Rapid Hydrolysis of Benzodiazepines in Urine

Alicia Zook[1] and Crystal Xander B.S.[2]

Cedar Crest College, Allentown, PA[1]

Health Network Laboratories, Allentown, PA[2]

Abstract:

Benzodiazepines are sedative/hypnotic drugs routinely prescribed to treat conditions such as anxiety, insomnia, and epilepsy. Patients using these drugs are often monitored to ensure that the drugs are being used properly, as they are common targets of abuse due to their sedative effects. The major metabolic pathway of benzodiazepines includes conjugation with glucuronides. Most laboratories choose to look for the free form of the drug rather than the conjugated metabolite; therefore, analysis of benzodiazepines requires enzyme hydrolysis of glucuronides for accurate detection of the drugs and drug metabolites in urine. A recombinant β-glucuronidase enzyme, IMCSzyme™, was used in this experiment to reduce hydrolysis time. Thomson eXtreme|FV ® 0.2µm PVDF (polyvinylidene fluoride filter vials) were used in place of solid phase extraction to further decrease the preparation time associated with analysis of benzodiazepines. Analysis of liquid chromatography and tandem mass spectrometry (LC/MS/MS) data indicated that 30 minutes of hydrolysis at 55°C was ideal to break the glucuronide linkages associated with benzodiazepine metabolites. In addition, the sample preparation method was changed from a more time consuming Solid Phase Extraction Method (SPE) to a simple filtration method. A three day validation study confirmed that the method may be applied to the analysis of patient samples.

Introduction:

Benzodiazepines are prescribed for their uses as anti-anxiety medications, anticonvulsants, or hypnotic drugs. The sedative effects of these drugs make them targets of abuse; therefore, there is a need for evaluation of such drugs. Benzodiazepines commonly monitored for such abuse include: nordiazepam, alprazolam (Xanax©), oxazepam (Serax©), clonazepam (Rivotril©), diazepam (Valium©), midazolam (Versed©), lorazepam (Ativan©), and temazepam (Restoril©). Zolpidem (Ambien©) is incorporated into benzodiazepine analysis at Health Network Laboratories due to its similar sedative effects; however, zolpidem is not a benzodiazepine.

Many analysis methods contain either the benzodiazepine parent drugs listed above or the metabolites of the parent drugs. The method used to monitor benzodiazepines in this laboratory consists of the following drugs: diazepam, nordiazepam (metabolite), α-hydroxyalprazolam (metabolite), oxazepam (parent drug or metabolite), 7-aminoclonazepam (metabolite), hydroxymidazolam (metabolite), lorazepam, temazepam (parent drug or metabolite), and zolpidem. Figure 1 depicts the relationship between parent drugs and metabolites that are used in benzodiazepine analysis at Health Network Laboratories. (Baselt, 2011)

Most benzodiazepine metabolites excreted in urine contain glucuronide linkages. These conjugated linkages must be hydrolyzed for acceptable detection of the benzodiazepines with LC/MS/MS. (Webster, 2007) Enzyme hydrolysis has previously been a time consuming process. Formerly, abalone β-glucuronidase was used to hydrolyze the glucuronide linkages. This process required a three hour incubation. The use of the recombinant β-

glucuronidase enzyme, IMCSzyme ™, significantly decreases the amount of time needed for enzyme hydrolysis. (Morris, Chester, Strickland, McIntire, 2014)

Analysis of benzodiazepines does require some type of sample preparation method after the enzyme hydrolysis to remove any particulate in the sample. Previously, solid phase extraction was used as the method of choice. Unfortunately, solid phase extraction requires a great deal of time and the consumption of many solvents and buffers. The use of the recombinant enzyme results in a cleaner sample, which allows for the use of a less stringent filtration method, such as filter vials. Use of the Thomson eXtreme|FV ® 0.2µm PVDF (polyvinylidene fluoride filter vials) significantly decreases the filtration time, as well as alleviating the need for additional solvents and buffers. (Thomson Instrument Company, 2015)

The application of the recombinant β-glucuronidase enzyme, IMCSzyme ™, and the Thomson eXtreme|FV ® was investigated to determine the effectiveness of the method in the analysis of benzodiazepines.

Materials and Methods:

Chemicals and Reagents

The IMCSzyme ™ enzyme kit, containing the recombinant enzyme and rapid hydrolysis buffer, was purchased from IMCS (Integrated Micro-Chromatography Systems). Thomson eXtreme|FV® 0.2µm PVDF with pre-slit red caps and a 48 position vial filter press were purchased from Thomson Instrument Company. HPLC grade methanol was purchased from Fisher Scientific. The 1 mg/mL or 100 µg/mL (Zolpidem) drug solutions used to make the working standards were purchased from Cerilliant Corporation. HPLC grade water was purchased from Acros Corporation. Formic Acid (96%) was purchased from the Sigma-Aldrich Company. Drug free urine was obtained from patient samples identified as negative in a screening method. Urine samples positive for benzodiazepines used for method validation were obtained from patient urine specimens previously identified as positive in a screening method. Control urine samples consisted of drug free urine spiked with the drug solutions obtained from Cerilliant Corporation.

Analytical Procedure

An LC check sample was created using 40% methanol, Standard #1, and the internal standard. The LC check was transferred to an auto sampler vial and set aside.

Internal standard (Table 3) was added to safe lock tubes containing 50 µL of rapid hydrolysis buffer for all samples. The calibration curve was made up of five samples labeled Levels 1 through 5. Levels 1 and 2 were spiked with Standard #1 (Table 1), and Levels 3 through 5 were spiked with Standard #2 (Table 2). The curves contained 75, 300, 1,000, 5,000 and 10,000 ng/mL (Level 1 to Level 5) of each drug, with the exception of Zolpidem. The Zolpidem curve contained the concentrations 75, 300, 500, 2,500, and 5,000 ng/mL (Level 1 to Level 5). All sample tubes were capped and vortexed for 30 seconds at 1750 rpm. After the addition of 40 µL of IMSC β-glucuronidase, the tubes were capped and vortexed for 2 minutes at 1750 rpm. All samples were incubated uncapped at 55°C±2°C for 30 minutes. The tubes were allowed to come to room temperature. The hydrolyzed urine was added to 40% methanol in Thomson eXtreme vials. The Thomson filter plungers were pressed approximately ¼ of the way down, and the tubes were vortexed at 2 minutes at 1750 rpm. The filter plungers were pressed the rest of the way down using the Thomson 48 position press.

Instrument Parameters

The separation was performed using a Shimadzu LC system with a Restek Ultra Biphenyl column (5µm 50 x 2.1 mm). The mobile phases were 0.1% formic acid in HPLC water (A), and 0.1% formic acid in methanol (B), and the flow rate was 0.5000 mL/min. The initial mobile phase condition was 40% B at 0.01 minutes, then 60% B at 1.50 minutes, which was ramped up to 85% B at 6.00 minutes, and further increased to 98% B at 6.80 minutes, and then brought back down to 40% at 6.50 minutes. The program ran for a duration of 8.00 minutes.

Analysis requiring mass spectrometry was done on the ABSciex 3200® with Analyst and Multi Quant 3.0 software. Ionization was carried out by Electron Spray Ionization (ESI), in conjunction with Multiple Reaction Monitoring (MRM). Two MRM transitions were monitored for each analyte and internal standard.

Validation

After a suitable hydrolysis time was established, a three day validation study was used to confirm that the method was suitable for application to patient samples. The validation utilized a calibration curve, limit of detection (LOD) and limit of quantitation (LOQ) samples in triplicate, low and high benzodiazepine concentration controls, and patient samples. Within run and between run accuracy and precision were evaluated. An ion suppression study was performed by adding drugs to urine samples pre extraction and post extraction for comparison.

Results and Discussion

Samples were initially analyzed using LC/MS/MS following a 15 minute hydrolysis at 55°C. Due to discrepancies between the glucuronidated quality control results obtained and those expected, it was concluded that a longer incubation time was needed to fully hydrolyze the glucuronide linkages. Samples were then incubated at 55°C for 30 minutes, which appeared to have hydrolyzed all glucuronide linkages.

Calibration curves were made for each drug using the Level 1 through 5 samples. The calibration curves yielded correlation coefficients in the range of $0.99735 < r < 0.99864$ for each drug. The values for each analyte can be found in Table 4.

Controls spiked with benzodiazepines used in the panel were within ±20% of their target range; meaning that the concentrations used in the calibration curves, and the calculated patient concentrations, were accurate. The percent coefficient of variation for the analytes ranged from 1.8 % - 9.5 % between the three days of validation. This indicates that the amount of dispersion seen in the control concentrations throughout validation was small.

Patient samples were analyzed, and the calculated concentrations were compared to those from the previous method using abalone β-glucuronidase and solid phase extraction. Table 4 contains the mean percent difference in the patient samples for each analyte. The patient concentrations using the new method were within ±20% of the patient concentrations using the older methodology, except for that of lorazepam, but most were greater than the older method indicating possibly more complete removal of the conjugated glucuronides by the new method.

Ion suppression studies indicated that there was no existence of a significant difference in the analyte recovery when the analytes underwent the extraction, compared to samples where the analytes were added post extraction. Table 5 shows that the percent recoveries for each analyte range from 93% to 115%; so the recoveries from each analyte addition method were similar. The percent ion suppression in Table 5 ranges from -11%-11%, which shows that the new methodology does not significantly suppress any analyte ions. Therefore, there are no interfering substances in the urine matrix remaining from the less stringent filtration method.

Conclusion

This method shows the potential for the use of the recombinant β-glucuronidase enzyme, IMCSzyme ™, in conjunction with Thomson eXtreme|FV ® 0.2µm PVDF, in the analysis of benzodiazepines in urine. The use of the recombinant enzyme leads to a more rapid hydrolysis time, and a cleaner sample, in comparison to other enzymes used for glucuronide hydrolysis.(3) The filter vials alleviate the need for solid phase extraction to clean up the samples before instrumental analysis. This method will dramatically decrease the amount of time needed to run benzodiazepine analysis in clinical and forensic toxicology settings.

Acknowledgements

I would like to thank Crystal Xander B.S., who provided direction and guidance throughout this project. I would also like to thank Dean Fritch, Ph.D. for his guidance, and assistance in data analysis.

References

Baselt, R. (2011). *Disposition of Toxic Drugs and Chemicals in Man, 9th Edition*. Seal Beach: Biomedical Publications.

IMCS. (2015). IMCSzyme™ Package Insert Pamphlet. Columbia, SC: IMCS.

Morris, A.A., Chester, S.A., Strickland, E.C., McIntire, G.L. (2014).Rapid Enzymatic Hydrolysis Using a Novel Recombinant β-Glucuronidase in Benzodiazepine Urinalysis. *Journal of Analytical Toxicology*, 38, 610-614.

Thomson Instrument Company. (2015). Filter Vial Catalog. Oceanside, CA: Thomson Instrument Company HTS labs.

Webster, C. (2007). Tandem Mass Spectroscopy Basics [Powerpoint Slides]. Retrieved from Slideshare http://www.slideshare.net/cwebster/tandem-mass-spectroscopy-basics?utm_source=slideshow02&utm_medium=ssemail&utm_campaign=share_slideshow_loggedout

Appendix

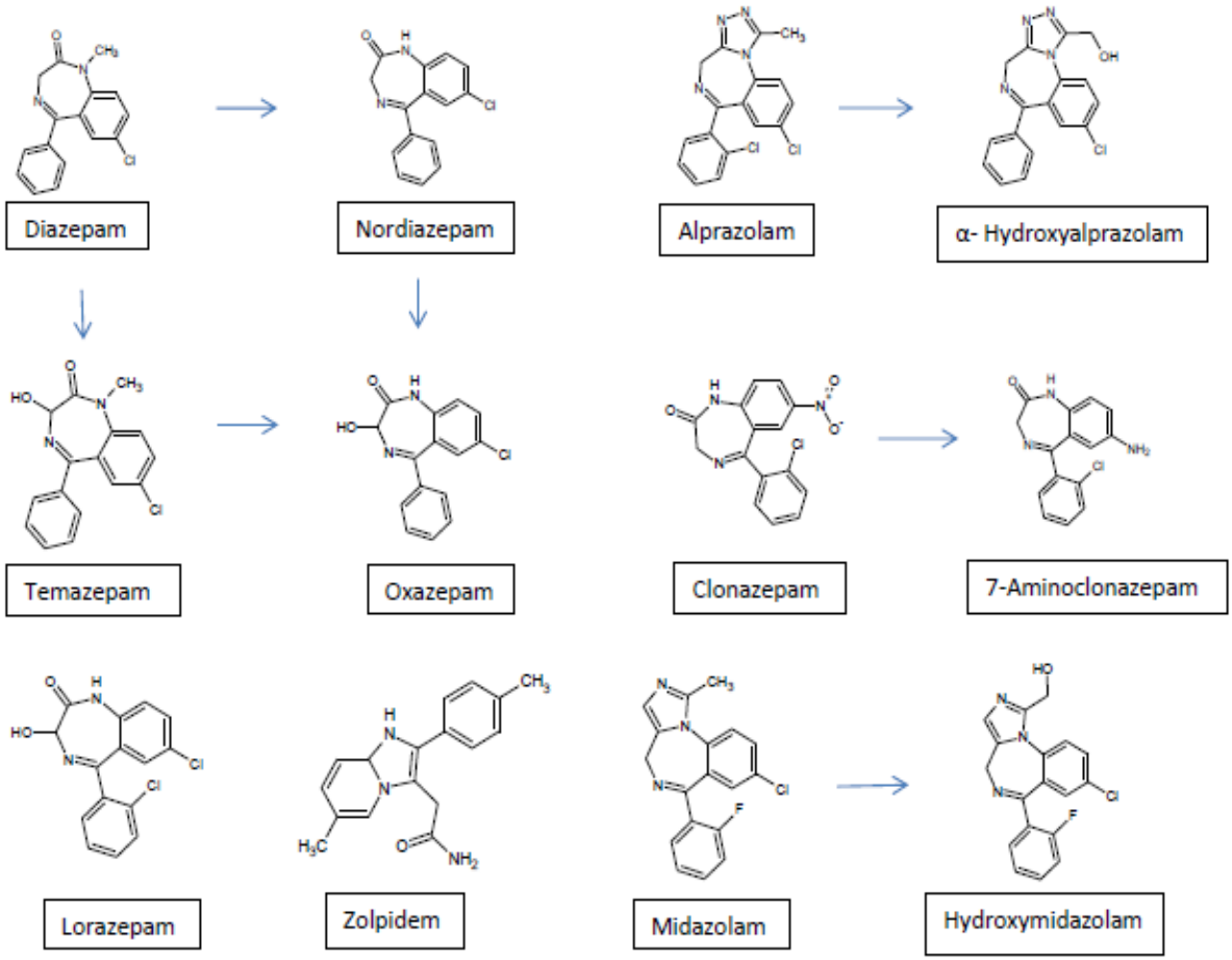

Figure 1- The parent benzodiazepines and their respective metabolites, as well as zolpidem.

Table 1- The benzodiazepines contained in the Standard #1 working solution.

Analyte	Final Concentration in Standard Solution (ng/µL)
Nordiazepam	3
Oxazepam	3
Diazepam	3
Lorazepam	3
Temazepam	3
α-Hydroxymidazolam	3
α- Hydroxyalprazolam	3
7-Aminoclonazepam	3
Zolpidem	3

Table 2- The benzodiazepines contained in the Standard #2 working solution.

Analyte	Final Concentration in Standard Solution (ng/µL)
Nordiazepam	40
Oxazepam	40
Diazepam	40
Lorazepam	40
Temazepam	40
α-Hydroxymidazolam	40
α- Hydroxyalprazolam	40
7-Aminoclonazepam	40
Zolpidem	20

Table 3- The deuterated benzodiazepines contained in the Internal Standard working solution.

Analyte	Final Concentration in Standard Solution (ng/µL)
Nordiazepam d_5	4
Oxazepam d_5	4
Diazepam d_5	4
Lorazepam d_4	4
Temazepam d_5	4
α- Hydroxyalprazolam d_5	4
7-Aminoclonazepam d_4	4
Zolpidem d_6	0.4

Table 4- The limit of detection (LOD), limit of quantitation (LOQ), linearity, correlation coefficients, expected control concentrations, percent coefficients of variation (%CV), patient comparison percent differences, and patient sample sizes for each analyte.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Linearity (ng/mL)	Correlation Coefficient	Expected Control Concentrations	% CV	Mean Patient Samples % Difference	# of Patients Tested
Diazepam	37.5	75	10,000	0.99829	110	4.2	N/A	0
					500	1.8		
α-Hydroxyalprazolam	37.5	75	10,000	0.99838	110	7.0	-5.05	4
					500	3.5		
Lorazepam	37.5	75	10,000	0.99864	110	5.7	24.2	1
					500	4.9		
Nordiazepam	37.5	75	10,000	0.99818	110	9.5	2.23	3
					500	2.4		
Oxazepam	37.5	75	10,000	0.99735	110	3.2	7.63	4
					500	4.7		
Temazepam	37.5	75	10,000	0.99782	110	6.8	2.42	3
					500	7.0		
Hydroxymidazolam	37.5	75	10,000	0.99808	N/A	N/A	-0.80	1
					N/A	N/A		
7-Aminoclonazepam	37.5	75	10,000	0.99810	N/A	N/A	8.87	3
					N/A	N/A		
Zolpidem	37.5	75	5,000	0.99746	110	1.8	18.50	1
					500	2.0		

Table 5- The percent recovery and percent ion suppression for each analyte.

Analyte	% Recovery	% Ion Suppression
Diazepam	94	6
α-Hydroxyalprazolam	89	11
Lorazepam	91	9
Nordiazepam	94	5
Oxazepam	92	8
Temazepam	91	8
Hydroxymidazolam	111	-11
7-Aminoclonazepam	99	0.6
Zolpidem	107	-6