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LC-MS/MS Analysis of Benzodiazepines in Urine with Room Temperature Hydrolysis and One-Step Clean Up Using Beta™-Gone

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Introduction

Benzodiazepines are a class of depressants that are used for sedation, to relieve anxiety, and reduce muscle spasms and seizures. Commonly prescribed benzodiazepines include Valium® (Diazepam) Xanax® (alprazolam), Halcion® (triazolam), Ativan® (lorazepam) and Klonopin® (clonazepam). These controlled substances can be habit forming. Misuse and dependence are a concern with these drugs. Toxicology labs quantitate benzodiazepines for drug monitoring, drug facilitated crime and impairment investigations, and cause of death determinations. Many labs use LC-MS/MS for analysis of benzodiazepines, due to its sensitivity and specificity. Modern LC-MS/MS instrumentation and chromatography using superficially porous "core-shell" LC columns can generate fast methods for high-throughput drug analysis workflows.

Urine is one of the most commonly used specimens for drug testing methods. Benzodiazepines are extensively metabolized via methylation and hydroxylation. They are also heavily conjugated prior to excretion in the urine. Therefore, most urine methods hydrolyze the urine prior to extraction and analysis by LC-MS/MS to convert glucuronide conjugates back to the parent drug or metabolite. Sample clean up to remove residual hydrolysis enzyme and other matrix components improves quantitation by LC-MS/MS. SPE and other extraction methods require multiple steps and significant method development time. They also require more time in a drug analysis workflow. Beta-Gone is a simple, single step extraction that cleans up hydrolyzed urine specimens. It requires no method development and can be easily incorporated into a urine toxicology drug testing workflow.

Sample Preparation

Eight calibrators at 20, 40, 100, 200, 500, 1000, 2000 and 2500 ng/mL, and three QC samples at 50, 125, and 800 ng/mL prepared in drug free urine were analyzed. Internal standard concentrations were 100 ng/mL for all analytes. All calibration curves used a quadratic fit, except lorazepam which used a linear calibration fit. All calibration curves were weighted 1/x. MRM parameters are listed in **Table 1.** Oxazepam glucuronide was used to measure hydrolysis efficiency.

For each sample, 50 µL of internal standard solution were added to 100 µL of urine. Next, 200 µL Kura® B-One® enzyme were added, and the samples were incubated for 30 min at room temperature. Samples were filtered through Beta-Gone 1 mL cartridges (part number 8B-S139-TAK) into a vial prior to analysis by LC-MS/MS.

LC Conditions

Column: Kinetex™ Phenyl-Hexyl
Dimensions: 50 x 4.6 mm, 2.6 µm
Part No.: 00B-4495-E0
Mobile Phase: A= 10mM Ammonium Formate and 0.05% Formic Acid
B= Methanol and 0.05% Formic Acid

Gradient:

Time (min)	% B
0.0	10
4.5	100
5.5	100
6.1	10
7.5	10

Flow Rate: 0.6 mL/min
Injection Volume: 3 µL
Temperature: 40 °C
LC System: Agilent® 1200 Series LC
Detection: LC-MS/MS
Detector: Sciex® 5500 Triple Quad™

MS/MS Conditions

Ion Source: Turbo Spray
Polarity: ESI Positive Mode
Source Temperature: 650 °C
GS1: 60
GS2: 50
CUR: 40
IS: 2500 V

Have questions or want more details on implementing this method? We would love to help!
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Table 1. MRM Conditions Retention Times, Recovery and Matrix Effect for Benzodiazepines

Analyte or Internal Standard	Retention Time (min)	% Recovery	Matrix Effect	Q1	Q3	Dwell	DP	CE	CXP
7-aminoclonazepam-d4	3.69	-	-	290.10	254.00	15.00	65.00	29.00	13.00
				290.10	226.00	15.00	65.00	26.00	13.00
7-aminonitrazepam	3.48	107%	44%	252.10	121.00	15.00	40.00	23.00	35.00
				252.10	146.00	15.00	40.00	25.00	53.00
7-aminoclonazepam	3.7	103%	41%	286.10	250.00	15.00	80.00	19.00	13.00
				286.10	222.00	15.00	80.00	27.00	13.00
7-aminoflunitrazepam	3.94	94%	29%	284.10	135.10	15.00	51.00	23.00	39.00
				284.00	227.20	15.00	51.00	23.00	49.00
alpha-OH Alprazolam-d5	4.74	-	-	330.10	302.20	15.00	51.00	30.00	31.00
				330.10	284.00	15.00	51.00	36.00	31.00
alpha-OH Midazolam	4.8	96%	30%	342.10	203.10	15.00	60.00	26.00	10.00
				342.10	168.10	15.00	60.00	43.00	11.00
alpha-OH Triazolam	4.62	106%	49%	359.10	331.10	15.00	90.00	27.00	12.00
				359.10	239.00	15.00	90.00	50.00	12.00
alpha-OH Alprazolam	4.75	103%	34%	325.10	297.10	15.00	60.00	30.00	15.00
				325.10	299.10	15.00	60.00	25.00	15.00
Oxazepam-d5	4.74	-	-	292.20	246.20	15.00	30.00	20.00	35.00
				292.20	274.00	15.00	30.00	19.00	35.00
Oxazepam	4.75	100%	27%	287.10	241.10	15.00	76.00	31.00	18.00
				287.10	269.20	15.00	76.00	14.00	22.00
Triazolam-d4	4.75	-	-	347.20	312.20	15.00	90.00	30.00	50.00
				347.20	243.00	15.00	90.00	40.00	50.00
Lorazepam	4.68	109%	34%	321.10	275.10	15.00	80.00	30.00	10.00
				321.10	229.10	15.00	80.00	30.00	10.00
Temazepam-d5	4.91	-	-	306.00	260.10	15.00	70.00	15.00	35.00
				306.00	177.00	15.00	70.00	40.00	35.00
2-OH Ethylflurazepam	4.74	100%	29%	333.10	211.20	15.00	51.00	25.00	53.00
				333.10	109.20	15.00	56.00	29.00	41.00
Temazepam	4.92	102%	34%	301.10	255.10	15.00	70.00	15.00	8.00
				301.10	177.10	15.00	70.00	40.00	10.00
Nordiazepam-d5	4.95	-	-	276.10	140.00	15.00	100.00	30.00	15.00
				276.10	213.10	15.00	100.00	30.00	15.00
Nordiazepam	4.96	106%	22%	271.10	140.10	15.00	100.00	30.00	15.00
				271.10	165.10	15.00	100.00	30.00	15.00

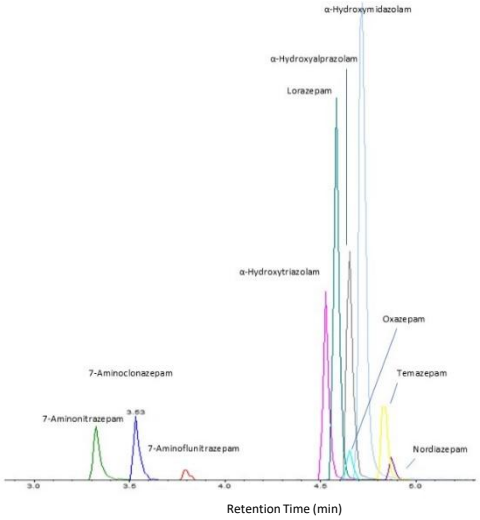
Results and Discussion

Analyte retention time, matrix effect and recovery are reported in **Table 1**. Matrix effect was between 29% and 49%. Recovery ranged from 96% to 109% with %CVs of ±15% for analytes with a corresponding isotopically labeled internal standard, and within ±20% for analytes without a matching internal standard (n = 12). **Figure 1** shows the separation of 11 benzodiazepines using a the Phenomenex Kinetex™ Phenyl-Hexyl LC column. Hydrolysis efficiency was 100%.

Conclusions

The fast 30-minute room temperature hydrolysis using Kura B-One® and a simple one step extraction using Beta-Gone™ produced very clean samples with high recovery, low matrix effect and excellent hydrolysis efficiency. The fast 5-minute LC separation and LC-MS/MS analysis using a SCIEX® 5500 MS/MS system provided accurate and reliable quantitation of benzodiazepines in urine.

Figure 1. Separation of benzodiazepines on a Kinetex Phenyl-Hexyl column



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