Rapid Hydrolysis of Benzodiazepines in Urine

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Objective

Determine a more time effective method for the analysis of benzodiazepines in human urine.

- Use the recombinant β-glucuronidase enzyme, IMCSzyme ™, to reduce enzyme hydrolysis by 2.5 hours.
- Use Thomson eXtreme|FV ® 0.2µm PVDF to reduce sample preparation time.

Methods

Quality Control

The patient samples and control urines were run with a 5 point calibration curve. The curves were spiked with concentrations of 75, 300, 1,000, 5,000, and 10,000 ng/mL of all drugs except Zolpidem. The Zolpidem curve consisted of concentrations of 75, 300, 500, 2,500, and 5,000 ng/mL. The calibrators were obtained from 1 mg/mL standards or 100 µg/mL standards (Zolpidem) purchased from Cerilliant Corporation.

Sample Preparation

Urine specimens were hydrolyzed using a combination of 50µL of IMCSzyme™ rapid hydrolysis buffer and 40µL of ICMSzyme™ enzyme per sample at 55°C for 30 minutes. Thomson eXtreme|FV ® 0.2µm PVDF containing 40% methanol in HPLC water and 50µL of hydrolyzed urine were pressed to filter out any particulate in the samples. Internal standards were incorporated into every sample. Patient samples were prepared, in addition to controls and calibration curve samples.

LC/MS/MS

An ABSciex 3200® MS/MS coupled with a Shimadzu Prominence LC system was used for analysis of the samples. Electrospray Ionization (ESI) was used in conjunction with Multiple Reaction Monitoring (MRM) for mass spectrometry analysis.

Table 1- 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 number of patients tested for each analyte.

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Linearity (ng/mL)	Correlation Coefficient	Expected Control Concentrations	% CV	Mean Patient Comparisons % 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	24.20	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	0.9981	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		

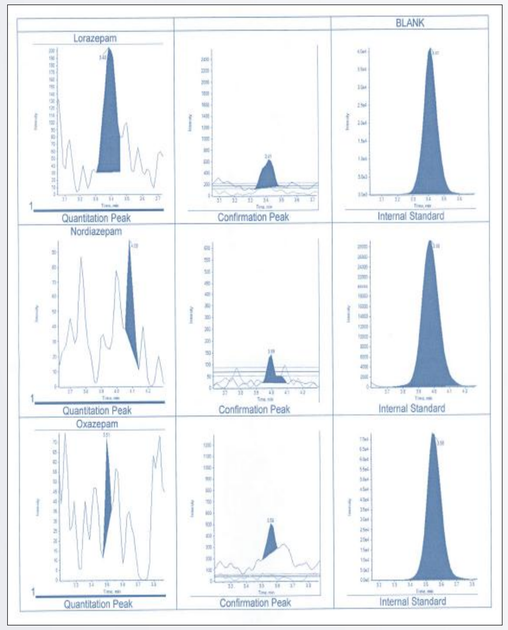

Figure 1

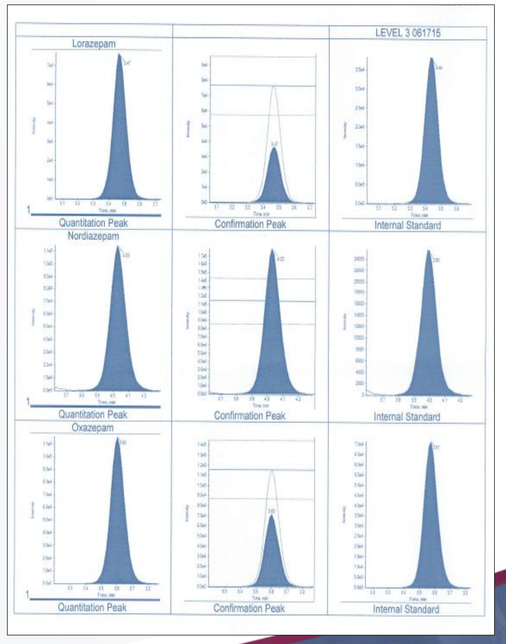

Figure 2

Results

Validation studies were run on three different days. Method validation was accomplished using a calibration curve, LOD and LOQ samples run 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 also done to compare analyte recovery with the extraction method versus adding the analyte post extraction. The percent recovery ranged from 91%-111%, and the ion suppression ranged from -11%-11% for the analytes.

Figure 1 shows chromatograms for a negative result for the drugs lorazepam, nordiazepam, and oxazepam.

Figure 2 shows chromatograms for a positive result for the drugs lorazepam, nordiazepam, and oxazepam

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. This method will dramatically decrease the amount of time needed to run benzodiazepine analysis in clinical and forensic toxicology settings.

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