

Designer and Pharmaceutical Benzodiazepine Analysis from Urine using In-Well Hydrolysis Solid Phase Extraction (SPE) and LC-MS/MS

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Overview

Designer benzodiazepines are a rapidly increasing threat as an alternative to prescription benzodiazepines and due to their complex structures and sample matrices, clean-up techniques can be difficult to develop. Designer benzos provide many analytical challenges due to the limited knowledge of their metabolites and the similarities to traditional pharmaceutical benzodiazepines, such as Diazepam and its metabolite, Lorazepam. This can make clean-up and separation a problematic step in the method development process.

In traditional sample preparation methods, the hydrolysis step must be performed prior to the extraction step and analytes must be converted back to their non-conjugated form, which often requires long wait time and high temperatures by either ovens or water baths. Once the hydrolysis has been performed, samples must be transferred to an extraction product which could cause cross-contamination or loss of sample in the transfer step. By utilizing a room temperature beta-glucuronidase enzyme that is designed for fast hydrolysis without the need of high temperatures and by using solid phase extraction (SPE), this can save significant time in the sample preparation workflow. Strata®-X Drug B Plus is a mixed-mode polymeric SPE product with in-well hydrolysis capabilities, saving significant sample processing time and additional lab equipment needs. Utilizing Strata-X Drug B Plus as a SPE solution also does not require condition or equilibration steps, further reducing the time spent on sample preparation while not sacrificing high recoveries of analytes or removal of matrix interferences.

The objective of this project was to develop a fast and complete quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) method utilizing a room temperature hydrolysis combined with an advanced SPE product for the analysis of designer and pharmaceutical benzodiazepines in urine.

Materials and Methods

Reagents and Chemicals

Alprazolam, N-desmethyloclobazam, Clonazepam, 7-amino-clonazepam, Clonazolam, Diazepam, Estazolam, Etizolam, Flualprazolam, Flubromazepam, Flubromazolam, Flunitrazolam, Lorazepam, Nordiazepam, Oxazepam, Phenazepam, and Temazepam reference standards as well as Diazepam-D5, Nordiazepam-D5, Oxazepam-D5, Temazepam-D5 deuterated internal standards were purchased from Cayman Chemical (Ann Arbor, MI). Clobazam and Deschloretizolam reference standards and Clobazam-13C6 internal standard were purchased from Cerilliant® Corporation (Round Rock, TX). Optima LC-MS grade formic acid, water, and methanol were ordered from Fisher Scientific (Pittsburgh, PA). Beta-glucuronidase enzyme was purchased from IMCS Inc. (Irmo, SC). Certified drug free urine was sourced from UTAK (Valencia, CA).

Sample Preparation

Pre-treatment

Combine 100 µL spiked urine, 30 µL IMCS Room Temp buffer, 270 µL water, and 10 µL of IMCSzyme RT onto the plate. Incubate for 15 minutes at room temperature.

Solid Phase Extraction Method

96-Well Plate: Strata-X Drug B Plus, 30 mg/well
Part No.: 8E-S128-TGB-P
Condition: Not required
Equilibration: Not required
Load: After incubation time is up, switch vacuum manifold on quickly to facilitate flow through the SPE sorbent
Wash 1: 1 mL of 1.0 % Formic acid in Water
Wash 2: 1 mL of Water/Methanol (70:30)
Dry: 10 minutes at high vacuum
Elute: 2x 0.5 mL 5 % Ammonium hydroxide in Methanol/Acetonitrile (50:50)
Dry Down: To dryness at 40 °C under a gentle stream of Nitrogen
Reconstitute: 100 µL 0.1 % Formic acid in Water/0.1 % Formic acid in Methanol (85:15)

HPLC Conditions

Column: Kinetex® 2.6 µm Biphenyl
Dimensions: 100 x 3.0 mm
Part No.: 00D-4622-Y0
Mobile Phase: A: 0.1 % Formic acid in Water
B: 0.1 % Formic acid in Methanol

Gradient:	Time (min)	% B
	0	60
	1	60
	2	70
	5	95
	6	95
	6.1	60
	8	60

Flow Rate: 0.6 mL/min
Injection Volume: 1 µL
Temperature: 40 °C
System: Agilent® 1260
Detection: MS/MS (SCIEX® Triple Quad™ 4500, ESI+)
Detector: 6500 QTRAP® (SCIEX)
Backpressure: ~160 bar (250 bar at max)

MS/MS Conditions

Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	ID	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
286.1	121	50	7-Aminoclonazepam (1)	85	10	35	12
286.1	222.2	50	7-Aminoclonazepam (2)	85	10	35	16
309.1	205.1	50	Alprazolam (1)	0	10	55	6
309.1	281.1	50	Alprazolam (2)	85	10	30	10
313	314.2	50	Alprazolam-D5	85	10	10	6
301.1	259.1	50	Clobazam	80	10	35	8
307	265	50	Clobazam-13C6	80	10	25	20
316.2	270.2	50	Clonazepam (1)	75	10	35	20
316.2	214.2	50	Clonazepam (2)	70	10	50	6
354	308	50	Clonazolam (1)	80	10	35	20
354	280	50	Clonazolam (2)	85	10	45	18
309.1	280.1	50	Deschloretizolam (1)	80	10	30	10
309.1	225.1	50	Deschloretizolam (2)	85	10	45	12
285.3	193.2	50	Diazepam (1)	40	10	40	12
285.3	154.2	50	Diazepam (2)	80	10	35	10
290.2	154.2	50	Diazepam-D5	85	10	40	12
319	227	50	Diclazepam (1)	85	10	40	16
319	154	50	Diclazepam (2)	85	10	40	10
295	205	50	Estazolam (1)	50	10	45	14
295	267.1	50	Estazolam (2)	45	10	35	10
343.1	314.1	50	Etizolam (1)	85	10	30	10
343.1	138.2	50	Etizolam (2)	80	10	40	10
327	299	50	Flualprazolam (1)	85	10	30	10
327	223	50	Flualprazolam (2)	40	10	50	14
333	226	50	Flubromazepam (1)	0	10	35	14
333	206	50	Flubromazepam (2)	0	10	50	10
333	184	50	Flubromazepam (3)	40	10	50	12
371	292.1	50	Flubromazolam (1)	85	10	35	10
371	223	50	Flubromazolam (1)	80	10	35	14
338	292	50	Flunitrazolam (1)	45	10	35	10
338	264	50	Flunitrazolam (2)	40	10	45	14
321.3	275.3	50	Lorazepam (1)	0	10	35	14
321.3	229.1	50	Lorazepam (2)	70	10	40	14
287	245	50	N-Desmethyloclobazam	80	10	25	8
271.3	140.3	50	Nordiazepam (1)	0	10	40	10
271.3	164.9	50	Nordiazepam (2)	40	10	35	12
276.3	140.1	50	Nordiazepam-D5	65	10	35	10
287.1	241.1	50	Oxazepam (1)	75	10	30	12
287.1	246.3	50	Oxazepam (2)	75	10	20	10
292.3	246.3	50	Oxazepam-D5	65	10	20	12
351	179	50	Phenazepam	70	10	55	12
301.3	255.2	50	Temazepam (1)	70	10	30	8
301.3	177.1	50	Temazepam (2)	40	10	50	6

Table 1.
Analyte Retention Times

Analyte Name	Retention Time (min)
7-Aminoclonazepam	1.47
Alprazolam ⁺	5.15
Alprazolam-D5	5.15
Clobazam [*]	4.79
Clobazam-13C6	4.79
Clonazepam	4.21
Clonazolam	4.51
Deschloretizolam ⁺	5.66
Diazepam	5.56
Diazepam-D5	5.52
Diclazepam	5.33
Estazolam	5.00
Etizolam	5.57
Flualprazolam	4.83
Flubromazepam	4.49
Flubromazolam	5.01
Flunitrazolam	4.31
Lorazepam	3.93
N-Desmethyloclobazam [^]	4.03
Nordiazepam	4.71
Nordiazepam-D5	4.67
Oxazepam [^]	4.21
Oxazepam-D5	4.18
Phenazepam	4.67
Temazepam [*]	5.08

Legend:

* Isomeric Pair

+ Isobaric Pair

^ Isomeric Pair

Parameter	Setting
Curtain Gas (CUR)	20 psi
Collision Gas (CAD)	6 psi
IonSpray Voltage (IS)	4500 V
Temperature (TEM)	550 °C
Ion Source Gas 1 (GS1)	40 psi
Ion Source Gas 2 (GS2)	60 psi

Figure 1.
Representative Chromatogram, 400 ng/mL Neat Standard

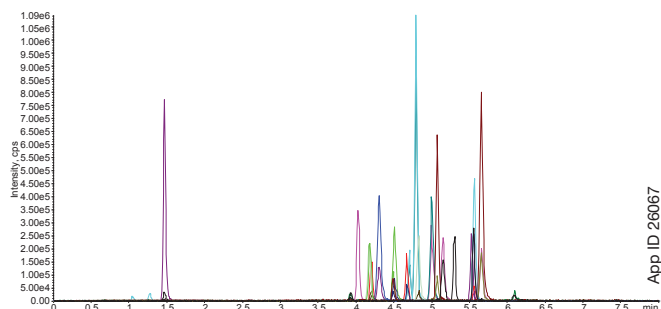


Figure 2.
Oxazepam (1,000 ng/mL) and N-desmethyloclobazam (50 ng/mL) in Urine

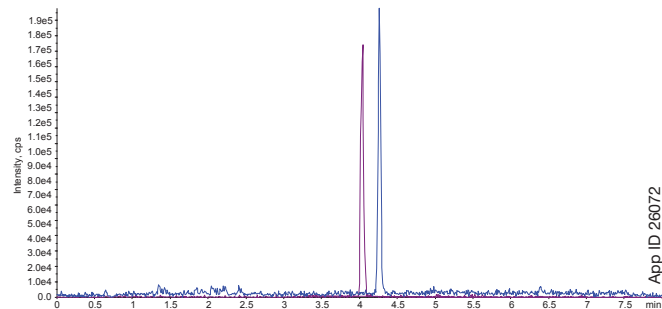


Figure 3.
Clobazam (10 ng/mL) and Temazepam (25 ng/mL) in Urine

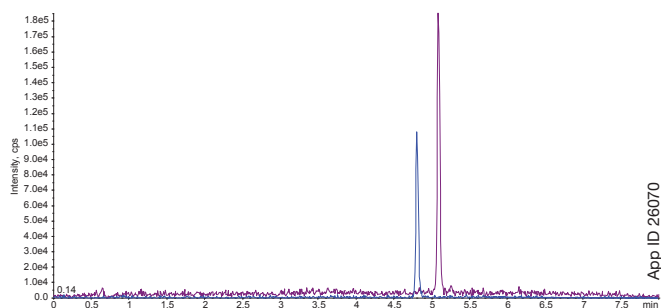


Figure 4.
Oxazepam (1,000 ng/mL) and N-desmethyloclobazam (50 ng/mL) in Urine

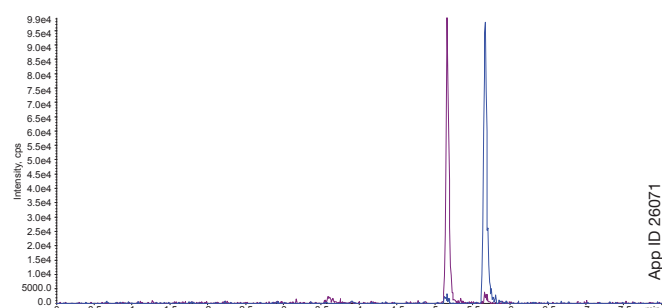


Table 2.
Calibration Curves (5 to 1,000 ng/mL)

Analyte	Calibrator Concentration (ng/mL)							
	5	10	25	50	100	250	500	1,000
7-Aminoclonazepam Accuracy (%)	80	80.2	102	102	98.1	104	96.4	95.2
Alprazolam Accuracy (%)	89	99.8	104	110	99.7	103	100	101
Clobazam Accuracy (%)	81.2	82.7	101	112	103	104	100	99.6
Clonazepam Accuracy (%)	103	95.9	96.4	101	95.1	102	93.2	103
Clonazolam Accuracy (%)	89.4	101	101	105	97.1	99.2	97.9	99
Deschloretizolam Accuracy (%)	84.3	94.4	98.2	115	107	104	100	99.4
Diazepam Accuracy (%)	109	86.4	102	115	102	99.6	100	99.6
Estazolam Accuracy (%)	84.7	105	102	107	102	100	97.8	97.8
Etizolam Accuracy (%)	79.8	98.7	106	111	104	103	98.8	101
Flualprazolam Accuracy (%)	81.8	106	103	110	101	106	100	101
Flubromazepam Accuracy (%)	91.3	99.1	99.8	103	96.2	101	97.7	102
Flubromazolam Accuracy (%)	80	87.1	117	118	109	97.3	102	101
Flunitrazolam Accuracy (%)	90	88.8	100	106	99.5	106	97.3	98.6
Lorazepam Accuracy (%)	108	119	109	116	99.5	104	107	101
N-Desmethyloclobazam Accuracy (%)	102	89.3	98.9	102	94.9	101	95.2	102
Nordiazepam Accuracy (%)	89.8	119	96.9	98.2	98.6	100	96.9	98
Oxazepam Accuracy (%)	117	104	119	98.1	99.2	104	107	98.7
Phenazepam Accuracy (%)	119	70.8	93.1	104	96.1	95.3	95.8	102
Temazepam Accuracy (%)	93.5	87.2	98.7	104	97.3	99.7	98.6	98.5

Table 3.
Quality Controls

Analyte	QC Low (2.0 ng/mL)				QC High (250 ng/mL)			
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
7-Aminoclonazepam	98.3	84.8	102	10	99.3	97.2	93.8	3
Alprazolam	100	102	98.7	2	98.8	96.9	90.8	4
Clobazam	111	112	106	3	99.7	103	103	2
Clonazepam	88.3	88.1	85.8	2	91.3	91.9	90.2	1
Clonazolam	91.4	81.7	99.5	10	89	88.4	90.8	1
Deschloretizolam	87.8	107	115	14	97.4	102	103	3
Diazepam	88.5	85.9	79.9	5	95.9	91.2	92.3	3
Estazolam	90.8	91.8	94.5	2	100	96.5	94	3
Etizolam	90	107	118	13	98.2	101	102	2
Flualprazolam	82.8	103	106	13	100	102	104	2
Flubromazepam	85.2	108	114	15	98	98.6	101	2
Flubromazolam	97.1	91.1	96.7	4	95.5	97	106	6
Flunitrazolam	90.4	101	112	11	89.2	99	87.3	7
Lorazepam	97.6	117	122	11	101	101	94.2	4
N-Desmethyloclobazam	93.2	88.4	87.7	3	91.9	92.7	89.2	2
Nordiazepam	98.4	102	102	8	99	102	106	3
Oxazepam	98.4	98.3	88.3	6	89.7	86.5	92	3
Phenazepam	93.5	93.3	98.2	3	100	101	97.5	2
Temazepam	87.3	101	107	10	99.9	96.9	96.4	2

Table 4.
Recovery and Matrix Effects at 25 ng/mL

Analyte	% Recovery	% Matrix Effects
7-Aminoclonazepam	117	105
Alprazolam	109	110
Clobazam	112	107
Clonazepam	107	116
Clonazolam	96	110
Deschloretizolam	109	100
Diazepam	110	120
Estazolam	105	110
Etizolam	98	122
Flualprazolam	105	125
Flubromazepam	107	111
Flubromazolam	101	121
Flunitrazolam	80	121
Lorazepam	86	119
N-Desmethyclobazam	112	107
Nordiazepam	101	105
Oxazepam	113	104
Phenazepam	108	108
Temazepam	100	115

Discussion

All isomeric and isobaric pairs were baseline resolved or better and chromatographically separated in 8 minutes (**Figures 2, 3, and 4**), which pairs well with the fast sample preparation product and subsequent method.

A linear regression with 1/x weighting was used for all calibration curves. The linear range was 5 ng/mL to 1,000 ng/mL (**Table 2**). Accuracy ranged from 80% to 119% for all calibrators, and curves were linear with $r^2 \geq 0.99$ (**Figure 5**). Precision ranged from 1% to 15% CV, and accuracy ranged from 81% to 118% for quality controls (**Table 3**).

Recoveries and matrix effects were evaluated (**Table 4**) and calculated according to Matuszewski et al. Recoveries ranged from 80% to 122% for all analytes. Matrix effects ranged from 99% to 125% for all analytes.

The fast and room temperature hydrolysis procedure simplified the traditional hydrolysis workflow and increased sample throughput by greatly reducing the sample processing time without negatively affecting the end results.

By combining the hydrolysis and SPE procedure into one method using the Strata®-X Drug B Plus 96-well plate, both time and resources can be saved through the elimination of the need for conditioning and equilibration steps and eliminating a transfer step post-hydrolysis for minimal analyte loss.

Conclusion

This work presents a fast, simplified, reproducible, and sensitive LC-MS/MS method for identification and analysis of 20 analytes, including 8 designer benzodiazepines from urine. By utilizing an in-well, room temperature hydrolysis workflow, this method provides a more efficient workflow through the elimination of transfer and conditioning steps for a faster, more cost-effective solution. Sample processing time was under 20 minutes for the hydrolysis and extraction of a 96-well plate.

Ordering Information

Strata®-X-Drug B Plus SPE

Sorbent Mass	Part No.	Unit
96-Well Plate		
10 mg	8E-S128-AGB-P	2 Plates/Box
30 mg	8E-S128-TGB-P	2 Plates/Box

Strata-X-Drug B SPE

Sorbent Mass	Part No.	Unit
Tube		
10 mg	8B-S128-AAK	1 mL (100/box)
30 mg	8B-S128-TAK	1 mL (100/box)
30 mg	8B-S128-TBJ	3 mL (50/box)
60 mg	8B-S128-UBJ	3 mL (50/box)
60 mg	8B-S128-UCH	6 mL (30/box)
60 mg	8B-S128-UCL	6 mL (200/bag)
Giga™ Tube		
100 mg	8B-S128-EDG	12 mL (20/box)
96-Well Plate		
10 mg	8E-S128-AGB	2 Plates/Box
30 mg	8E-S128-TGB	2 Plates/Box
60 mg	8E-S128-UGB	2 Plates/Box

Kinetex® Core-Shell LC Columns

2.6 µm Minibore Columns (mm)	SecurityGuard™ ULTRA Cartridges*				
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
Biphenyl	00A-4622-AN	00B-4622-AN	00D-4622-AN	00F-4622-AN	AJO-9209 for 2.1mm ID

2.6 µm MidBore™ Columns (mm)	SecurityGuard ULTRA Cartridges*			
Phases	50 x 3.0	100 x 3.0	150 x 3.0	3/pk
Biphenyl	00B-4622-Y0	00D-4622-Y0	00F-4622-Y0	AJO-9208 for 3.0mm ID

2.6 µm Analytical Columns (mm)	SecurityGuard ULTRA Cartridges*			
Phases	50 x 4.6	100 x 4.6	150 x 4.6	3/pk
Biphenyl	00B-4622-E0	00D-4622-E0	00F-4622-E0	AJO-9207 for 4.6mm ID

* SecurityGuard ULTRA Cartridges require holder, Part No. AJO-9000

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