Automated, High-Throughput Analysis of Benzodiazepines from Urine and Plasma using Strata™-X 96-Well Plate Solid Phase Extraction (SPE) and Kinetex® Core-Shell Technology HPLC/UHPLC Columns

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Eight benzodiazepines were extracted from both urine and plasma using an automated solid phase extraction (SPE) procedure and a polymer-based Strata-X SPE sorbent. The method proved to be reproducible and produced excellent recoveries for the target analytes from both sample matrices. When coupled with a rapid (<3 minute) LC/MS/MS method utilizing a Kinetex core-shell HPLC column, this workflow provides the opportunity to substantially increase sample throughput and simultaneously decrease costs per each analysis.

Introduction

Benzodiazepines have been prescribed historically to treat anxiety, insomnia, agitation, seizures, muscle spasms, alcohol withdrawal, and are even used as premedication for medical and dental procedures. Unfortunately, like many beneficial drugs, benzodiazepines are also widely abused and these drugs are now regulated by the Substance Abuse and Mental Health Services Administration (SAMHSA) in the United States, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), and other regulating bodies around the world. Admissions into treatment programs specifically for benzodiazepine abuse have tripled from 1998 to 2008, which is a huge increase when compared to the 11 % rise in treatment admissions for all substances[1]. Because of the widespread use and abuse of benzodiazipines, it is important that clinical, forensic, and toxicological testing laboratories have reliable, reproducible methods to rapidly screen for benzodiazepines and their metabolites in matrices such as urine and plasma. With many labs turning to automation to ensure that their cleanup and sample handling is consistent, we have developed an automated solid phase extraction (SPE) method followed by rapid LC/MS/MS analysis using Kinetex Core-Shell Technology that can provide the accurate, rapid, and reproducible results that are required in these testing environments.

Materials and Methods

Sample pretreatment

500 µL of urine or plasma was first diluted with 1 mL of water. The diluted sample was then subjected to an automated SPE protocol which was run on a PerkinElmer MultiPROBE® II.

Solid Phase Extraction

SPE Protocol	
96-Well Plate:	Strata-X 30 mg/well
Part No.:	8E-S100-TGB
Condition:	1 mL methanol
Equilibrate:	1 mL water
Load:	500 µL urine (or plasma) diluted with 1 mL water
Wash 1:	0.8 mL water
Wash 2:	0.8 mL Acetonitrile/Water (20:80)
Dry:	3 to 4 minutes at maximum vacuum
Elute:	800 µL Ethylacetate/Isopropyl alcohol (85:15)
Blow Down:	To dryness under a slow stream of nitrogen at 45 °C
Reconstitute:	500 µL of 0.1 % Formic acid in Water/0.1 % Formic acid in Methanol (55:45)

LC/MS/MS

Extracts were analyzed by LC/MS/MS using an Agilent® 1200 SL LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an API 4000™ LC/MS/MS detector (AB SCIEX, Framingham, MA, USA). MRM transitions and MS/MS parameters are listed in **Table 1**.

Column: Kinetex 2.6 µm C8
Dimensions: 50 x 2.1 mm
Part No.: 00B-4497-AN
Guard: SecurityGuard™ ULTRA C8
Guard Part No.: AJ0-8784
Mobile Phase: A: 0.1 % Formic acid in water
B: 0.1 % Formic acid in methanol

Gradient:

Time (min)	B(%)
0	45
2	95
3.5	95
3.51	45
6	45

Flow Rate: 400 µL/min
Temperature: Ambient
Detection: API 4000 MS/MS, ESI Positive (ESI+)

Source/Gas Parameters	
(positive polarity)	
CAD:	6
CUR:	20
GS1:	55
GS2:	50
IS:	5500
TEM:	625

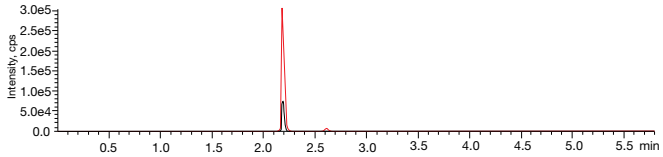
Table 1.
MRM Transitions and MS/MS Parameters

Compound	Q1	Q3	Dwell Time	DP	EP	CE	CXP
Nordiazepam	271.3	140.3	25	46	10	39	10
Nordiazepam-2	271.3	164.9	25	46	10	39	10
Nordiazepam-D5	276.3	140.1	25	56	10	41	10
Diazepam	285.3	193.2	25	51	10	45	10
Diazepam-2	285.3	154.2	25	51	10	45	10
Oxazepam	287.1	241.1	25	61	10	25	10
Oxazepam-2	287.1	268.9	25	61	10	25	10
Diazepam-D5	290.2	154.2	25	51	10	45	10
Oxazepam-D5	292.3	246.3	25	61	10	25	10
Temazepam	301.3	255.2	25	69	10	29	10
Temazepam-2	301.2	177.1	25	69	10	47	10
Temazepam-D5	306.3	260.2	25	35	10	31	10
Alprazolam	309.1	205.1	25	49	10	45	10
Alprazolam-2	309.1	281.1	25	49	10	33	10
Alprazolam-D5	314.2	210.2	25	51	10	59	10
Clonazepam	316.2	270.2	25	75	10	35	10
Clonazepam-2	316.2	214.2	25	75	10	47	10
Clonazepam-D4	320.1	274.1	25	75	10	35	10
Lorazepam	321.3	275.3	25	46	10	29	10
Lorazpeam-2	321.3	229.1	25	46	10	37	10
Lorazepam-D4	325.1	279.2	25	46	10	29	10
α-Hydroxyalprazolam	325.2	279.2	25	56	10	32	10

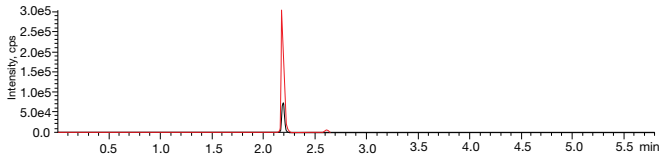
Note: for α-Hydroxyalprazolam, Lorazepam-D4 was used as an internal standard (IS) to create a linearity curve.

Figure 1.
Benzodiazepines Extracted from Urine

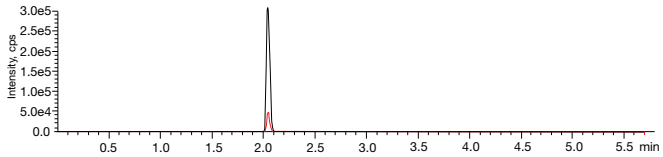
Alprazolam



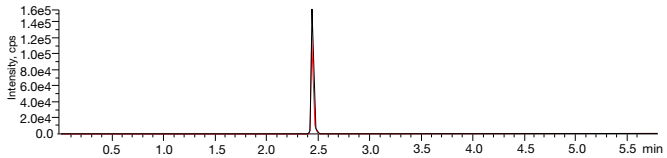
α-Hydroxyalprazolam



Clonazepam



Diazepam



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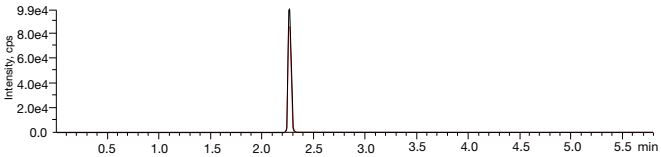
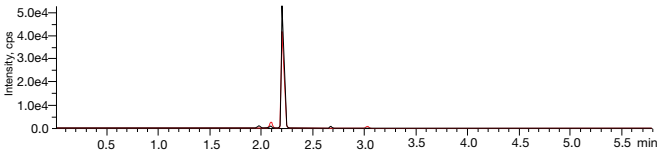
Nordiazepam



Lorazepam



Oxazepam

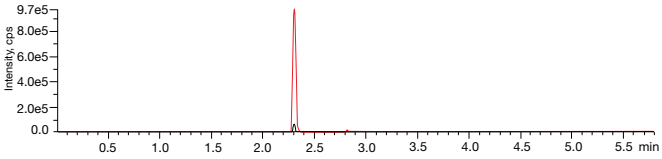


Table 2.
Recoveries and % CV of Benzodiazepines from Urine at 50 ng/mL and 500 ng/mL using Strata™-X SPE

	50 ng/mL	% CV	500 ng/mL	% CV
Alprazolam	103.1	1.1	99.6	1.6
α-OH-Alprazolam	94.4	3.2	82.9	2.8
Clonazepam	104.2	1.3	101.5	1.8
Diazepam	103.4	0.5	101.9	3.1
Nordiazepam	105.7	2.5	99.2	2.0
Lorazepam	108.2	4.0	98.9	1.6
Oxazepam	102.4	3.9	100.8	1.5
Temazepam	102.2	1.0	104.0	3.1

Table 3.
Recoveries and % CV of Benzodiazepines from Plasma at 50 ng/mL and 500 ng/mL using Strata™-X SPE

	50 ng/mL	% CV	500 ng/mL	% CV
Alprazolam	96.6	5.9	100.3	2.0
α-OH-Alprazolam	76.1	5.1	72.8	4.6
Clonazepam	98.9	1.2	98.6	2.2
Diazepam	103.0	2.3	92.0	12.7
Nordiazepam	100.1	3.8	95.9	3.0
Lorazepam	101.0	2.7	94.6	2.8
Oxazepam	101.9	2.1	104.3	4.1
Temazepam	99.0	2.2	104.7	5.4

Results and Discussion

Benzodiazepines comprise a broad range of structurally-related molecules that can be basic, acidic, or neutral in charge depending upon the specific functional groups present. This wide variance in chemistry can make it difficult to effectively recover benzodiazepines using a single SPE extraction method and sorbent. For this reason, we chose to develop our extraction method using the Strata™-X SPE sorbent because it provides a wide range of retention mechanisms which can aid in the retention and recovery of this broad class of molecules. Strata-X is a polymeric SPE sorbent that contains a benzene ring coupled with an N-vinylpyrrolidone. This structure provides 3 mechanisms of retention for target compounds: π – π bonding, hydrogen bonding via dipole-dipole interactions, and hydrophobic interactions (**Figure 2**), making it an excellent choice for our eight benzodiazepine compounds. The sorbent is also available in a variety of formats including 96-well plates for high-throughput cleanups which addressed our goal to develop an automated sample preparation method.

Automation within high-throughput laboratories is becoming more and more popular due to the many benefits that it can introduce into an analysis. Automated procedures can result in increased productivity as well as time and cost savings, particularly the cost of labor. Results are also more precise and lab-to-lab variation is minimized. These benefits were demonstrated in our automated SPE procedure to extract and concentrate benzodiazepines from both urine and plasma. Using a polar reversed phase sorbent, Strata-X, in 96-well plates we were able to consistently achieve high recoveries of all eight benzodiazepines from both urine and plasma at two different concentrations (**Tables 2** and **3**). Recovery values for the benzodiazepines exceeded 80 %, even at ULOQ, when extracted from urine samples and were greater than 70 %, even at ULOQ, when extracted from plasma samples. The slightly lower recoveries in the plasma samples are most likely due to matrix effects such as protein binding.

Not only was our extraction method accurate when used to extract benzodiazepines from both plasma and urine, the method was also reproducible. **Figure 3** shows the linearity of selected benzodiazepines using a 6 point SPE extracted calibration curve in both urine and plasma, all of which demonstrate that our automated extraction method is reproducible.

Figure 2.
Retention Mechanisms of Strata-X SPE Sorbent

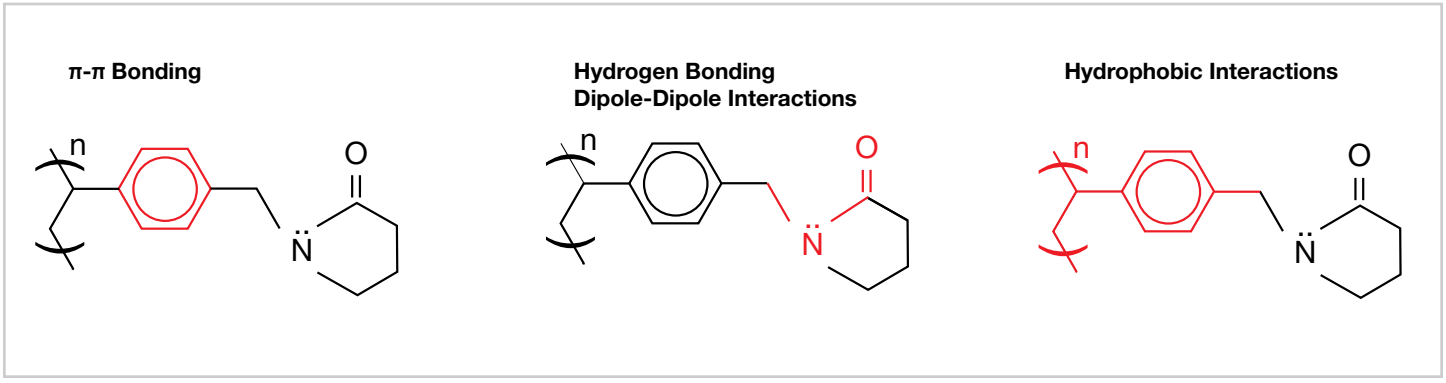
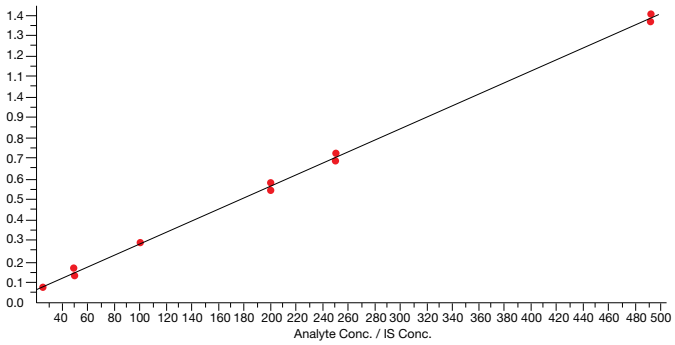
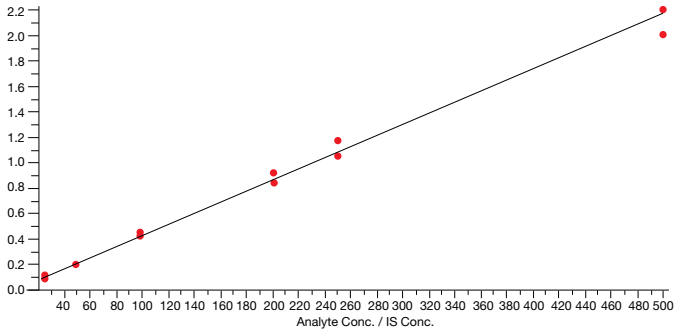


Figure 3.
Linearity Across 25, 50, 100, 200, 250, and 500 ng/mL Extracted Calibration Curve

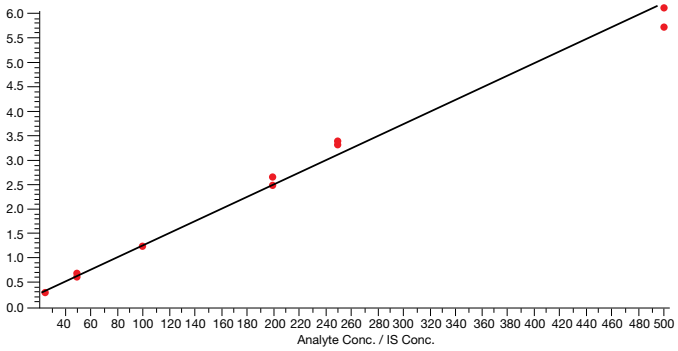
Diazepam in Urine, R = 0.9992



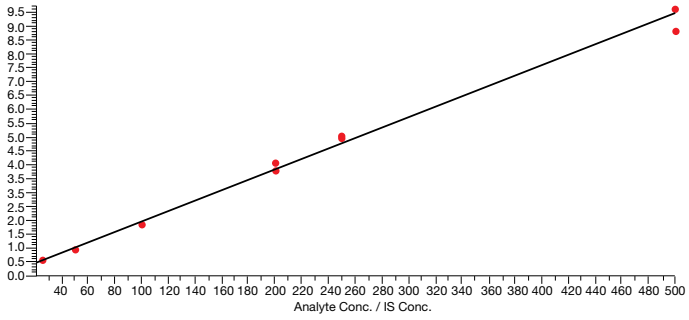
Oxazepam in Urine, R = 0.9971



Diazepam in Plasma, R = 0.9972



Oxazepam in Plasma, R = 0.9980



After cleanup using Strata™-X SPE, our urine and plasma extracts were separated using a Kinetex® C8 2.6µm Core-Shell Technology HPLC/UHPLC Column. The Kinetex column was chosen because the core-shell particle design was able to rapidly separate all target benzodiazepine compounds in under 3 minutes without sacrificing resolution (**Figure 1**). The core-shell particle design also resulted in backpressures that are acceptable for HPLC systems while providing UHPLC performance.

Conclusion

The automated Strata-X SPE extraction of benzodiazepines from both urine and plasma extracts proved to be reproducible, efficient, and provided both time and cost savings. By automating the procedure, high-throughput laboratories can implement our proposed extraction method without training technicians, which provides additional time savings.

After extraction, our analytical methods for both ULOQ and LLOQ level showed good recoveries and reproducibility of our targeted benzodiazepine probes in both urine and plasma samples. While the method validation was attempted using 6 points calibration, all except for α-hydroxyalprazolam in plasma and lorazepam in urine gave good linearity over the working range of the calibration curve (as per correlation coefficient value). This may be a result of matrix derived stability issues for α-hydroxyalprazolam and lorazepam since they each performed well in at least one matrix that we studied.

References

1. SAMHSA News Release, June 9, **2011**.

APPLICATIONS

Ordering Information

Kinetex® Core-Shell HPLC/UHPLC Columns

2.6 µm Analytical Columns (mm)

Phases	50 x 4.6	75 x 4.6	100 x 4.6	150 x 4.6	SecurityGuard™ ULTRA Cartridges‡ 3/pk
C8	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	AJ0-8770 for 4.6 mm ID

2.6 µm MidBore™ Columns (mm)

Phases	30 x 3.0	50 x 3.0	75 x 3.0	100 x 3.0	150 x 3.0	SecurityGuard ULTRA Cartridges‡ 3/pk
C8	00A-4497-Y0	00B-4497-Y0	00C-4497-Y0	00D-4497-Y0	00F-4497-Y0	AJ0-8777 for 3.0 mm ID

2.6 µm Minibore Columns (mm)

Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard ULTRA Cartridges‡ 3/pk
C8	00A-4497-AN	00B-4497-AN	00D-4497-AN	00F-4497-AN	AJ0-8784 for 2.1 mm ID

1.7 µm MidBore Columns (mm)

Phases	30 x 3.0	50 x 3.0	100 x 3.0	SecurityGuard ULTRA Cartridges‡ 3/pk
C8	00A-4499-Y0	00B-4499-Y0	00D-4499-Y0	AJ0-8777 for 3.0 mm ID

1.7 µm Minibore Columns (mm)

Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	SecurityGuard ULTRA Cartridges‡ 3/pk
C8	00A-4499-AN	00B-4499-AN	00D-4499-AN	00F-4499-AN	AJ0-8784 for 2.1 mm ID

‡SecurityGuard ULTRA cartridges require holder, Part No.: AJ0-9000

Strata™-X SPE

Sorbent Mass	Part No.	Unit
Tube		
30 mg	8B-S100-TAK	1 mL (100/box)
30 mg	8B-S100-TBJ	3 mL (50/box)
60 mg	8B-S100-UBJ	3 mL (50/box)
100 mg	8B-S100-EBJ	3 mL (50/box)
100 mg	8B-S100-ECH	6 mL (30/box)
200 mg	8B-S100-FBJ	3 mL (50/box)
200 mg	8B-S100-FCH	6 mL (30/box)
500 mg	8B-S100-HBJ	3 mL (50/box)
500 mg	8B-S100-HCH	6 mL (30/box)
Giga™ Tube		
500 mg	8B-S100-HDG	12 mL (20/box)
1 g	8B-S100-JDG	12 mL (20/box)
1g	8B-S100-JEG	20 mL (20/box)
2 g	8B-S100-KEG	20 mL (20/box)
5 g	8B-S100-LFF	60 mL (16/box)
96-Well Plate		
10 mg	8E-S100-AGB	2 Plates/Box
30 mg	8E-S100-TGB	2 Plates/Box
60 mg	8E-S100-UGB	2 Plates/Box

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