Journal of Analytical Toxicology 2014;38:610–614
doi:10.1093/jat/bku083

Technical Note

# Rapid Enzymatic Hydrolysis Using a Novel Recombinant β-Glucuronidase in Benzodiazepine Urinalysis

Ayodele A. Morris*, Scot A. Chester, Erin C. Strickland and Gregory L. McIntire

Ameritox, Ltd., 486 Gallimore Dairy Road, Greensboro 27409, NC, USA

*Author to whom correspondence should be addressed. Email: ayodele.morris@ameritox.com

**Only trace amounts of parent benzodiazepines are present in urine following extensive metabolism and conjugation. Thus, hydrolysis of glucuronides is necessary for improved detection. Enzyme hydrolysis is preferred to retain identification specificity, but can be costly and time-consuming. The assessment of a novel recombinant β-glucuronidase for rapid hydrolysis in benzodiazepine urinalysis is presented. Glucuronide controls for oxazepam, lorazepam and temazepam were treated with IMCSzyme™ recombinant β-glucuronidase. Hydrolysis efficiency was assessed at 55°C and at room temperature (RT) using the recommended optimum pH. Hydrolysis efficiency for four other benzodiazepines was evaluated solely with positive patient samples. Maximum hydrolysis of glucuronide controls at 5 min at RT (mean analyte recovery ≥94% for oxazepam and lorazepam and ≥80% for temazepam) was observed. This was considerably faster than the optimized 30 min incubation time for the abalone β-glucuronidase at 65°C. Mean analyte recovery increased at longer incubation times at 55°C for temazepam only. Total analyte in patient samples compared well to targets from abalone hydrolysis after recombinant β-glucuronidase hydrolysis at RT with no incubation. Some matrix effect, differential reactivity, conjugation variability and transformation impacting total analyte recovery were indicated. The unique potential of the IMCSzyme™ recombinant β-glucuronidase was demonstrated with fast benzodiazepine hydrolysis at RT leading to decreased processing time without the need for heat activation.**

## Introduction

Benzodiazepines are widely prescribed drugs that are readily abused for their sedative effects and as such are very frequently targeted in therapeutic drug monitoring. Commonly monitored benzodiazepines include diazepam (Valium®); the 3-hydroxy benzodiazepines, oxazepam (Serax®), lorazepam (Ativan®) and temazepam (Restoril™); the triazolobenzodiazepine derivative, alprazolam (Xanax®); in addition to clonazepam (Klonopin®). Some benzodiazepines are indicated as antianxiety agents (diazepam, oxazepam, lorazepam and alprazolam), some as anticonvulsants (diazepam and clonazepam), some as antidepressants (alprazolam) and some as hypnotic drugs (temazepam) (1). For the diazepam, clonazepam and the 3-hydroxy benzodiazepines, only trace amounts of parent drug are present in urine due to extensive metabolism and conjugation. Conjugated metabolites in the form of glucuronides are major urinary species (1). With alprazolam, a significant percentage of drug is excreted unchanged in urine, but its metabolite α-hydroxyalprazolam glucuronide is also a major urinary species. Chemical structures of common benzodiazepines and their metabolites are shown in Figure 1. In drug testing, hydrolysis to cleave the glucuronide linkage prior to analysis is necessary for improved detection. Although attempts have been made to monitor intact glucuronides, such methods are limited by the availability of glucuronide reference standards (2) and the stability of these conjugates in the source of the mass spectrometer (3). Hydrolysis by enzyme is preferred over acid for benzodiazepine analysis, because the latter induces degradation and produces benzophenones, which convolute result interpretation (4, 5). Enzyme hydrolysis can be costly and time-consuming, with reported incubation times ranging from 0.5 to 20 h (5–7), depending on the enzyme source. β-Glucuronidase from abalone has been reported to be more effective than β-glucuronidase from other sources including bovine liver, *Helix pomatia*, *Escherichia coli* and *Patella vulgata* (8). Although reference of the application of abalone-derived enzyme as a hydrolytic agent is only recent and limited, this has been a traditional option for urinalysis pretreatment by the authors' laboratory.

The application of a novel recombinant β-glucuronidase for rapid hydrolysis in benzodiazepine urinalysis was investigated. The effectiveness of the recombinant enzyme relative to β-glucuronidase from abalone was assessed.

## Experimental

### *Materials*

β-Glucuronidase from abalone (catalog # DR2102, Lot # 40109-02) was purchased from Campbell Science (Rockford, IL, USA) as a solution containing minimum 100,000 Fishman units/mL β-glucuronidase and 8,000 Fishman units/mL sulfatase. Recombinant β-glucuronidase (catalog # 04-01F-050K, Lot # E13-1210, E13-1224, E14-0101, E14-0124, E13-1203 and E13-0924) was obtained from Integrated Micro-Chromatography Systems (Columbia, SC, USA) as a solution containing ~50,000 units/mL β-glucuronidase. Potassium phosphate buffer (0.2 M, pH 6.8) was obtained from Integrated Micro-Chromatography Systems. Sodium phosphate buffer (0.2 M, pH 6.8) was obtained from VWR (Ward Hill, MA, USA). Glacial acetic acid, formic acid (88%), ammonium hydroxide (14.8 N), ammonium formate (98% purity) and ammonium acetate (98.6% purity) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Potassium hydroxide (50%, w/v) was acquired from Ricca Chemical Company (Arlington, TX, USA). Methanol (OmniSolv grade) was obtained from EMD Chemicals (Billerica, MA, USA). Acetone and 2-propanol (Optima HPLC Grade) were purchased from Fisher Scientific. The following compounds were purchased from Cerilliant (Round Rock, TX, USA): oxazepam (1 mg/mL), oxazepam glucuronide (100 μg/mL), oxazepam D5 (1 mg/mL), temazepam (1 mg/mL), temazepam glucuronide lithium salt (100 μg/mL), temazepam D5 (1 mg/mL), lorazepam (1 mg/mL), lorazepam glucuronide (100 μg/mL), alphahydroxyalprazolam (1 mg/mL), alphahydroxyalprazolam D5 (1 mg/mL), nordiazepam (1 mg/mL), nordiazepam D5 (1 mg/mL), 7-aminoclonazepam (1 mg/mL) and 7-aminoclona

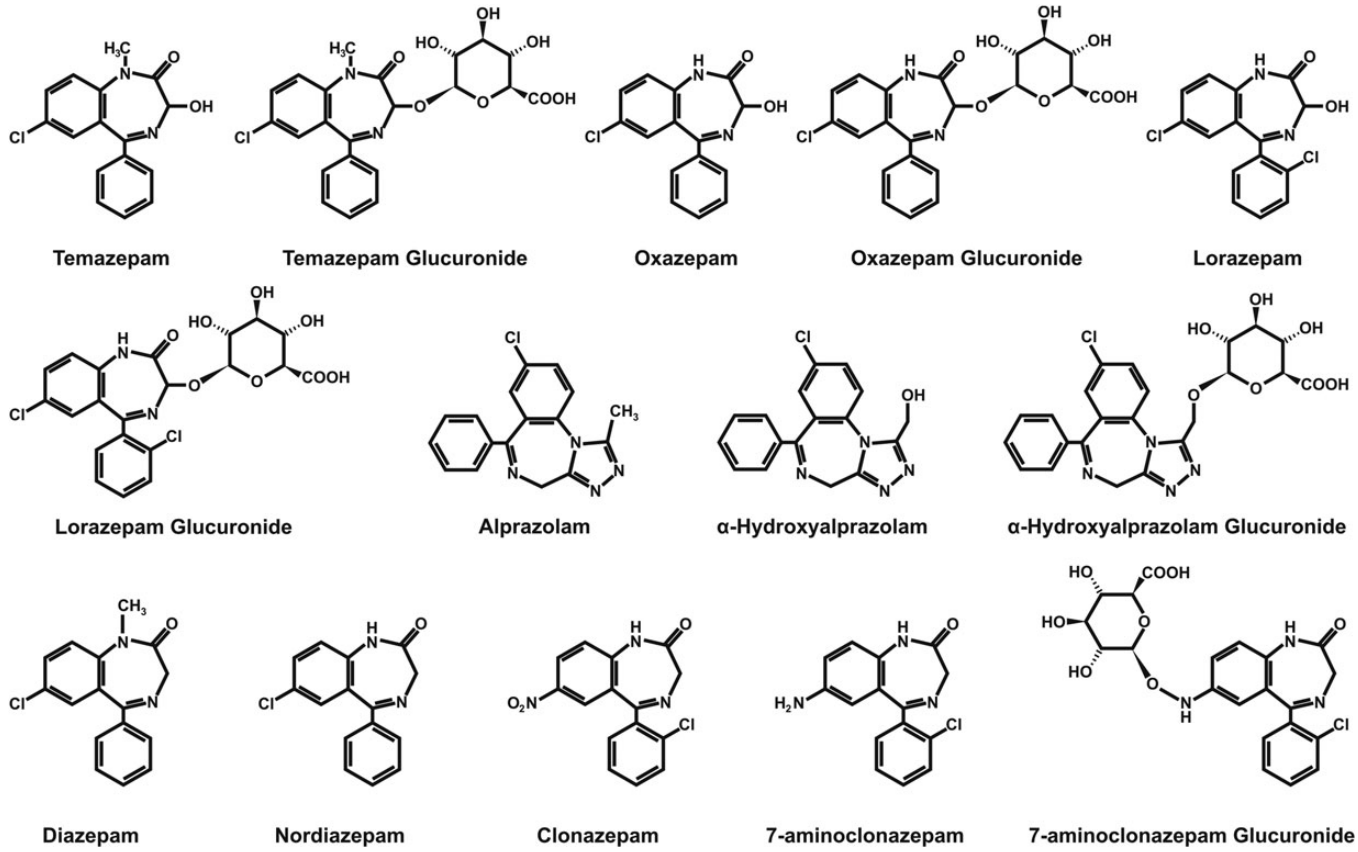


**Figure 1.** Chemical structures of targeted benzodiazepines and associated conjugates.

zepam D4 (1 mg/mL). Certified drug negative normal human urine was obtained from UTAK Laboratories, Inc. (Valencia, CA, USA).

### *Standards, calibrators and controls and urine samples*

Aliquots of drug-free urine were fortified separately with glucuronides of oxazepam, lorazepam and temazepam at 2,500 ng/mL for use in hydrolysis optimization studies. A mixed control containing all three glucuronides was also prepared at 2,500 ng/mL. The internal standard solution consisted of oxazepam D5, temazepam D5, α-hydroxyalprazolam D5, nordiazepam D5 and 7-aminoclonazepam D4 prepared in deionized water. Internal standard concentration was 1,000 ng/mL for all deuterated compounds, except for 7-aminoclonazepam D4 at 5,000 ng/mL. Working solutions were prepared using the undeuterated analytes. Multi-point calibration curves were prepared in normal human urine with the following calibrators in established linear range for each analyte (20–5,000 ng/mL): 20, 50, 75, 250, 500, 1,000, 2,500 and 5,000 ng/mL. The calibration curves underwent the same dilution and sample preparation conditions as specimens. Randomly selected authentic benzodiazepine urine specimens that were previously confirmed positive for oxazepam, temazepam, lorazepam, alprazolam, α-hydroxyalprazolam, nordiazepam and/or 7-aminoclonazepam using a 20 ng/mL cut off ($n = 20$ per analyte) were sequestered and stored at 4°C until analysis. The initial confirmation involved hydrolysis using abalone-derived enzyme.

## Methods

### *Sample analysis*

*Hydrolysis methods*

IMCSzyme™ recombinant β-glucuronidase was buffered to the recommended optimum (pH 6.8) (9) and the hydrolysis conditions were evaluated using glucuronide controls and authentic urine specimens (Table I). The hydrolysis efficiency was assessed in triplicate with individual glucuronide controls for oxazepam, lorazepam and temazepam at the recommended optimum temperature of 55°C (9) at incubation times of 5, 15, 30 and 60 min and at room temperature (RT) at incubation times of 0, 5, 10 and 15 min. The zero-time point and all others included an ~10 min delay for injection. Hydrolysis efficiency for α-hydroxyalprazolam glucuronide and conjugates of nordiazepam, alprazolam and 7-aminoclonazepam were evaluated solely with patient samples positive for that compound in lieu of unavailable reference standards. A lot-to-lot comparison with three different lots of IMCSzyme™ recombinant β-glucuro nidase was also completed on a mixed glucuronide control co ntaining all three analytes at the chosen production hydrolysis conditions. To minimize the number of steps involved in sample aliquoting, the viability of the use of a mastermix solution co mprised of enzyme, buffer and internal standard was evaluated. Authentic urine specimens (250 µL) were hydrolyzed at the chosen production hydrolysis conditions by incubation with a mastermix solution (150 µL) containing internal standard

**Table I**
Hydrolysis Conditions

| | Evaluation hydrolysis (recombinant β-glucuronidase) | Reference hydrolysis (abalone β-glucuronidase) |
|---|---|---|
| Enzyme | 12.5 μL of >50,000 Fishman units/mL | 20 μL of >20,000 Fishman units/mL |
| Sample | 250 μL urine | 250 μL urine |
| Optimal buffer | 40 μL of 0.2 M potassium phosphate, pH 6.8 | 40 μL of 0.8 M potassium acetate, pH 4.8 |
| Internal standard | 50 μL of 1,000/5,000 ng/mL aqueous solution[a] | 50 μL of 1,000/5,000 ng/mL aqueous solution[a] |
| Optimal temperature | 55°C[b] | 65°C |
| Incubation time | Evaluated 5–60 min | 30 min |

[a]All deuterated compounds in internal standard solution at 1,000 ng/mL except 7-aminoclonazepam D4 at 5,000 ng/mL.
[b]Hydrolysis was also further evaluated at RT.

(360 ng/mL of each deuterated compound, except for oxazepam D5 and 7-aminoclonazepam D4 present at 1,800 ng/mL) and the IMCSzyme™ recombinant β-glucuronidase (minimum 625 Fishman units), buffered to pH 6.8 with 0.2 M sodium phosphate buffer as diluent. Following hydrolysis, samples were centrifuged and then 10 μL was injected onto the instrument. Target co ncentrations for the authentic specimens were derived from sample processing involving reference hydrolysis of sample with β-glucuronidase from abalone diluted 5-fold in water under previously validated optimal conditions (Table I).

*HPLC–MS/MS*

For benzodiazepine confirmation using a 20 ng/mL reporting cut off, urine specimens were hydrolyzed and analyzed on a TLX-4 Multiplexed HPLC with Agilent 1,200 Series Binary Pumps coupled to a Thermo Scientific™ TSQ Quantum Ultra™ Triple-Stage Quadrupole Mass Spectrometer using a previously validated multiplexed method. Analytes were separated chromatographically in a six minute gradient on a Hypersil Gold aQ, (3.0 × 50 mm, 5 μm) analytical column following online sample purification using Cohesive Technologies Cyclone-P 0.5 × 50 mm Turboflow™ column. Mobile phases included loading solvents A–D 50 mM ammonium acetate$_{(aq)}$, 6.25 mM ammonium acetate$_{(aq)}$, 60 mM ammonium acetate in methanol, 2-propanol : acetone (50 : 50) with 0.3% formic acid, respectively. Eluting solvents A and B were 10 mM ammonium formate$_{(aq)}$ with 0.1% formic acid and methanol with 0.1% formic acid, respectively. Mass spectral data were acquired in positive electrospray ionization mode with two selected transition ions for all analytes and internal standards in a 1 min data window. Oxazepam, oxazepam D5, temazepam, temazepam D5, lorazepam, α-hydroxyalprazolam, α-hydroxyalprazolam D5, nordiazepam, nordiazepam D5, 7-aminoclonazepam and 7-aminoclonazepam D4 were monitored. In the HPLC–MS-MS confirmation method, the limit of detection (LOD) was defined as the lowest concentration at which the identity of the analyte could be accurately established in terms of retention time and ion ratio. The limit of quantitation (LOQ) was defined as the lowest concentration at which both the identity and concentration of the analyte could be accurately established. LODs and LOQs were 20 ng/mL for all analytes. Upper limits of linearity and carryover were 5,000 ng/mL for all analytes. Calibration curve correlation coefficients ($R^2$) exceeded 0.99. Inter/intra-assay precision did not exceed 7% coefficient of variation and accuracy was within 11% of the target concentrations for the seven compounds.

The hydrolysis efficiency was calculated by dividing the resulting analyte concentration by the target concentration of the test compound in the sample and multiplying by 100%. Statistical analyses were completed using GraphPad Prism 6 software.

## Results and discussion

At 0 min (no incubation time but 10 min delay for injection on instrument), mean analyte recovery >91% from hydrolyzed oxazepam and lorazepam glucuronide controls and mean analyte recovery >80% from hydrolyzed temazepam glucuronide controls were observed at RT (Figure 2).

Maximum hydrolysis of the glucuronide controls was observed at 45 min at 55°C (mean analyte recovery ≥94%) and at 5 min at RT (mean analyte recovery ≥94% for oxazepam and lorazepam and ≥80% for temazepam). Variations in enzyme hydrolysis efficiency with analyte are expected due to the influence of chemical structure on reactivity with the enzyme. Recovery of temazepam from the glucuronide control improved with heat activation, but was still satisfactory in the absence of heat. The maximum hydrolysis of oxazepam and lorazepam glucuronide controls at RT equivalent to measurements at 55°C provided convenience with the elimination of heat activation and was considerably faster than the optimized incubation time of 30 min for the abalone β-glucuronidase. It is believed that the improved hydrolysis was not due to the presence of more enzyme in the prepared sample (~625 Fishman units of recombinant enzyme versus 400 Fishman units of abalone), given the enzymes were in excess and the vast difference in temperatures. Mean analyte recovery for all glucuronide controls did not increase at longer incubation times at both temperatures. Because of the potential impact on increased productivity, the recombinant enzyme was further evaluated at RT with 0 min incubation time with authentic urine specimens. Slightly better overall recoveries were observed using the mastermix solution as opposed to separate aliquots of reagents for hydrolysis, possibly due to its lower viscosity allowing more accurate and reproducible aliquoting. Excellent reproducibility of the hydrolysis was observed across three different lots of the recombinant enzyme; % coefficient of variation did not exceed 2.9%.

In patient samples, total oxazepam, lorazepam and temazepam compared well between the two enzyme treatments ($R^2$ = 0.9116–0.9969, Table II). Temazepam still registered a mean percent hydrolysis representing complete hydrolysis (99%), although hydrolysis was completed at RT with no incubation time, which was not noted as optimal for temazepam. Total oxazepam concentrations had an overall negative bias, whereas total nordiazepam concentrations were elevated with a relatively high standard deviation and statistically significantly different

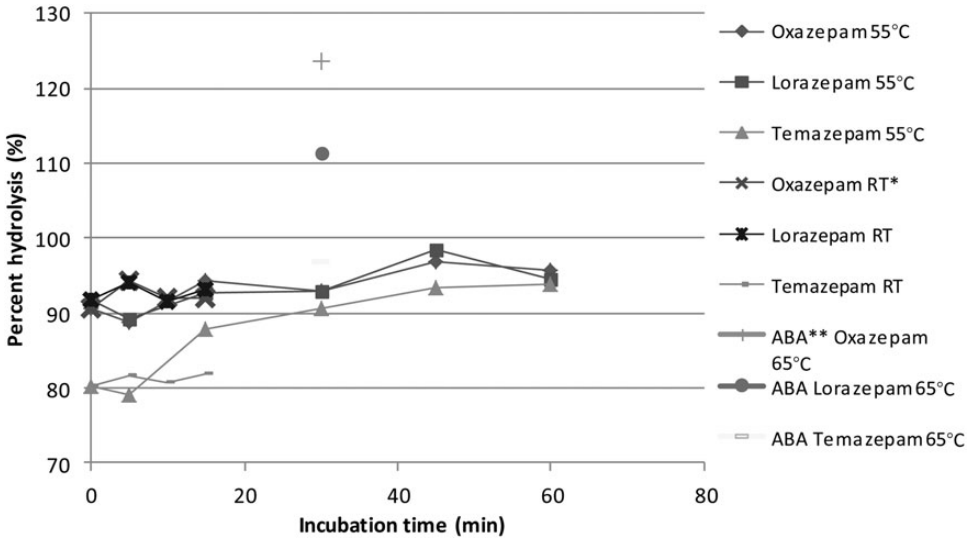


**Figure 2.** Effect of incubation time and temperature on the hydrolysis of glucuronides of oxazepam, lorazepam and temazepam with recombinant β-glucuronidase. *RT, room temperature; **ABA, reference abalone optimum; ***hydrolysis was further investigated at RT from 5 to 15 min after satisfactory hydrolysis was observed at zero-time during initial evaluation at 55°C.

**Table II**
Analyte Recovery after Hydrolysis of Authentic Urine Specimens with Recombinant β-Glucuronidase

| Analyte ($n = 20$ per compound) | Abalone target range (ng/mL) | IMCSzyme™ range (ng/mL) | Mean % hydrolysis[a] ± SD | Correlation ($R^2$) | *P*-value at 95% confidence interval |
|---|---|---|---|---|---|
| Oxazepam | 45–2,788 | 38–2,708 | 90.5 ± 10.1 | 0.9969 | 0.057 |
| Lorazepam | 79–3,838 | 37–5,808 | 87.6 ± 29.3 | 0.9495 | 0.854 |
| Temazepam | 33–4,105 | 32–4,361 | 99.1 ± 7.8 | 0.9968 | 0.078 |
| α-Hydroxy-alprazolam | 34–3,016 | 54–5,039 | 156.3 ± 80.2 | 0.9953 | 0.106 |
| Alprazolam | 38–1,131 | 31–1,437 | 110.7 ± 20.3 | 0.9868 | 0.099 |
| Nordiazepam | 24–1,219 | 32–1,512 | 150.8 ± 89.7 | 0.9116 | [b]0.009 |
| 7-Aminoclonazepam | 49–1,882 | 25–1,740 | 86.0 ± 15.9 | 0.9947 | 0.065 |
| Subset of authentic patient comparison | | | | | |
| Analyte | Sample ID | Abalone (ng/mL) | IMCSzyme™ (ng/mL) | % Deviation | |
| Oxazepam | Oxa1 | 518 | 421 | −23 | |
| | Oxa2 | 45 | 38 | −20 | |
| | Oxa3 | 1,817 | 1,908 | 5 | |
| | Oxa4 | 2,788 | 2,708 | −3 | |
| Lorazepam | Lor1 | 3,279 | 2,633 | −25 | |
| | Lor2 | 631 | 518 | −22 | |
| | Lor3 | 295 | 226 | −31 | |
| | Lor4 | 191 | 162 | −17 | |
| Temazepam | Tem1 | 373 | 381 | 2 | |
| | Tem2 | 440 | 410 | −7 | |
| | Tem3 | 43 | 40 | −8 | |
| | Tem4 | 4,105 | 4,361 | 6 | |
| α-Hydroxy-alprazolam | Aha1 | 90 | 133 | 32 | |
| | Aha2 | 40 | 54 | 27 | |
| | Aha3 | 114 | 153 | 26 | |
| | Aha4 | 511 | 677 | 25 | |
| Alprazolam | Alp1 | 1,131.1 | 1,437.3 | 21.3 | |
| | Alp2 | 339.9 | 368.2 | 7.7 | |
| | Alp3 | 41.2 | 40.8 | −0.9 | |
| | Alp4 | 167.2 | 174.9 | 4.4 | |
| Nordiazepam | Nor1 | 182 | 261 | 30 | |
| | Nor2 | 77 | 100 | 22 | |
| | Nor3 | 549 | 690 | 20 | |
| | Nor4 | 24 | 32 | 26 | |
| 7-Aminoclonazepam | 7Ac1 | 1,882 | 1,522 | −24 | |
| | 7Ac2 | 57 | 49 | −16 | |
| | 7Ac3 | 4,463 | 3,598 | −24 | |
| | 7Ac4 | 132 | 118 | −12 | |

[a]% hydrolysis relative to target concentrations using abalone-derived enzyme.
[b]Significant statistical difference.

from target. Transformation of oxazepam to nordiazepam under several hydrolysis conditions has been reported and variability of nordiazepam conjugates due to the less stable amine glucuronide linkage has been suggested (8, 10). The occurrence of the former was demonstrated to be minor with other enzyme sources compared with what was observed in the presence of the recombinant enzyme in this study. It is possible that nordiazepam conjugates are more reactive with this enzyme compared with abalone, resulting in the substantially higher recovery of nordiazepam relative to decreased recovery of oxazepam. Also, the time between dosing and sample collection and/or varied patient metabolic profiles may also have had an impact. Further studies with a larger sample pool are needed to confirm the extent of oxazepam transformation in addition to nordiazepam reactivity with this enzyme. Mean recovery of α-hydroxyalprazolam was 50% higher using the recombinant β-glucuronidase versus that from abalone, highlighting the differential hydrolysis of α-hydroxyalprazolam by the abalone enzyme under conditions optimal for the other analytes. Total lorazepam concentrations had an overall negative bias and a relatively high standard deviation, but there was no statistically significant difference between the sample sets. The high standard deviation may have resulted from patient to patient matrix variability, the effect of which could not be minimized in the absence of a matching deuterated internal standard. Mean recovery of 7-aminoclonazepam also had an overall negative bias with no statistically significant difference between the sample sets, but a relatively low standard deviation. This scenario points towards the reactivity of the compound with abalone possibly being better than with the recombinant enzyme under the chosen hydrolysis conditions. Overall, percent hydrolysis exceeded 86% for all target analytes in patient samples.

The IMCSzyme™ recombinant β-glucuronidase appears to be a cleaner extract. As such, it is possible that on column filtration may not be necessary, assuming the enzyme does not adversely impact the analytical column. The maximum number of analytical column injections of recombinant enzyme-treated samples is expected to exceed that seen with abalone-treated samples and early anecdotal evidence supports this approach. A detailed evaluation to confirm this is underway.

## Conclusion

The unique potential of the IMCSzyme™ recombinant β-glucuronidase was demonstrated with fast benzodiazepine hydrolysis which is complete at RT before the samples can be injected onto the HPLC–MS-MS instrument. The use of this enzyme will decrease processing time due to an absence of incubation time, requiring no heat activation and thus removing a processing step from the analysis of benzodiazepines in urine. Work applying this enzyme to other analyses is underway.


## Acknowledgments

The authors thank Dr L. Andrew Lee of Integrated Micro-Chromatography Systems for guidance on use of the IMCSzyme™ recombinant β-glucuronidase.


## References


1. Baselt, R.C. *Disposition of Toxic Drugs and Chemicals in Man*; 7th edition. Chemical Toxicology Institute, Foster City, CA, 2004. pp. 35–37, 621–623, 824–825, 1063–1065.
2. McIntire, G. L., Smith, J., Park, C., Sadjadi, S., Layne, J. *Eliminating hydrolysis for pain management monitoring of benzodiazepines by LC/MS/MS*; Poster presented at the annual meeting of The Association for Mass Spectrometry: Applications for the Clinical Laboratory, San Diego, CA, 2013. February.
3. Enders, J. R., Hitchcock, J. C., Park, C. W., Morris, A. A., McIntire, G. L. *A quantitative inspection of ESI glucuronide degradation*; Poster presented at the annual meeting of the American Society of Mass Spectrometry, Minneapolis, MN, 2013, June.
4. Levine, B. (2010) Central nervous system depressants. In Levine, B. (ed), *Principles of Forensic Toxicology*, 3rd edition, Chapter 11. American Association for Clinical Chemistry, Inc., Washington, DC, pp. 178–182.
5. Miki, A., Tatsuno, M., Katagi, M., Nishikawa, M., Tsuchihashi, H. (2002) Simultaneous determination of eleven benzodiazepine hypnotics and eleven relevant metabolites in urine by column-switching liquid chromatography-mass spectrometry. *Journal of Analytical Toxicology*, **26**, 87–93.
6. Borrey, D., Meyer, E., Duchateau, L., Lambert, W., Van Peteghem, C., De Leenheer, A. (2002) Enzymatic hydrolysis improves the sensitivity of emit screening for urinary benzodiazepines. *Clin. Chem.*, **48**, 2047–2049.
7. O'Neal, C., Poklis, A. (1996) Validation of benzodiazepine β-glucuronide primary reference materials for hydrolysis and quality assurance controls. *Forensic Sci. Int.*, **79**, 69–81.
8. Malik-Wolf, B., Vorce, S., Holler, J., Bosy, T. (2014) Evaluation of abalone β-glucuronidase substitution in current urine hydrolysis procedures. *Journal of Analytical Toxicology*, **38**, 171–176.
9. IMCSzyme™ Package Insert, Catalog No. 04-E1F-050K (2014) Integrated Micro-Chromatography Systems, Columbia, SC.
10. Fu, S., Lewis, J., Wang, H., Keegan, J., Dawson, M. (2010) A novel reductive transformation of oxazepam to nordiazepam observed during enzymatic hydrolysis. *Journal of Analytical Toxicology*, **34**, 243–251.