Extraction of Prescription and Designer Benzodiazepines Using Clean Screen® BNZ

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UCT Featured Products

CSBNZ206
Clean Screen® BNZ
200 mg, 6 mL

SPHPHO6001-10
Select pH Buffer Pouches
100mM Phosphate pH 6.0

SCS27-DA1021
SelectraCore® DA
100 x 2.1 mm, 2.7 µm

SCS27-DAGDC21
SelectraCore® DA Guard Column
5 x 2.1 mm, 2.7 µm

SLGRDHLDR-HPOPT
Selectra® Direct
Connect Guard Holder

Abstract:

Benzodiazepines are a class of drugs prescribed to treat anxiety, insomnia, muscle spasms, and seizures. Designer benzodiazepines (DBZD) are illicitly sold compounds that are structurally and pharmacologically similar to benzodiazepines. This application note outlines a LC-MS-MS method for the determination of prescription and designer benzodiazepines from blood and urine using UCT's Clean Screen® BNZ specialized solid phase extraction (SPE) column for sample preparation. The unique chemistry of Clean Screen® BNZ demonstrated superior retention of benzodiazepines compared to Clean Screen® DAU, particularly for the 7-amino metabolites. Notably, the recovery of compounds in blood using Clean Screen® BNZ ranged from 58%-95%, while in urine, recovery ranged from 64%-103%.

Introduction:

Benzodiazepines are a class of compounds characterized by a shared chemical structure, consisting of a benzene ring fused to a diazepine ring. The diazepine ring is a seven-membered ring containing two nitrogen atoms at the 1 and 4 positions, with additional substituents at various positions on the ring.[1] Benzodiazepines are primarily used to treat conditions such as anxiety, insomnia, and seizures.[2] Commonly prescribed benzodiazepines include diazepam (Valium), alprazolam (Xanax), and lorazepam (Ativan). Benzodiazepines work by enhancing the effects of the neurotransmitter gamma-aminobutyric acid (GABA), producing calming effects on the central nervous system.[2] They are classified as short-acting or long-acting depending on their pharmacokinetic properties. Short-acting benzodiazepines, such as alprazolam (Xanax) and lorazepam (Ativan), typically have a rapid onset of action and a relatively brief duration of effect, making them useful for treating acute anxiety or insomnia. These medications may carry a higher potential for abuse due to their fast-acting nature. Long-acting benzodiazepines, like diazepam (Valium) and clonazepam (Klonopin), have a slower onset of action, remain active in the body for an extended period, and can result in stable blood concentrations throughout the day.[3] These medications are often used to treat chronic anxiety, seizures, or muscle spasms. However, there is a risk of cumulative sedation or tolerance with long-term use.

Designer benzodiazepines (DBZD) are novel psychoactive substances (NPS) that are structurally similar to traditional benzodiazepines but are often designed to evade legal restrictions, or to produce more potent or prolonged effects.[4] These substances, such as etizolam, flubromazepam, and diclazepam, are sold illicitly as recreational drugs or as substitutes for more commonly known benzodiazepines. As these substances are not regulated, the chances of inconsistent dosage and impurities increase, which in turn increases the risk of harmful side effects or fatal toxicity. Additionally, many designer benzodiazepines are used in conjunction with other drugs, such as opioids or alcohol, amplifying the risk of respiratory depression and overdose.[4] The emergence of these substances presents significant challenges for forensic toxicologists, who must remain vigilant in detecting and identifying these novel compounds in biological samples, as traditional drug tests may not always detect them. This study outlines a method for the simultaneous identification and quantification of prescription (alprazolam, clonazepam, diazepam, nordiazepam, oxazepam, temazepam, flunitrazepam, lorazepam, adinazolam, 7-aminoclonazepam, 7-aminoflunitrazepam) and designer benzodiazepines (etizolam, flualprazolam, clonazolam, α-hydroxyalprazolam, deschloroetizolam, bromazolam, flubromazepam) in blood and urine samples.

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Experimental:

a) Sample Preparation

SPE Product:

Clean Screen® BNZ 200 mg, 6 mL

Sample Pretreatment:

0.5 mL sample* + 1.5 mL 100 mM phosphate buffer (pH 6.0) + ISTDs. Vortex and centrifuge.

*Include a hydrolysis procedure to deconjugate glucuronidated analytes in urine samples.

SPE Extraction:

1. **Condition:** apply 3 mL methanol, followed by 3 mL 100 mM phosphate buffer (pH 6.0).
2. **Sample Load:** load sample at a flow rate of 1 to 2 mL/minute.
3. **Wash:** apply 3 mL DI H_2O, followed by 3 mL 5% ACN in H_2O.
4. **Dry:** for at least 10 minutes under full pressure or vacuum.
5. **Elute:** apply 3 mL EtOAc:IPA (90:10 v/v).
6. **Evaporate:** Evaporate eluate at 35°C.
7. **Reconstitute:** 0.5 mL H_2O:MeOH (60:40 v/v).

b) Analytical Conditions

Table 1. LC-MS-MS parameters

LC-MS-MS parameters	
LC-MS/MS	Shimadzu Nexera LC-30AD with MS-8050
UHPLC Column	SelectraCore® DA Column 100 x 2.1 mm, 2.7 µm
Guard Column	SelectraCore® DA Guard Column 5 x 2.1 mm, 2.7 µm
Column Temperature	40°C
Flow Rate	0.4 mL/min
Injection Volume	1 µL
Mobile Phase A	5 mM ammonium formate + 0.1% formic acid in water
Mobile Phase B	5 mM ammonium formate + 0.1% formic acid in methanol

Gradient Program		
Time (min)	**Mobile Phase A (%)**	**Mobile Phase B (%)**
0	60	40
9-12	0	100
12.1-15	60	40

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Table 2. MRM Transitions

Compound	Retention Time (min)	Precursor Ion	Product Ion	Collision Energy
7-aminoclonazepam	2.70	285.7	222.1 250.0	-25 -21
7-aminoflunitrazepam	3.56	283.6	135.0 227.0	-27 -26
Adinazolam	4.13	351.7	58.0	-23
Lorazepam	4.90	320.6	275.0 302.9	-23 -17
Oxazepam	5.04	286.6	241.1 269.0	-25 -13
Clonazepam	5.11	315.6	370.0 214.0	-27 -39
Clonazolam	5.56	353.7	308.0 280.0	-28 -37
α-hydroxyalprazolam	5.52	324.6	297.0 216.0	-38 -39
Flubromazepam	5.56	334.6	226.0 186.0	-30 -31
Nordiazepam	5.76	370.7	140.1 208.1	-28 -28
Flunitrazepam	5.95	313.7	268.0 239.0	-27 -36
Flualprazolam	6.01	326.6	299.0 223.0	-29 -43
Temazepam	6.11	300.6	255.0 283.0	-21 -15
Alprazolam	6.34	308.7	281.0 205.0	-27 -43
Bromazolam	6.59	354.8	326.9 338.3	-28 -11
Diazepam	6.83	284.7	193.1 154.0	-32 -28
Etizolam	6.98	342.5	314.0 259.0	-27 -34
Deschloroetizolam	7.18	308.5	280.0 255.1	-25 -26

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Chromatogram:

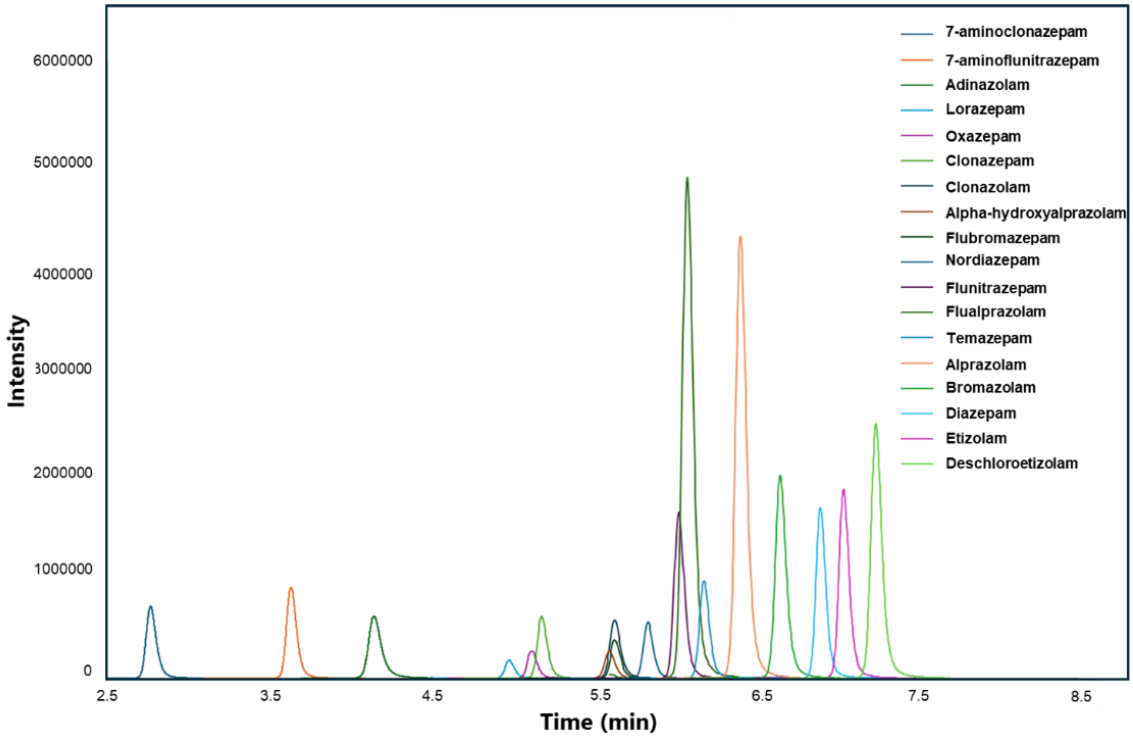

Figure 1. Chromatogram of 300 ng/mL extracted urine sample.

Calibration Curves:

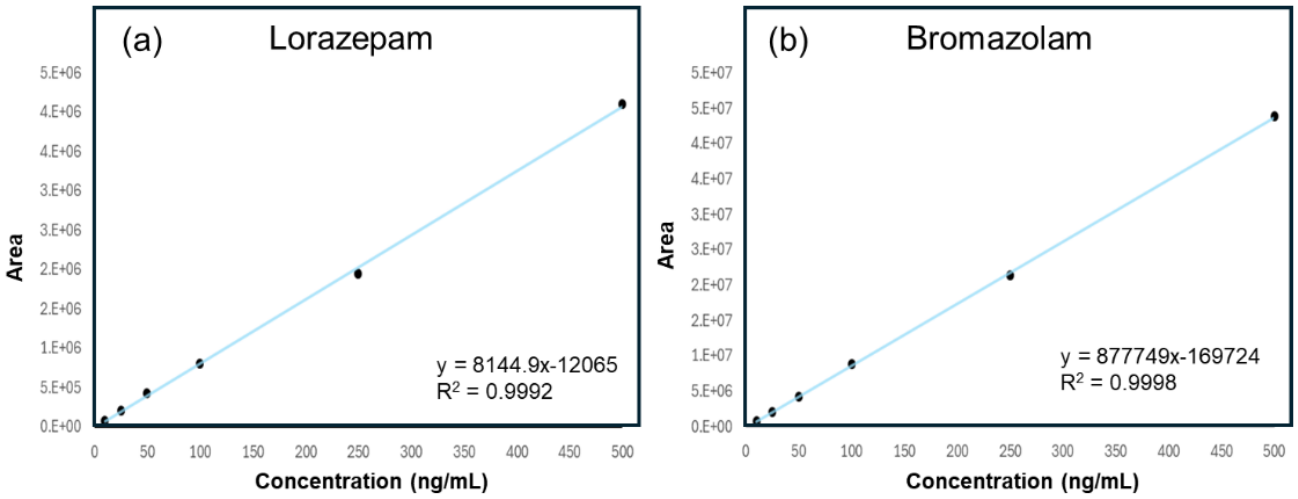

Figure 2. Calibration curves for (a) Lorazepam and (b) Bromazolam

Results & Discussion:

Method evaluation was conducted by assessing recovery, matrix effect, and relative standard deviation for samples at both high and low concentrations. For blood samples (n=3) recovery and matrix effect ranged from 58% to 95% and -30% to 17%, respectively. The relative standard deviation was equal to or less than 10%. Urine samples (n=3) recovery and matrix effect range from 64% to 103% and -23% to 0%, respectively. Relative standard deviation ranged from 0% to 17%.

For both matrices, temazepam, oxazepam, and lorazepam resulted in lower recovery and higher relative standard deviation. These compounds are the only ones in the panel with a chlorine substitution on the fused benzene ring and a hydroxyl substitution on the seven-membered diazepine ring. Results (**Figure 3**) indicate the sorbent chemistry of Clean Screen® BNZ has lower retention of these compounds compared to other benzodiazepines. Higher recovery of these compounds was achieved using the Clean Screen® DAU SPE columns. However, Clean Screen® BNZ had higher recoveries for all other compounds, most notably for the 7-amino metabolites. It is theorized that the large discrepancy in adinazolam recovery between Clean Screen® DAU and BNZ may be due to the retention of the compound on the benzene sulfonic acid portion of the copolymerized DAU sorbent. Adinazolam has a basic pka of 6.3, at pH 6 about half of the compound would be ionized and may have been retained on the ion exchange component of the sorbent. Clean Screen® BNZ is a proprietary sorbent, however, this data shows that its chemistry retains benzodiazepines better than Clean Screen® DAU except for lorazepam, oxazepam, and temazepam.

Table 3. Average recovery, matrix effect, and precision values obtained in spiked blood samples.

Blood						
Analytes	30 ng/mL (n=3)			300 ng/mL (n=3)		
	Recovery (%)	Matrix Effects (%)	RSD (%)	Recovery (%)	Matrix Effects (%)	RSD (%)
7-Aminoclonazepam	95	-30	10	95	-29	8
7-Aminoflunitrazepam	94	-21	8	93	-18	7
Adinazolam	70	17	6	77	-1	4
Lorazepam	69	2	9	78	0	5
Oxazepam	68	0	6	79	-2	5
Clonazepam	80	3	6	86	0	4
Clonazolam	80	6	6	89	2	4
α-hydroxyalprazolam	80	2	6	87	-1	4
Flubromazepam	81	-8	8	87	-2	3
Nordiazepam	90	-12	8	88	-7	4
Flunitrazepam	90	7	6	89	3	4
Flualprazolam	79	7	6	87	5	4
Temazepam	58	4	9	71	3	6
Alprazolam	79	6	6	87	2	5
Bromazolam	79	4	6	86	0	5
Diazepam	79	0	6	86	-3	3
Etizolam	78	8	5	85	4	4
Deschloroetizolam	79	8	6	86	4	4

Table 4. Average recovery, matrix effect and precision values obtained in spiked urine samples.

Urine						
Analytes	30 ng/mL (n=3)			300 ng/mL (n=3)		
	Recovery (%)	Matrix Effects (%)	RSD (%)	Recovery (%)	Matrix Effects (%)	RSD (%)
7-Aminoclonazepam	105	-11	2	96	-19	3
7-Aminoflunitrazepam	103	-1	1	93	-9	3
Adinazolam	100	-1	1	82	-5	2
Lorazepam	83	-8	6	77	-6	1
Oxazepam	81	-12	7	75	-7	3
Clonazepam	98	-6	1	89	-6	1
Clonazolam	97	-4	1	86	-2	1
α-hydroxyalprazolam	94	-7	3	84	-6	0
Flubromazepam	95	-15	2	89	-6	0
Nordiazepam	102	-23	2	90	-11	2
Flunitrazepam	98	-3	2	89	-2	0
Flualprazolam	98	-2	1	86	0	1
Temazepam	64	-11	17	65	-6	3
Alprazolam	99	-6	3	92	-3	0
Bromazolam	100	-10	2	85	-6	1
Diazepam	100	-11	2	88	-8	1
Etizolam	100	-7	0	86	-3	2
Deschloroetizolam	101	-7	0	85	-3	2

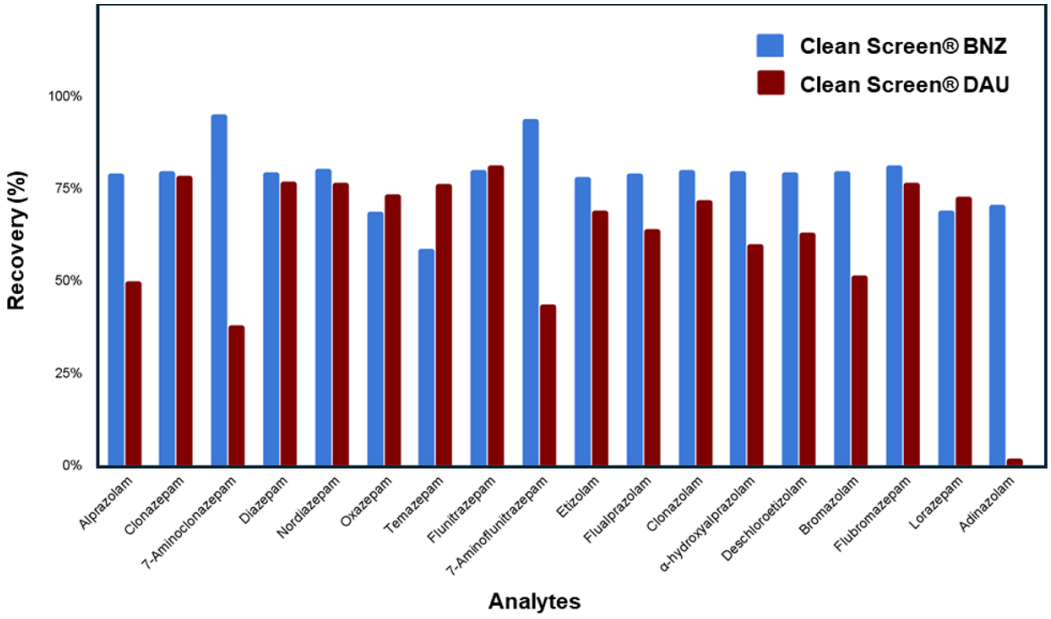

Figure 3. Recovery of benzodiazepines from 30 ng/mL blood samples (n=3) using Clean Screen® BNZ versus Clean Screen® DAU.

Conclusion:

In this application, a size-by-side sorbent comparison demonstrated that Clean Screen® BNZ resulted in higher recovery for the majority of the target analytes compared to Clean Screen® DAU, particularly for the 7-amino metabolites. Although lorazepam, oxazepam, and temazepam showed lower recovery with Clean Screen® BNZ, a successful method for simultaneous extraction and detection of prescription and designer benzodiazepines was developed. This makes Clean Screen® BNZ SPE columns an effective choice for confirmation analysis.

References:

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