LC/MS/MS ANALYSIS OF URINARY BENZODIAZEPINES AND Z-DRUGS VIA A SIMPLIFIED, MIXED-MODE SAMPLE PREPARATION STRATEGY

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INTRODUCTION

Benzodiazepines are commonly prescribed drugs used for their sedative, anxiolytic, and hypnotic properties.[1] Nationally, overdose deaths from benzodiazepines have risen 600% from under 1,600/year in 2001 to 8,000 in 2014, greater than any other drug class with the exception of heroin.[2] So-called "Z-drugs" (zolpidem and zopiclone) are commonly used sleep aids that act in a similar manner to benzodiazepines.[1] While the use of LC/MS/MS for benzodiazepine analysis has increased in recent years, many published techniques still rely on labor intensive liquid-liquid extraction techniques.[3-5]

The objective of this study was to develop a simplified sample preparation and LC/MS/MS analysis strategy for these compounds. Strong cation exchange micro elution plates were used to rapidly extract these compounds from urine samples. All sample preparation steps, including enzymatic hydrolysis, were performed within the wells of the µElution plates, and the extraction method is simplified by eliminating conditioning and equilibration steps.

This method analyzes 18 benzodiazepine drugs and metabolites, along with zolpidem, zopiclone and N-desmethyl zopiclone.

METHODS

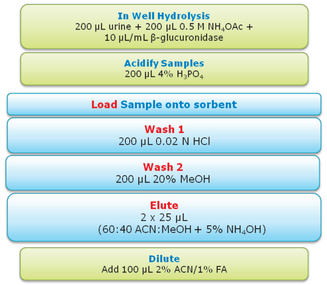
Extraction Procedure

200 µL of urine was added to individual wells of a Waters Oasis® MCX µElution SPE plate, along with internal standards, hydrolysis buffer and *β*-glucuronidase enzyme. 20 deuterated internal standards were used for quantification. Samples were incubated for 1 hr. at 50 °C. After incubation, samples were quenched with 200 µL of 4% H_3PO_4 and directly loaded onto the sorbent bed by vacuum. All samples were subsequently washed with 200 µL of 0.02 N HCl, and 200 µL of 20% MeOH. Samples were eluted with 2 x 25 µL of 60:40 ACN:MeOH containing 5% strong ammonia solution and then diluted with 100 µL of sample diluent (2% ACN:1% formic acid in water).

5 µL of each sample was injected and analyzed by UPLC/MS/MS using a Waters' Cortecs C18+ column (1.6 µm; 2.1 x 100) and a Xevo® TQ-S micro mass spectrometer.

Calibrators were prepared in blank urine at concentrations ranging from 0.5-500 ng/mL. Quality control samples were prepared at 4 concentrations than covered the calibration range.

Figure 1. Extraction procedure for benzodiazepines and Z-drugs



LC Conditions

LC System	ACQUITY I-Class (FL)
Column	Waters CORTECS C18+ Column, 1.6 µm, 2.1 x 100 mm
Column Temp	30 °C
Sample Temp	10 °C
Injection Vol.	5 µL
Flow Rate	0.5 mL/min
Mobile Phase A	0.01% formic acid in water
Mobile Phase B	0.01% formic acid in ACN

Table 1. Mobile phase gradient

Time (min)	Flow (mL/min)	% MPA	% MPB
initial	0.5	90	10
5.00	0.5	50	50
5.25	0.5	5	95
6.00	0.5	5	95
6.10	0.5	90	10
7.50	0.5	90	10

MS Conditions

MS System	Waters Xevo® TQ-S micro
Ionization Mode	ESI Positive
Capillary Voltage	0.5 kV
Desolvation Temp	500 °C
Desolvation Flow	150 L/hr
Source Temp	150 °C
MRM Conditions	Optimized for individual compounds

MS Conditions and Retention Times

Waters Xevo® TQ-S micro

	Compound	RT	M+H+	MRM Product Ions	Cone Voltage	Collision Energy
1	N-desmethyl Zopiclone	1.06	375.1	245.0	6	14
2	Zopiclone	1.12	389.1	245.0	8	12
3	Zolpidem	1.61	308.1	235.1	34	32
4	7-amino-clonazepam	1.91	286.1	121.0	50	26
5	Flurazepam	2.31	388.2	315.1	40	26
6	7-amino-flunitrazepam	2.35	284.1	135.0	34	26
7	Chlordiazepoxide	2.34	300.0	227.0	34	20
8	Midazolam	2.53	326.0	291.0	16	36
9	α-OH-midazolam	2.90	342.0	203.0	2	24
10	α-OH-triazolam	3.76	359.0	176.0	28	24
11	α-OH-alprazolam	3.77	325.1	297.1	50	25
12	Oxazepam[1]	3.84	289.0	103.9	50	30
13	Nitrazepam	3.86	282.1	180.1	50	36
14	Lorazepam	4.00	321.0	277.0	50	20
15	Clonazepam	4.09	316.0	214.1	54	42
16	Alprazolam	4.35	309.1	205.0	50	40
17	Nordiazepam	4.36	271.0	140.0	50	30
18	Flunitrazepam	4.41	314.1	239.2	50	30
19	Temazepam	4.44	301.1	177.0	36	46
20	Triazolam	4.47	343.0	308.0	28	24
21	Diazepam	5.13	285.1	154.0	50	26

Table 2. Individual MS conditions and retention times

RESULTS

Chromatography

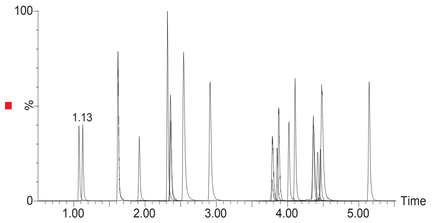


Figure 2. Chromatography of benzodiazepines and Z-drugs from an extracted calibration standard. Conditions are detailed in Materials and Methods. Retention times are listed in Table 2.

Separation of critical pairs

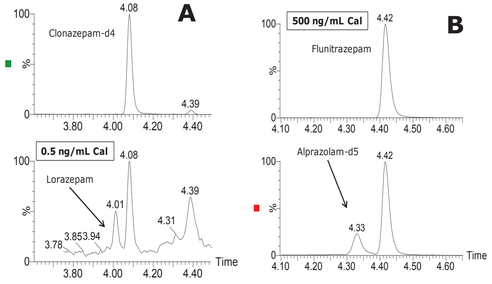


Figure 3. Selected chromatograms from the Cortecs C18+ column. **A.** Clonazepan-d4 does not interfere with lorazepam, even at the lowest calibrator. **B.** Flunitrazepam, even at 500 ng/mL does not interfere with alprazolam-d5.

Extraction Recovery

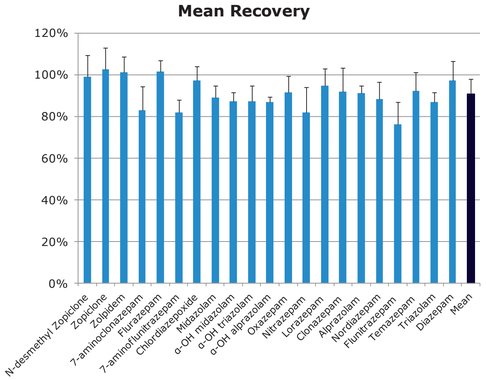


Figure 4. Extraction recovery of benzodiazepines and Z-drugs using Oasis MCX µElution plates. *N* = 4 separate extractions

Representative Calibration Curves

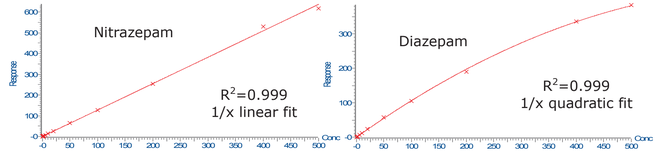


Figure 5. Calibration curves ranged from 0.5-500 ng/mL. The minimum r^2 value was 0.997 and all mean % deviations from the curves were less than 10%.

Inter-batch Quality Control Results

	QC 1.5		QC 7.5		QC 75		QC 300		
Name	**Mean**	**%CV**	**Mean**	**%CV**	**Mean**	**%CV**	**Mean**	**%CV**	**Mean**
N-desmethyl zopiclone	99.2%	3.8%	96.7%	2.4%	96.6%	2.9%	97.1%	4.7%	**97.4%**
Zopiclone	97.7%	3.2%	96.7%	3.4%	98.0%	2.8%	96.2%	3.5%	**97.2%**
Zolpidem	99.4%	3.4%	98.8%	1.5%	95.8%	1.1%	91.7%	1.6%	**96.4%**
7-aminoclonazepam	100.4%	1.9%	95.6%	1.0%	93.8%	2.4%	95.1%	2.0%	**96.2%**
Flurazepam	103.6%	7.1%	97.6%	4.3%	99.3%	7.4%	97.6%	5.0%	**99.5%**
7-aminoflunitrazepam	99.3%	2.3%	93.7%	2.3%	96.1%	4.7%	97.0%	3.2%	**96.5%**
Chlordiazepoxide	100.5%	1.1%	100.3%	2.1%	99.3%	1.5%	98.4%	3.2%	**99.6%**
Midazolam	103.7%	4.4%	104.2%	5.4%	102.1%	3.1%	98.9%	2.0%	**102.2%**
α-OH midazolam	103.4%	4.3%	102.5%	4.7%	100.8%	5.0%	99.1%	2.5%	**101.4%**
α-OH triazolam	101.5%	8.4%	98.8%	4.9%	98.3%	4.9%	95.1%	2.6%	**98.4%**
α-OH alprazolam	104.4%	9.6%	101.4%	2.2%	99.1%	5.9%	97.7%	2.4%	**100.7%**
Oxazepam	100.4%	4.3%	98.5%	4.1%	98.2%	4.7%	97.6%	4.6%	**98.7%**
Nitrazepam	102.0%	6.2%	95.8%	1.3%	95.7%	2.4%	98.1%	1.8%	**97.9%**
Lorazepam	100.3%	6.9%	100.2%	4.2%	100.8%	5.4%	98.7%	4.9%	**100.0%**
Clonazepam	102.0%	4.9%	98.2%	3.0%	97.5%	3.3%	95.2%	4.5%	**98.2%**
Alprazolam	107.0%	8.7%	94.6%	4.7%	95.0%	4.6%	98.8%	4.5%	**98.9%**
Nordiazepam	106.1%	9.0%	106.7%	3.7%	101.7%	4.6%	95.4%	5.2%	**102.5%**
Flunitrazepam	101.8%	8.1%	98.2%	2.8%	96.3%	2.6%	96.3%	7.8%	**98.1%**
Temazepam	102.9%	7.3%	101.6%	1.2%	97.5%	2.8%	94.7%	1.8%	**99.2%**
Triazolam	104.4%	8.4%	102.4%	2.3%	99.9%	3.2%	98.2%	3.4%	**101.2%**
Diazepam	104.3%	6.5%	103.8%	2.1%	99.6%	4.1%	94.9%	7.6%	**100.6%**
Mean	**102.1%**		**99.3%**		**98.2%**		**96.8%**		

Table 3. Quality control results from 4 separately extracted batches. Mean values show the average for each compound and the average for all compounds at each QC level. Individual batches had accuracies mostly within 10% of target values and % CVs under 10%

CONCLUSIONS

- Accurate, quantitative analysis of a broad panel of benzodiazepines and z-drugs
- Rapid, simplified sample preparation of urinary benzodiazapines
- Baseline separation of all critical analyte pairs
- All sample pretreatment and extraction performed in-well, eliminating transfer steps
- Concentration on the SPE device. No need for evaporation and reconstitution
- High and consistent recovery for all compounds
- Excellent accuracy and reproducibility

References

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