A Novel Reductive Transformation of Oxazepam to Nordiazepam Observed during Enzymatic Hydrolysis

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Abstract

β-Glucuronidase is an enzyme often employed to de-conjugate β-glucuronides during urinary drug testing for benzodiazepines. It is commonly accepted that use of β-glucuronidase is a preferred method of hydrolysis over acid-catalysed hydrolysis, which is known to induce benzodiazepine degradation and transformation. Literature to date, however, has not reported any cases of benzodiazepine transformation initiated by commercial β-glucuronidase products. In this study, urine specimens containing either oxazepam or oxazepam glucuronide were incubated with β-glucuronidase enzymes obtained from *Escherichia coli*, *Helix pomatia*, and *Patella vulgata* under various incubation conditions. After liquid-liquid extraction, the extract was analysed by both liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS) for the presence of benzodiazepines. All three enzyme preparations examined were capable of reducing oxazepam or oxazepam glucuronide into nordiazepam (desmethyldiazepam). Nordiazepam formation was positively correlated with incubation temperature, incubation

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time, oxazepam concentration and enzyme concentration. Under all enzymatic hydrolysis conditions investigated, the percentage of nordiazepam formation is <2.5% relative to the amount of oxazepam present in the system. The findings of this study have both clinical and forensic implications and it is clear that the detection of nordiazepam in biological samples subjected to testing involving enzyme-catalysed hydrolysis should be interpreted with care.

Introduction

Benzodiazepines are widely prescribed for their sedative, hypnotic, muscle relaxant and anticonvulsant properties. They are also associated with misuse and abuse and implicated in crimes such as drug-facilitated sexual assault (1,2). Consequently, benzodiazepines are among the most frequently encountered substances in clinical and forensic toxicological analyses. Concentrations of benzodiazepines and their metabolites are highest in urine than in other biological specimens such as blood, oral fluids and hair, making urine one of the preferred matrices for drug analysis. Since the majority of metabolites of benzodiazepines present in urine are in the form of glucuronide conjugates, a hydrolysis step to cleave these conjugates prior to either GC-MS analysis or LC-MS/MS analysis has been adapted by many laboratories involved in urinary drug testing of benzodiazepines. It has been well documented that hydrolysis by β-glucuronidases obtained from sources such as *Escherichia coli (E. coli)*, *Helix pomatia (H. pomatia)*, *Patella vulgata (P. vulgata)* or bovine liver (3,4) is preferred over hydrolysis by acid, as the latter decomposes some benzodiazepines into common benzophenones and thus renders unequivocal identification of target drugs impossible (5-7).

Many benzodiazepine metabolites are also biologically active and these active metabolites are marketed as drug products in their own right. For example, the drug substance in

Serepax® is oxazepam which is a metabolite of diazepam (marketed as Valium®). Detection of oxazepam in a biological fluid may not necessarily be an indication of oxazepam use, as it could be a metabolite of temazepam, diazepam and many other drugs such as clorazepate, prazepam, and medazepam (8). Diazepam, on the other hand, can be a thermal degradation product of ketazolam (9), as well as a metabolite of tetrazepam (10). These observations reveal the challenging nature of interpreting benzodiazepine drug testing results.

In this study, the authors wish to report a novel reduction reaction of oxazepam to nordiazepam (desmethyldiazepam) observed during enzymatic treatment of urine specimens. The findings of this study further highlight the complexity of interpreting drug testing results associated with benzodiazepines.

Material and methods

Chemicals and reagents

Individual ampoules of oxazepam and nordiazepam (at 1 mg/mL in methanol) and α-hydroxy alprazolam-d_5 (HO-Alp-d_5, as internal standard, at 0.1 mg/mL) were supplied from Cerilliant (Austin, TX). Three β-glucuronidase preparations were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia): *E. coli* Type VII-A, lyophilized powder, 12,495,000 units/g protein; *P. vulgata* Type L-II, lyophilized powder, 2,262,000 units/g solid; *H. pomatia* Type H-3, aqueous solution, 99,000 units/mL. Derivatizing reagent bistrimethylsilyltrifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was purchased from United Chemical Technologies (Bristol, PA).Water was purified by a Milli-Q system obtained from Millipore (Sydney, Australia). All solvents and other chemicals were analytical grade or better.

Enzymatic hydrolysis of urine specimen

All enzyme stock solutions were prepared at a concentration of 50,000 units/mL with buffer solutions. *E. coli* β-glucuronidase was dissolved in 1 M sodium phosphate buffer pH 6.8. *P. vulgata* β-glucuronidase was prepared in 1 M ammonium acetate buffer pH 4.5. *H. pomatia* β-glucuronidase was diluted with 1 M ammonium acetate buffer pH 4.5.

A blank urine specimen was obtained from a donor without any medication of benzodiazepines. Positive urine samples for oxazepam were obtained from the Toxicology Unit, Pacific Laboratory Medicine Services, Macquarie Hospital, North Ryde, NSW, Australia. The samples were residual specimens from patients on oxazepam medication and were supplied after de-identification. All patient samples had been kept refrigerated by the testing laboratory for 1 to 2 weeks before they were used in this study.

Into 1mL of the above urine specimens in a 10-mL screw-cap test tube were added 250 ng of HO-Alp-d_5 (internal standard, 50 μL of 5 μg/mL HO-Alp-d_5 solution) and 1500 units of β-glucuronidase (30 μL of 50,000 units/mL enzyme solutions). After addition of 1 mL of 1 M sodium phosphate buffer pH 6.8 for *E. coli* enzyme, or 1M ammonium acetate buffer pH 4.5 for *H. pomatia* enzyme and *P. vulgata* enzyme, the samples were incubated under the specified conditions.

To assess the effect of β-glucuronidase on reduction of oxazepam (as free base), the following experimental procedures were followed. Into 1mL of blank urine in a 10-mL screw-cap test tube were added 250 ng of HO-Alp-d_5, 100,000 ng of oxazepam (100 μL of 1mg/mL stock solution) and 1500 units of β-glucuronidase unless otherwise specified. After

addition of 1 mL of 1 M sodium phosphate buffer pH 6.8 for *E. coli* enzyme, or 1M ammonium acetate buffer pH 4.5 for *H. pomatia* enzyme and *P. vulgata* enzyme, the samples were incubated under the specified conditions.

Liquid-liquid extraction

Into the above enzyme-treated samples were added 0.5 mL 2 M sodium hydroxide solution and 5 mL extracting solvent dichloromethane/isopropanol (9:1). The samples were mixed on a vortex mixer for 2 min and centrifuged at 3000 rpm for 10 min. After aspirating the top aqueous layer into waste, the bottom organic layer was treated with anhydrous sodium sulphate. The organic layer was transferred into a clean set of tubes and dried under a gentle stream of nitrogen at 30°C. The dried extract was then reconstituted in 200 µL of acetonitrile for both GC-MS and LC-MS/MS analyses.

GC-MS

Trimethylsilyl (TMS) derivatives of benzodiazepines were prepared for GC-MS analysis. Aliquots of 50 µL of the above acetonitrile extract were transferred into a high recovery MS vial (1.5mL, Agilent Technologies, Melbourne, Australia). After addition of 50 µL of BSTFA with 1% TMCS into each sample, the vial was capped and heated at 75ºC for 20 min. A previously published GC-MS method (11) was modified and adapted in this study. Briefly, GC-MS analysis was carried out on an Agilent 6890/5973 GC-MS equipped with an autoinjector and a HP-5MS 5% phenyl methyl siloxane cross-linked capillary GC column (30 m × 0.25-mm i.d., 0.25-µm film thickness, Agilent Technologies, Forest Hill, Australia). Injections (2 µL) were made in splitless mode with helium as a carrier gas at a constant flow of 1 mL/min. The inlet temperature was set to 240°C and the transfer line to 280°C. The oven temperature was maintained at 160°C for 1 min and then programmed at 30°C/min to a final

temperature of 325°C, which was held for 5 min. The MS was operated in selective ion monitoring (SIM) mode. The ions monitored included m/z 341, 342, 327 for nordiazepam (RT 5.04 min), 429, 341, 313 for oxazepam (RT 5.52 min), and 386, 401, 351 for HO-Alp-d_5 (RT 9.16 min). The first ion for each compound was used as the quantifying ion, and others were used as the qualifying ions. For positive identification of analytes, the retention time and ion ratios must be within ±2% and ±20%, respectively, when compared to those of the calibrating standards.

LC-MS/MS

Aliquots of the above acetonitrile extract (50 μL) were mixed with an equal volume of 5 mM ammonium acetate aqueous solution for LC-MS/MS study. Analyses were performed on a PerkinElmer SCIEX API365 LC-MS/MS instrument equipped with a Turbo IonSpray source, a PerkinElmer Series 200 autosampler, a micro PerkinElmer series 200 LC pump, and an on-line vacuum degasser. The Alltima C18 column (150 mm x 2.1-mm i.d., 5-μm particle size, Grace Davison Discovery Sciences, Sydney, Australia) was maintained at 28ºC. The mobile phase consisted of 25% water containing 5 mM ammonium acetate and 75% methanol. The flow rate was 0.5 mL/min and the injection volume was 5 μL.

The MS was run in selected reaction monitoring (SRM) mode using the Sample Control 1.4 program. Nitrogen was used as the nebulizing gas of the Turbo IonSpray, curtain gas and collision gas. A flow rate of 1.25 L/min was set for both the nebulizing gas and the curtain gas. The electrospray ion source voltage was +5000 V, the source temperature was 350°C, and the orifice voltage was +46 V. The ring voltage was +210 V, the Q0 energy was -10 V, and the RO2 energy was -48 V in order to give collision energy of 38 V. The SRM transitions monitored were m/z 271.0 (Q1) → 140.0 (Q3), m/z 271.0 (Q1) → 165.0 (Q3) for

nordiazepam; m/z 287.0 (Q1) → 241.0 (Q3), m/z 287.0 (Q1) → 269 (Q3) for oxazepam; and m/z 330.0 (Q1) → 302.0 (Q3) for HO-Alp-d_5. The first transition for each compound was used for quantitation. The dwell time for each transition was 400 msec. The retention times for HO-Alp-d_5, oxazepam and nordiazepam were at 2.51 min, 2.78 min and 3.52 min, respectively. For positive identification of analytes, the retention time and ion ratios must be within ±2% and ±20%, respectively, when compared to those of the calibrating standards.

Method validation

The liquid-liquid extraction method employed in this study yielded >95% recovery of the benzodiazepines when tested on pure standards (500 ng/mL in urine) without undergoing enzymatic hydrolysis procedures. However, the recoveries varied significantly when an enzymatic hydrolysis step was included. For example, when the benzodiazepines were incubated with *H. pomatia* enzyme (pH 4.5) at 50°C for 18h, the recoveries were found at 55%, 88% and 93% for oxazepam, nordiazepam and HO-Alp-d_5, respectively. The recoveries become 85%, 90%, and 94% when they were incubated with *E. coli* enzyme (pH 6.8) at 22°C for 24h.

A four-point calibration was performed at concentrations of 50, 100, 300, and 500 ng/mL benzodiazepines for both GC-MS and LC-MS/MS methods. Into 1 mL of blank urine was added 5, 10, 30 and 50 μL of 10 μg/mL mixed benzodiazepine standards containing oxazepam and nordiazepam to give a final concentration of 50, 100, 300, and 500 ng/mL benzodiazepines, correspondingly. After adding 250 ng HO-Alp-d_5 (internal standard) and 1500 units of β-glucuronidase into each sample, enzymatic hydrolysis and extraction procedures described above were followed. To determine the limit of detection (LOD) and the linearity range, a series of standards with concentrations both below the lowest calibrator

(50 ng/mL) and above the highest calibrator (500 ng/mL) were prepared and analysed together with the calibration standards. For both oxazepam and nordiazepam, the limit of detection (LOD) was at 2 ng/mL in the GC-MS method and 5 ng/mL in the LC-MS/MS method. The lower limit of quantitation (LLOQ) was found at 5 ng/mL in the GC-MS method and 10 ng/mL in the LC-MS/MS method. The upper limit of quantitation (ULOQ) was found at 5,000 ng/mL in both the GC-MS and the LC-MS/MS methods. The LLOQ and ULOQ were determined following conventional definitions, that is, acceptable retention times (± 2%), ion ratios (± 20%) and quantitative values (± 20% of the nominal values). The LOD was subject to all criteria except the ± 20% quantitative adherence. Therefore the linearity range determined was 5 ng/mL to 5,000 ng/mL for the GC-MS method and 10 ng/mL to 5,000 ng/mL for the LC-MS/MS method. In cases where oxazepam concentrations were above the ULOQ, the samples were re-analysed after appropriate dilution with blank urine so that the concentrations were within the linearity range.

Due to the different recoveries of the three benzodiazepines under different incubation conditions observed above, a 4 point calibration curve was needed for each incubation condition investigated in the study. Calibration curves were linear with correlation coefficient values ranging from 0.9940 to 0.9999 for all hydrolysis conditions employed and for both GC-MS and LC-MS/MS methods. Two quality control (QC) specimens were also included whenever a calibration curve was constructed in the study. The first QC sample contained 200 ng/mL of nordiazepam and oxezapam. The second QC sample contained 2,000 ng/mL of nordiazepam and oxazepam. The QC samples were prepared by adding appropriate amounts of nordiazepam and oxazepam standards into blank urine. All QC results were within ±20% of the expected values for each enzymatic hydrolysis condition investigated.

To investigate the matrix effect, benzodiazepine standards including oxazepam, nordiazepam and HO-Alp-d_5 (250 ng each) were added to 200 μL acetonitrile (solvent) or to 200 μL acetonitrile extract of a urine blank (urine). Analyses of the two samples revealed that there was no noticeable difference in absolute peak areas of the SRM signals for each compound between the two samples. The same results were obtained on 3 repeat experiments. Peak areas for urine samples were calculated at 92% for nordiazepam, 95% for oxazepam and 99% for HO-Alp-d_5 relative to those of the solvent alone. These results indicated that there was no obvious matrix effect associated with the LC-MS/MS method developed in this study.

Results

Formation of nordiazepam during β-glucuronidase catalysed hydrolysis of oxazepam glucuronide in patient urines

A urine sample from a patient on oxazepam medication was analysed for the presence of benzodiazepines by using both GC-MS and LC-MS/MS methods. Urine specimens were hydrolysed with 1500 units of β-glucuronidase (+Enzyme) from 3 different preparations, i.e. *E. coli*, *H. pomatia*, and *P. vulgata*. The samples were incubated at 22°C for 24 h, 37°C for 24 h, and 60°C for 2 h for each enzyme studied. To assess the amount of free benzodiazepines in the specimens, enzyme-free specimens (-Enzyme) were included in the study for each incubation condition and were analysed in the same manner. The results are summarized in Table I. Total oxazepam in the sample after enzymatic hydrolysis (Oxazepam+Enzyme) was determined at around 200,000 ng/mL. Free oxazepam in the sample was around 2,500 ng/mL judged from the values obtained when no enzyme was introduced into the specimen (Oxazepam-Enzyme). The nordiazepam concentration averaged 20 ng/mL (14-26 ng/mL) when no enzyme was present in the hydrolysis step. Its

concentration was elevated significantly in samples where enzyme was added. For instance, when the samples were incubated with *H. pomatia* β-glucuronidase at 22°C for 24 h, 37°C for 24 h and 60°C for 2 h, the concentrations of nordiazepam were detected at 66 ng/mL, 854 ng/mL, and 550 ng/mL, respectively, when measured by LC-MS/MS. The identity of nordiazepam found in these enzyme treated specimens was also confirmed by GC-MS analysis in SCAN mode of the extracted samples. Its full scan (100-300amu) spectrum matched very well with that of nordiazepam reference standard (Figure 1). The identification of nordiazepam was further confirmed by co-eluting of added nordiazepam to the extracted positive samples (Figure1, TIC trace C).

To assess the effect of enzyme treatment on the formation of nordiazepam from oxazepam, more urine specimens of oxazepam users were collected and analysed. Three enzymatic hydrolysis conditions i.e. 22°C for 24 h with *E. coli,* 60°C for 2 h with *H. pomatia,* and 50°C for 18 h with *H. pomatia* were investigated in this experiment. The nordiazepam detected relative to the oxazepam present in the same sample under these hydrolysis conditions was in the range of 0.02%-0.03%, 0.20-0.41%, and 0.85%-2.50%, correspondingly. The individual results were summarized in Table II.

Formation of nordiazepam from incubation of oxazepam (free base) with β-glucuronidase enzymes

Oxazepam (as free base) at 100,000 ng/mL was incubated with *E. coli*, *P. vulgata*, and *H. pomatia* β-glucuronidase (1000 units/mL) at 50°C for 18 h. LC-MS/MS analyses of the samples showed that nordiazepam was produced at concentrations of 78 (±11), 354 (±38), and 876 (±82) ng/mL, respectively. The values were means from three experiments with standard deviation in brackets. *H. pomatia* produced the highest concentration of

nordiazepam, while *E. coli* yielded the least. GC-MS analyses gave very similar results (data not shown). No nordiazepam was detectable in the control experiments in which oxazepam (100,000 ng/mL) was incubated under the same conditions without the presence of enzymes for each enzymatic condition (LOD for nordiazepam 2 ng/mL in the GC-MS method and 5 ng/mL in the LC-MS method).

In a separate experiment, oxazepam standard (250,000 ng/mL) was prepared in acetonitrile and was checked for the presence of nordiazepam impurity by both the GC-MS and the LC-MS/MS methods without any enzymatic hydrolysis and extraction process. No nordiazepam was detectable using both methods, indicating that the oxazepam standard sourced from Cerilliant was free from nordiazepam contaminants.

Incubation temperature effect

Oxazepam at 100,000 ng/mL was incubated with *H. pomatia* β-glucuronidase (1500 units/mL) for 18 h at 22°C, 37°C and 50°C. LC-MS/MS analyses of the samples showed that nordiazepam formation was positively correlated with the incubation temperature (Figure 2). When the incubation temperature was at 22°C, only 10 ng/mL nordiazepam was produced. Nordiazepam production was increased to 475 ng/mL and 1450 ng/mL when the incubation temperature was at 37°C and 50°C, respectively. When enzyme was omitted from the incubation mixture, no nordiazepam was detectable for each incubation temperature studied. The negative results were collectively represented by the "No enzyme" condition shown in Figure 2.

Incubation time effect

Oxazepam at 100,000 ng/mL was incubated with *H. pomatia* β-glucuronidase (1500 units/mL) at 50°C for 0, 1, 2 and 5 h. LC-MS/MS analyses of the samples showed that nordiazepam formation was positively correlated with the incubation time (Figure 3). The concentrations of nordiazepam formed from the incubation mixture were 17, 67, and 375 ng/mL, which corresponded to 1, 2, and 5 h of incubation, respectively. No nordiazepam was detectable at 0 h incubation time point.

Enzyme concentration effect

Oxazepam at 100,000 ng/mL was incubated with various concentrations of *H. pomatia* β-glucuronidase at 50°C for 18 h. LC-MS/MS analyses of the samples showed that nordiazepam formation was positively correlated with the enzyme concentration. Nordiazepam was produced from the incubation mixture at 253 (±18), 1444 (±89), and 1874 (±113) ng/mL, when 250, 1500, and 2500 units/mL enzymes were used correspondingly. The values were means from three experiments with standard deviation in brackets. No nordiazepam was detectable in the control experiments in which oxazepam (100,000 ng/mL) was incubated under the same conditions without the presence of *H. pomatia* enzymes.

Oxazepam concentration effect

Oxazepam at 30,000 ng/mL, 50,000 ng/mL, and 100,000 ng/mL was incubated with *H. pomatia* β-glucuronidase (1500 units/mL) at 50°C for 18 h. LC-MS/MS analyses of the samples showed that nordiazepam was detected at 605 (±43), 813 (±60), 1450 (±75) ng/mL, respectively. The values were means from three experiments with standard deviation in brackets. When oxazepam was incubated under the same conditions without the addition of

H. pomatia β-glucuronidase, there is no detectable nordiazepam at any of the 3 oxazepam concentrations studied.

Discussion

It is a common knowledge that β-glucuronidase-mediated hydrolysis is preferred over acid-catalysed hydrolysis, which is known to induce benzodiazepine degradation and transformation. The commonly used β-glucuronidases are prepared from different sources such as *H. pomatia*, *P. vulgata*, *E. coli*, and bovine liver. The enzymatic hydrolysis conditions reported in the literature vary widely, with incubation temperature ranging from 22ºC to 65ºC and incubation time ranging from 30 min to 24 h (2,3,11-15). In this study, β-glucuronidase enzymes were obtained from three different sources, i.e. *E. coli, H. pomatia*, and *P. vulgata*. Several incubation conditions were also applied. Meatherall conducted a systematic investigation of enzymatic hydrolysis conditions and found that prolonged incubation at 56ºC led to a loss of benzodiazepines and not all benzodiazepines were destroyed at the same rate (3). This varying degree of degradation was also observed in this study. For example, the recoveries for oxazepam and nordiazepam were determined at 55% and 88% respectively when they were incubated with *H. pomatia* enzyme (pH 4.5) at 50°C for 18 h. The recoveries become 85% and 90% when they were incubated with *E. coli* enzyme (pH 6.8) at 22°C for 24 h. Under these circumstances, deuterated oxazepam and nordiazepam would be ideal internal standards to use so that these variations in recoveries can be compensated. Although oxazepam-d_5 and nordiazepam-d_5 are readily available commercially, they are not considered suitable for this study. Since the current study was focused on the conversion of oxazepam to nordiazepam mediated by β-glucuronidase treatment, conversion of oxazepam-d_5 to nordiazepam-d_5 would also be expected to take

place under the experimental conditions, which inevitably leads to analytical measurement inaccuracy. HO-Alp-d_5 is readily available and was chosen over other potential candidates based on two key considerations. Firstly, HO-Alp-d_5 shows a good recovery (around 94%) under all hydrolysis conditions investigated in the study. Secondly, HO-Alp-d_5 shows good peak shape and signal response in both GC-MS and LC-MS/MS. The major drawback of using HO-Alp-d_5 was the need to include a calibration curve and QC samples for each hydrolysis condition studied. This had increased considerably the volume of sample analysis and analytical time. However, the methods developed showed good reproducibility and linearity over a wide range and do not compromise analytical accuracy in any way. Another shortcoming of the method was the use of mixed standard solutions of oxazepam and nordiazepam in the preparation of both calibrators and QC specimens. There would be small conversion of oxazepam into nordiazepam in these samples during hydrolysis, leading to inaccurate nordiazepam calibration curves and QC results. Separate calibrators and QC samples containing just nordiazepam and just oxazepam would avoid this inaccuracy. However, the contributions were relatively insignificant (less than 2.5%) and were not to alter the results and conclusions of the study.

In the experiment to investigate the occurrence of nordiazepam in a urine specimen from a patient prescribed oxazepam, three incubation conditions were explored, i.e. 22ºC for 24 h, 37ºC for 24 h, and 60ºC for 2 h. As shown in Table I, the total oxazepam concentration in each hydrolysis conditions investigated was in the vicinity of 200,000 ng/mL. The free oxazepam in the sample was determined at around 2500 ng/mL, which accounts for less than 2% of the conjugated oxazepam glucuronide present in the urine sample. This result is in agreement with the current knowledge that oxazepam is extensively conjugated in urine as oxazepam glucuronide. O'Neal and Poklis (12) reported that incubation for 2 h at 60ºC or 24

h at 22ºC with *H. pomatia* enzyme was optimal for oxazepam and lorazepam glucuronides and hydrolysis was complete at both conditions. It is therefore plausible to suggest that a complete hydrolysis of oxazepam glucuronide was achieved under all hydrolysis conditions investigated in the current study. It is evident from the results summarized in Table I that nordiazepam levels were elevated in all samples that underwent enzymatic hydrolysis when compared to those in the enzyme-free control samples. Incubation for 24 h at 22ºC resulted in the least increase in nordiazepam concentration for all three β-glucuronidase enzymes examined. Incubation for 24 h at 37ºC led to the most increase in nordiazepam concentration. The increase in nordiazepam concentration were modest when hydrolyses were conducted at 60ºC for 2 h. Although the origin of the small quantity of nordiazepam (at approximately 20 ng/mL) detected in the urine sample without enzyme treatment is unknown, enterohepatic circulation of oxazepam and its reduction to nordiazepam by the action of intestinal bacteria as previously reported by Okamura and co-authors (16) might offer a feasible explanation. However this reductive conversion of oxazepam to nordiazepam by intestinal bacteria cannot account for the elevated levels of nordiazepam after enzymatic hydrolysis observed in this study (see Table I). Firstly, the degree of nordiazepam elevation is different among the hydrolysis conditions examined. Secondly, results from the experiments in which oxazepam (as free base instead of its glucuronide conjugate) was incubated with β-glucuronidase under various conditions support an alternative explanation that the enzyme preparations has the capacity to reduce oxazepam into nordiazepam (Figure 4). This reductive transformation is a novel discovery. As such, it was critical to unequivocally identify nordiazepam using 3 different analytical methods, GC-MS analysis of its TMS derivative in SIM mode, GC-MS analysis of the underivatized urine extract in SCAN mode (100-300amu), and LC-MS/MS analysis of the underivatized urine extract in SRM mode.

It is anticipated that the reduction reaction observed may take place on the glucuronide-bound oxazepam as well as on the free oxazepam released during the enzyme-catalysed hydrolysis. Although it is difficult to assess which form of oxazepam is the favoured substrate, it is relatively easy to demonstrate that the released oxazepam free base takes part in the observed reductive transformation process. It was therefore decided to source commercial oxazepam (free base) instead of oxazepam glucuronide for conducting further investigation. The oxazepam (free base) was purchased from Cerilliant and was found to be free of any detectable nordiazepam contaminants.

When oxazepam (free base) was incubated with β-glucuronidase for 18 h at 50ºC, formation of nordiazepam was observed in all three enzyme-treated samples. There was no detectable nordiazepam in the corresponding controls when enzymes were not present while all other parameters such as the incubation time, incubation temperature, and the pH condition were matched. The results indicate conclusively that formation of nordiazepam is not a result of thermolysis of oxazepam (17), but a direct action of enzyme treatment. The reduction reaction was positively correlated with the reaction temperature (Figure 2), incubation time (Figure 3), oxazepam concentration, and enzyme concentration under the experimental conditions described in this study. When the same units of enzymes were used, *E. coli* enzyme produced the least amount of nordiazepam, while *H. pomatia* enzyme yielded the most products. Based on the product information provided by Sigma-Aldrich, *E. coli* β-glucuronidase contains buffer salts and stabilizers, while *P. vulgata* enzyme contains sulfatase activity and *H. pomatia* enzyme possesses sulfatase and sometimes acid phosphatase activities. Since neither β-glucuronidase nor sulfatase was reported in the literature to be involved in mediating reductive reactions in a biological or a chemical system, a great caution should be exercised when trying to link the observed reaction to the hydrolytic

enzymes. It is clear further studies are needed to identify the responsible component(s) in the β-glucuronidase enzymes so that investigation into the reaction mechanisms can become possible.

Urinary drug testing for benzodiazepines in many laboratories in Australia is performed to Australian/New Zealand Standard AS/NZS 4308 (18). According to the standard, the confirmatory test cut-off level for reporting positive nordiazepam in urine is 200 ng/mL. It is clear from our study that enzymatic hydrolysis is a potential source of producing false positive results for nordiazepam. Depending on the hydrolysis condition used, varying amounts of nordiazepam artefact can be produced. As summarized in Table II, when incubating for 24 h at 22ºC with *E. coli* β-glucuronidase, the nordiazepam formed is <0.04% relative to the amount of oxazepam present and is less likely to produce a false positive result. When incubating for 2 h at 60ºC with *H. pomatia* enzyme, the percentage of nordiazepam formation ranged from 0.2% to 0.4%. A specimen containing 50,000 ng/mL oxazepam could yield a false positive nordiazepam result with a concentration greater than 200 ng/mL. If overnight incubation (18 h) at 50ºC with *H. pomatia* enzyme was employed, the artefact formation ranged from 0.9% to 2.5%. A specimen with as low as 8,000 ng/mL oxazepam is likely to produce a false positive nordiazepam result. Depending on the enzymatic hydrolysis conditions applied, nordiazepam artefact formation could have significant implication on benzodiazepine reporting.

Based on the results of the current study, the observed reduction of oxazepam to nordiazepam can be minimised if *E. coli* β-glucuronidases are used and the condition of 24 h incubation at 22ºC is applied. However, hydrolysis involving *E. coli* enzyme is known to give lower yields for other benzodiazepines such as lorazepam from their glucuronide conjugates (3). In drug

testing industries, many laboratories that perform urinary drug testing services for multiple drugs may choose to hydrolyse and extract these drugs, e.g. benzodiazepines, opiates and other basic substances in one single sample preparation step, in order to cut down costs and improve turn-around time. It is well known that opiate glucuronides, especially codeine glucuronide, are difficult to de-conjugate by β-glucuronidase and require incubation for longer period of time (16-24 h) at higher temperature e.g. 60ºC (19,20). Significant reduction of oxazepam when present at high concentrations in urine is expected to occur under such conditions. Due to variables in enzyme type, source of supply and amount used, as well as differences in incubation time and incubation temperature, it is advisable that laboratories perform their own assessment on the extend of nordiazepam artefact formation under their specific analytical conditions. It is clear that where high levels of oxazepam with corresponding low amounts of nordiazepam and no other benzodiazepines (e.g. temazepam) are identified, caution should be exercised in the interpretation of the presence of nordiazepam.

Conclusions

A novel transformation of oxazepam during enzyme hydrolysis has been discovered in this study. Incubation of commercial β-glucuronidase preparations with either oxazepam glucuronide in patient urines or oxazepam free base added to a blank urine results in formation of nordiazepam. The finding of this study adds another layer of complexity to the challenging field of urinary drug testing for benzodiazepines and will have both clinical and forensic implications. The presence of nordiazepam in biological samples subjected to enzyme-catalysed hydrolysis should be interpreted with care.

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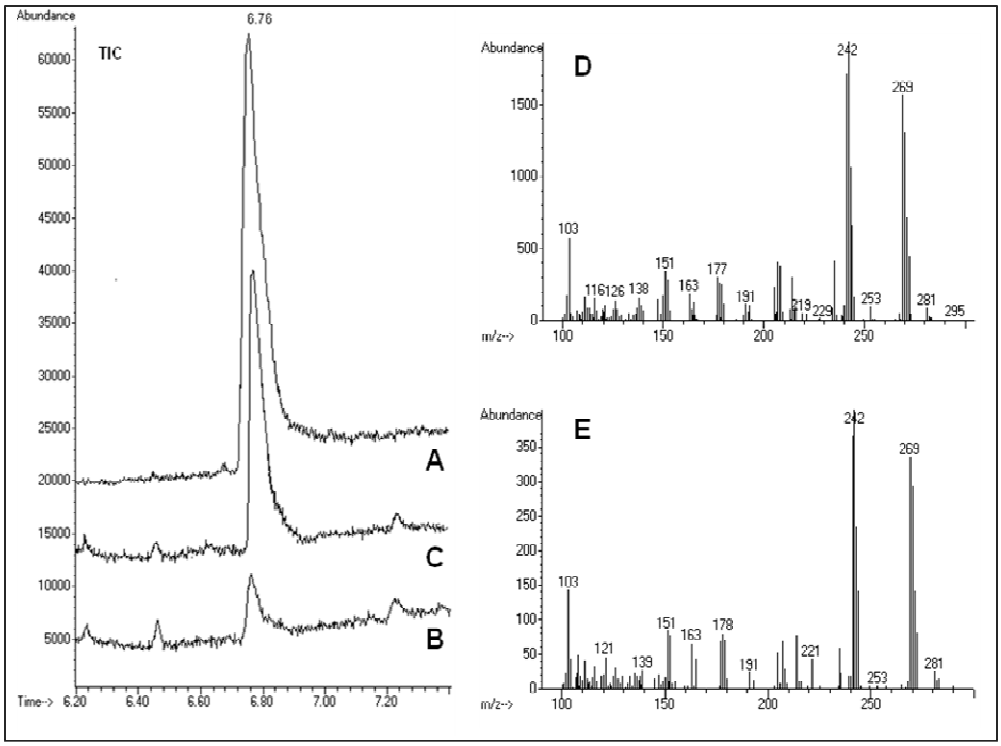

Figure 1. GC-MS identification of nordiazepam (non-derivatized) from a patient urine specimen following hydrolysis by *H. pomatia* enzyme at 60°C for 2 h. A) nordiazepam reference standard (20 µg/mL in methanol, RT 6.76 min); B) patient urine extract; C) mixture of samples A and B in equal volume; D) mass spectrum of nordiazepam standard from TIC trace A; E) mass spectrum of nordiazepam identified in patient urine specimen from TIC trace B. GC injection was done in split mode with a split ratio of 30:1 and an injection volume of 1 µL. The oven temperature was maintained at 200°C for 0.5 min and then programmed at 15°C/min to a final temperature of 300°C, which was held for 3 min. The MS was operated in SCAN mode (100-300 amu). All other GC-MS conditions were similar to those described under the section of Material and methods.

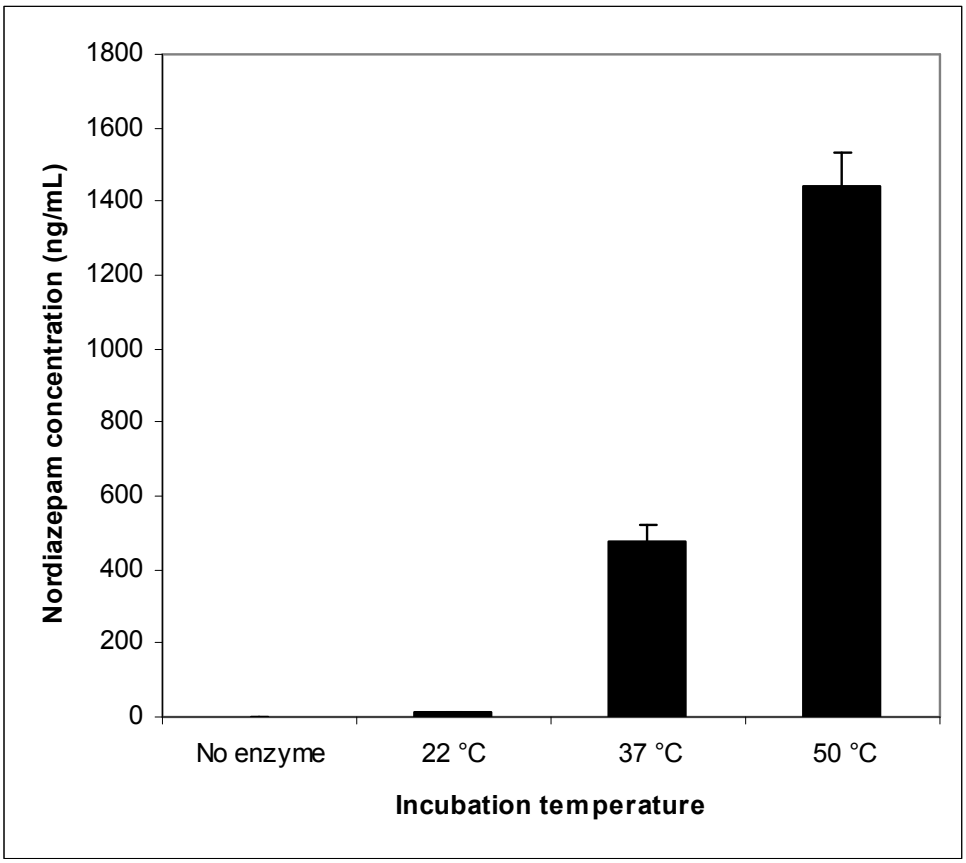

Figure 2. Formation of nordiazepam from incubation of oxazepam (at 100,000 ng/mL) with *H. pomatia* β-glucuronidase (1500 units/mL) for 18 h at various temperatures. Values were from LC-MS/MS analyses and were means of 3 measurements with standard deviation represented by the error bars. No nordiazepam was detectable in the enzyme-free control samples for each incubation condition investigated. The results were collectively represented by the "No enzyme" condition in the figure.

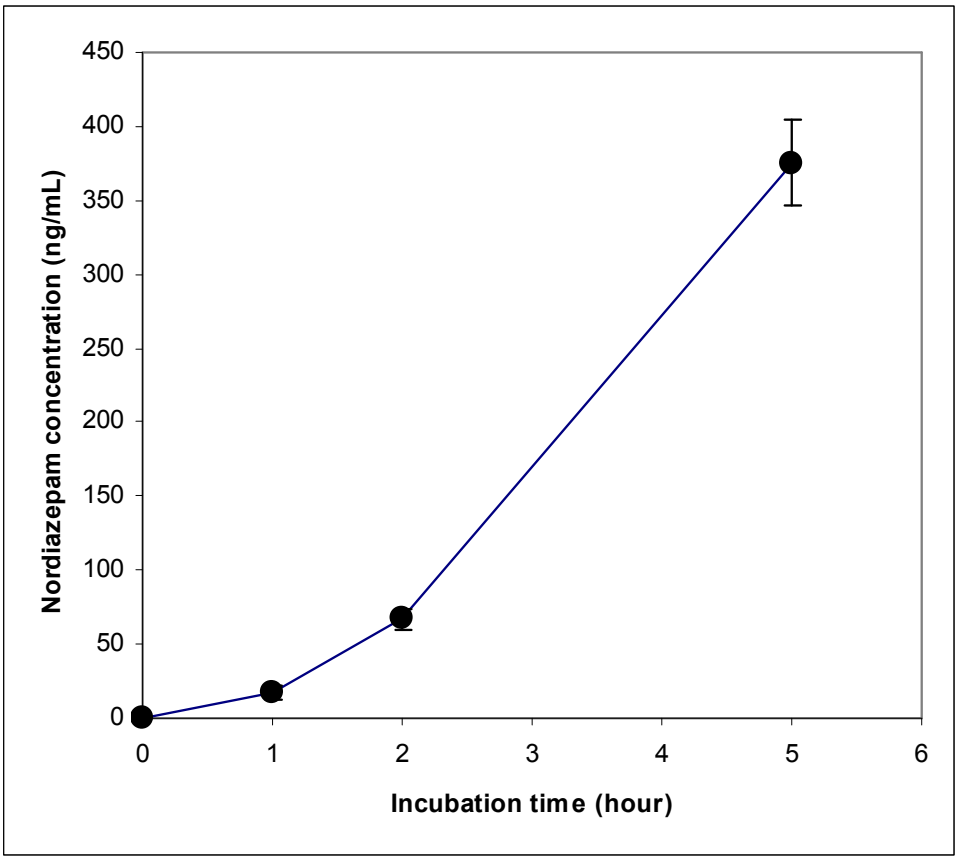

Figure 3. Formation of nordiazepam from incubation of oxazepam (at 100,000 ng/mL) with *H. pomatia* β-glucuronidase (1500 units/mL) at 50ºC. Values were from LC-MS/MS analyses and were means of 3 measurements with standard deviation represented by the error bars.

Oxazepam Nordiazepam

Figure 4. Reductive transformation of oxazepam to nordiazepam observed during enzyme hydrolysis employing commercial β-glucuronidase preparations.

Table I. Detection of benzodiazepines in a urine specimen from a patient on oxazepam medication

Hydrolysis Condition*	Oxazepam (+Enzyme)**		Oxazepam (-Enzyme)**		Nordiazepam (+Enzyme)**		Nordiazepam (-Enzyme)**	
	GC-MS	LC-MS/MS	GC-MS	LC-MS/MS	GC-MS	LC-MS/MS	GC-MS	LC-MS/MS
E. coli								
22°C_24h	201123 (20130)	214508 (10936)	2374 (160)	2611 (191)	31 (4)	33 (2)	13 (2)	15 (1)
37°C_24h	190472 (11515)	207659 (16492)	2519 (176)	2433 (145)	83 (10)	76 (7)	19 (3)	22 (2)
60°C_02h	187737 (19294)	193100 (15385)	2354 (205)	2507 (170)	58 (10)	62 (9)	17 (2)	20 (2)
H. pomatia								
22°C_24h	200173 (18945)	213608 (11061)	2398 (166)	2558 (132)	58 (8)	66 (4)	15 (2)	18 (1)
37°C_24h	185773 (17205)	196370 (15264)	2278 (128)	2380 (136)	789 (86)	854 (92)	22 (2)	20 (2)
60°C_02h	180636 (21759)	186933 (19239)	2866 (255)	3032 (183)	563 (62)	584(60)	21 (2)	25 (2)
P. vulgata								
22°C_24h	185271 (16485)	194207 (17271)	2214 (182)	2447 (208)	37 (3)	36 (1)	15 (2)	17 (1)
37°C_24h	180172 (19370)	189007 (14800)	2537 (148)	2729 (75)	329 (37)	351 (32)	20 (2)	22 (2)
60°C_02h	181905 (19034)	184974 (15837)	3042 (290)	3331 (217)	265 (34)	254 (30)	23 (3)	26 (2)

* Urine specimens were hydrolyzed with 1500 units of β-glucuronidase from 3 different preparations, *Escherichia coli (E. coli), Helix pomatia (H. pomatia),* and *Patella vulgata (P. vulgata)*.

** Benzodiazepine concentrations are expressed in ng/mL and are mean values from 3 measurements with standard deviation in brackets.

Table II. Urinary benzodiazepines detected in patients on oxazepam*

Sample	*E. coli* enzyme, 22°C_24h			*H. pomatia* enzyme, 60°C_02h			*H. pomatia* enzyme, 50°C_18h		
	Oxazepam	Nordiazepam	% Nordiazepam**	Oxazepam	Nordiazepam	% Nordiazepam**	Oxazepam	Nordiazepam	% Nordiazepam**
1	566	NQ	-	510	NQ	-	525	NQ	-
2	1105	NQ	-	985	NQ	-	913	NQ	-
3	2580	NQ	-	2150	NQ	-	2351	20	0.851%
4	4692	NQ	-	4315	15	0.348%	4229	51	1.206%
5	7732	NQ	-	7535	21	0.279%	7254	65	0.896%
6	9680	NQ	-	9500	25	0.263%	9398	216	2.298%
7	13670	NQ	-	15650	31	0.198%	14061	130	0.925%
8	15790	NQ	-	18764	63	0.336%	15438	160	1.036%
9	30367	NQ	-	27005	75	0.278%	28385	436	1.536%
10	38470	NQ	-	35123	135	0.384%	36768	601	1.635%
11	46982	NQ	-	48960	156	0.319%	45899	594	1.294%
12	59320	14	0.024%	63450	260	0.410%	59935	1496	2.496%
13	89320	25	0.028%	95678	329	0.344%	90058	1945	2.160%
14	156469	41	0.026%	143885	421	0.293%	154391	3802	2.463%
15	169347	56	0.033%	185640	519	0.280%	173906	2468	1.419%

* Urine specimens were hydrolyzed with 1500 units of β-glucuronidase per sample. Concentrations of benzodiazepines were expressed in ng/mL and were from a single determination by LC-MS/MS method. NQ means “not quantifiable”, referring to concentration below LOQ (10 ng/mL for nordiazepam).

** %Nordiazepam represents the ratio of nordiazepam to oxazepam in hydrolyzed urine specimens.