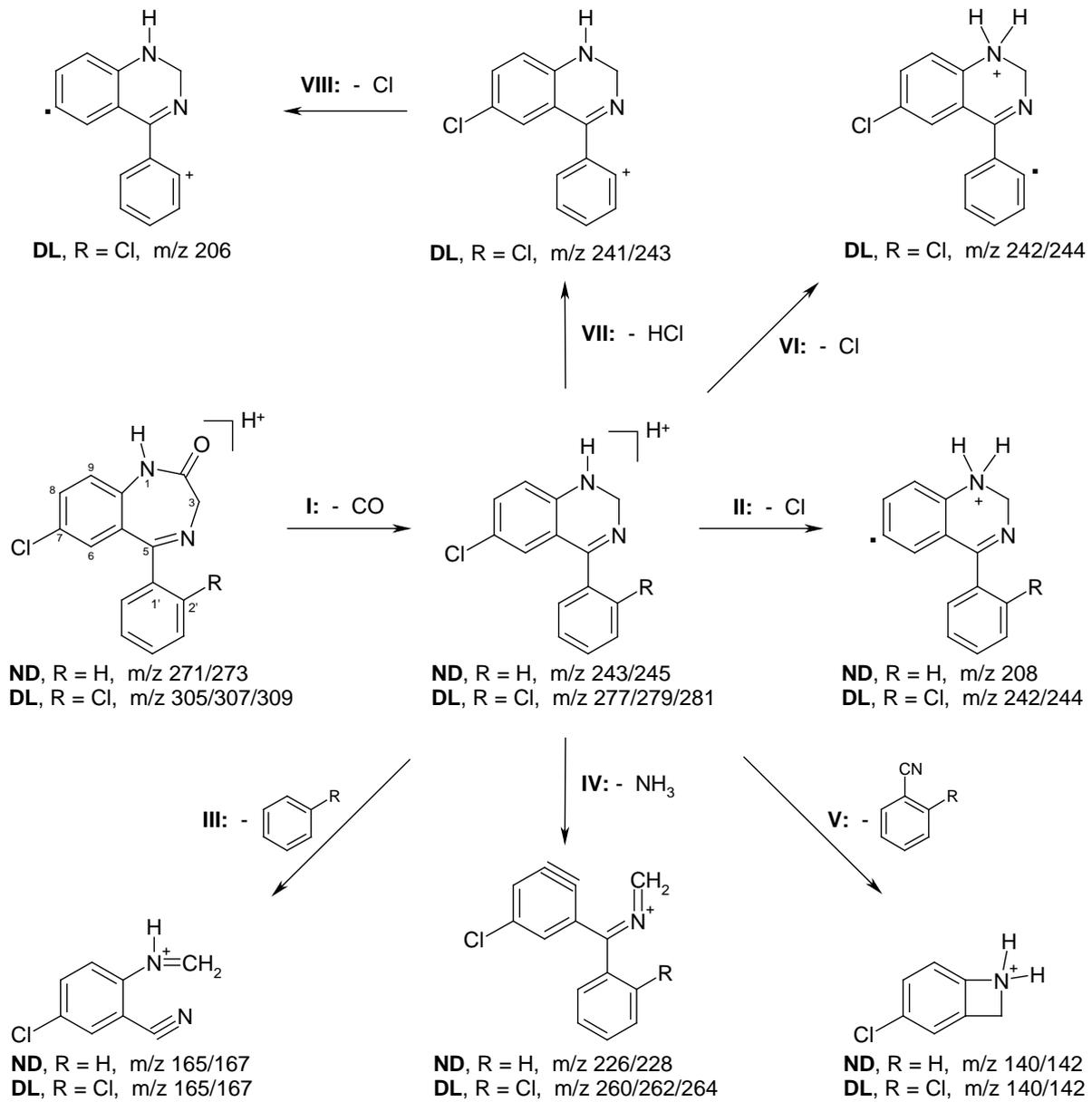
Scheme I



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1 **Scheme II**

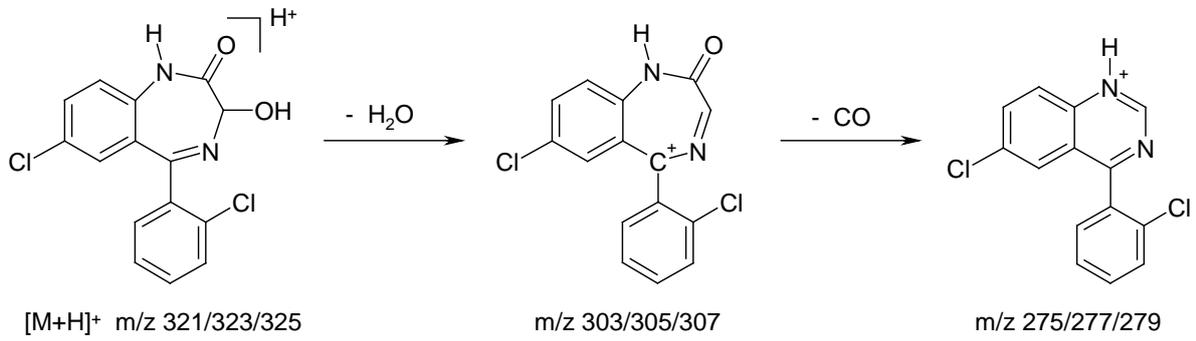
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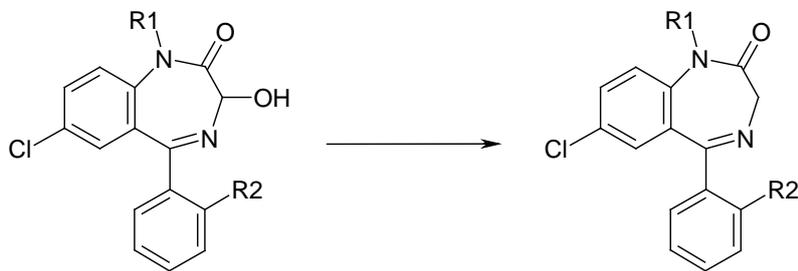
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1 **Scheme III**

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14 **Scheme IV**



Oxazepam R1=H, R2=H
Temazepam R1=CH₃, R2=H
Lorazepam R1=H, R2=Cl

Nordiazepam R1=H, R2=H
Diazepam R1=CH₃, R2=H
Delorazepam R1=H, R2=Cl