Simultaneous Determination of Prescription and Designer Benzodiazepines in Urine by SPE and LC-MS/MS

UCT Part Numbers

CSXCE106
Clean Screen® XCEL I
130 mg, 6 mL

SPHACE5001-5
Select pH Buffer Pouches
100 mM Acetate pH 5.0

BETA-GLUC-50
Selectrazyme® 50 mL beta-glucuronidase enzyme liquid form

SLDA100ID21-3UM
Selectra® DA Column,
100 x 2.1mm, 3µm

SLDAGDC21-3UM
Selectra® DA Guard Column,
10 x 2.1mm, 3µm

SLGRDHLDR
Guard Cartridge Holder

Summary:

Benzodiazepines, frequently referred to as "Benzos" , are prescribed for the treatment of anxiety, insomnia, muscle spasms, alcohol withdrawal and seizure-prevention on account of their ability to depress the central nervous system. Generally, these drugs are deemed safe and highly effective when used properly and for short durations of time. However, long term use can lead to both physical and psychological dependence consequentially triggering abuse[1].

Benzodiazepines are also recurrently utilized as illegal recreational drugs. In this case, they may be ground to a powder, mixed with water and injected, as well as being swallowed as pills. Their administration is often accompanied by the use of other drugs, such as alcohol and opioids for an enhanced overall effect. Similar to other commonly abused compounds, such as cannabinoids or amphetamines, "legal" alternatives have been developed for Benzos as well in an attempt to bypass the controlled substances act. These new designer drugs are structural or functional analogs of the controlled substance designed to not only mimic the pharmacological effects of the original drug, but also avoid illegal classification and/or detection in a standard drug test[2].

Keeping up with the ever changing designer drug market has proven to be a real challenge for laboratories across the country. Given that these compounds are derived from " template structures" , it will prove valuable for labs to have a method that can not only target current metabolites of interest, but also the latest ones being formulated.

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Procedure:

Sample Pretreatment

To 1 mL of urine sample, add 1 mL of 100 mM acetate buffer (pH= 5) and 25-50 µL of concentrated Selectrazyme™ β-glucuronidase (BETA-GLUC-50). Vortex and heat for 1-2 hours at 65° C; Allow sample to cool. Do not adjust pH~ sample is ready to be added to the extraction column.

SPE Method

1. Attach SPE cartridges (**CSXCE106**) to a glass block manifold or positive pressure manifold.
2. Load the pretreated sample, adjust vacuum or pressure for a slow dropwise sample flow (1-2 mL/min).
3. Wash the SPE cartridges with 3 mL of acetate buffer pH 5 (**SPHACE5001-5**). Dry the SPE cartridges under full vacuum or pressure for 5 minutes.
4. Repeat the wash with 3 mL methylene chloride. Dry the SPE cartridges under full vacuum or pressure for 10 min.
5. Insert collection rack with test tubes to the manifold, and elute the retained analytes with 3 mL of ethyl acetate:NH_4OH (98:2).
6. Evaporate the eluate to dryness at 45 °C under a gentle stream of nitrogen, and reconstitute with 100 µL of 50% MeOH in DI water.
7. Vortex the extract for 30 sec and transfer to 200-µL inserts held in 2-mL vials.

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HPLC Conditions	
Instrumentation	Agilent 1200 Binary Pump SL
HPLC column	UCT Selectra® DA, 100 × 2.1 mm, 3 µm (p/n: **SLDA100ID21-3UM**)
Guard column	UCT Selectra® DA, 10 × 2.0 mm, 3 µm (p/n: **SLDAGDC21-3UM**)
Guard column holder	p/n: **SLGRDHLDR**
Column temp.	40 °C
Mobile phase A	Water + 0.1% formic acid
Mobile phase B	Methanol + 0.1% formic acid
Flow rate	300 µL/min
Gradient	0 min, 70% B; 1-6 min, 100% B; 6-9 min, 100% B; 9.01-13 min, 70% B
Injection volume	10 µL
Autosampler temp.	10 °C
Wash solvent	Methanol

MS Conditions	
Instrumentation	API 4000 QTRAP MS/MS
Ionization mode	ESI^+
Spray voltage	4200 V
Vaporizer temperature	650 °C
Capillary temperature	350 °C
Sheath gas pressure	40 arbitrary units
Auxiliary gas pressure	5 arbitrary units
Ion sweep gas	0 arbitrary units

MRM Transitions (ESI positive, dwell time: 50 ms)

	Compound	Retention Time (min.)	MRM Transitions		
			Q1	Q3 ion 1	Q3 ion 2
1	**7-Aminoclonazepam**	1.56	286.1	222.3	250.2
2	**Midazolam**	1.79	326.0	291.0	222.0
3	**Lorazepam**	2.38	321.1	303.3	275.0
4	**Oxazepam**	2.54	287.1	241.3	104.2
5	**Clonazepam**	2.66	316.1	270.2	241.2
6	**Flubromazepam**	3.02	335.0	226.1	186.0
7	**Alpha-Hydroxy-Alprazolam**	3.10	325.2	297.1	216.3
8	**Nordiazepam**	3.19	271.1	104.1	165.2
9	**Phenazepam**	3.26	352.0	185.9	206.0
10	**Pyrazolam**	3.45	356.0	206.1	167.2
11	**Temazepam**	3.57	301.1	255.2	177.2
12	**Flubromazolam**	4.11	372.9	345.0	292.2
13	**Alprazolam**	4.22	309.2	205.3	281.2
14	**Diclazepam**	4.49	321.0	229.1	154.1
15	**Diazepam**	4.80	285.1	193.2	154.1
16	**Etizolam**	5.46	345.06	316.1	291.1

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Chromatogram:

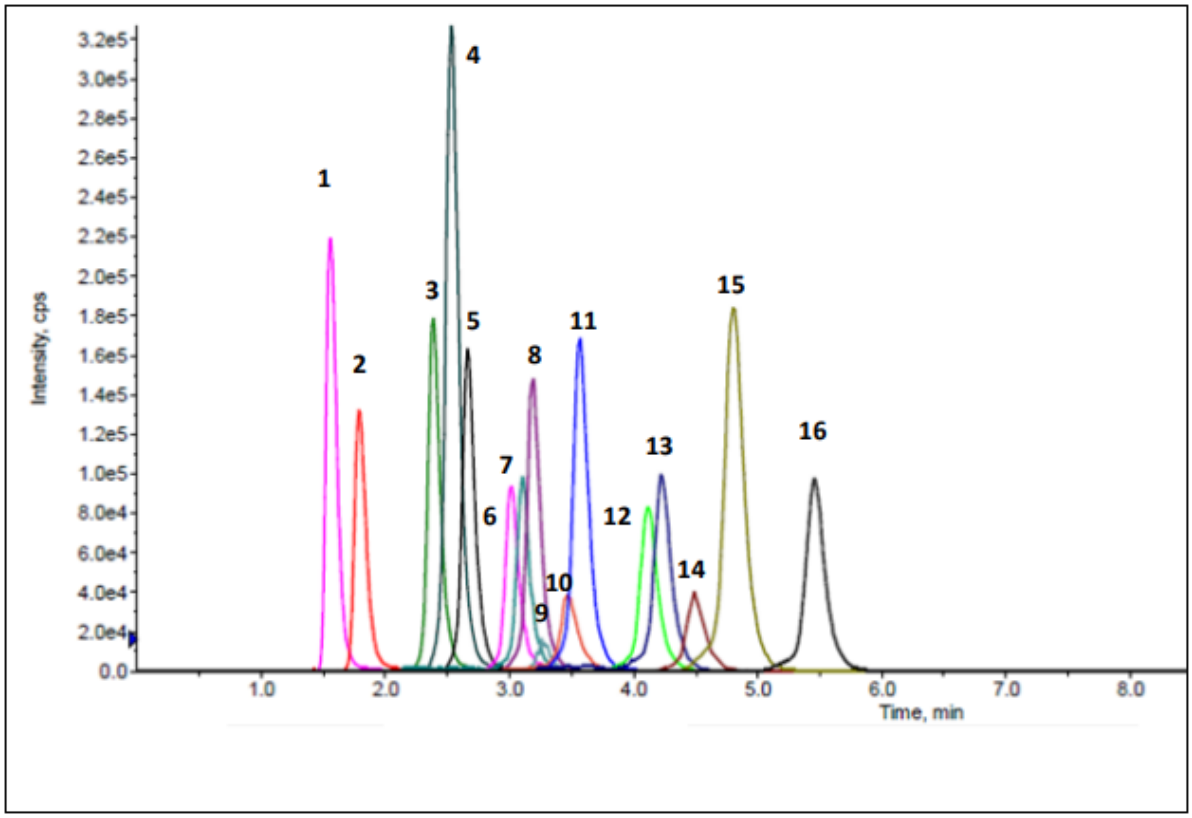

Figure 1: Chromatogram of 100 ng/mL solvent standard

Matrix Matched Calibration Curve:

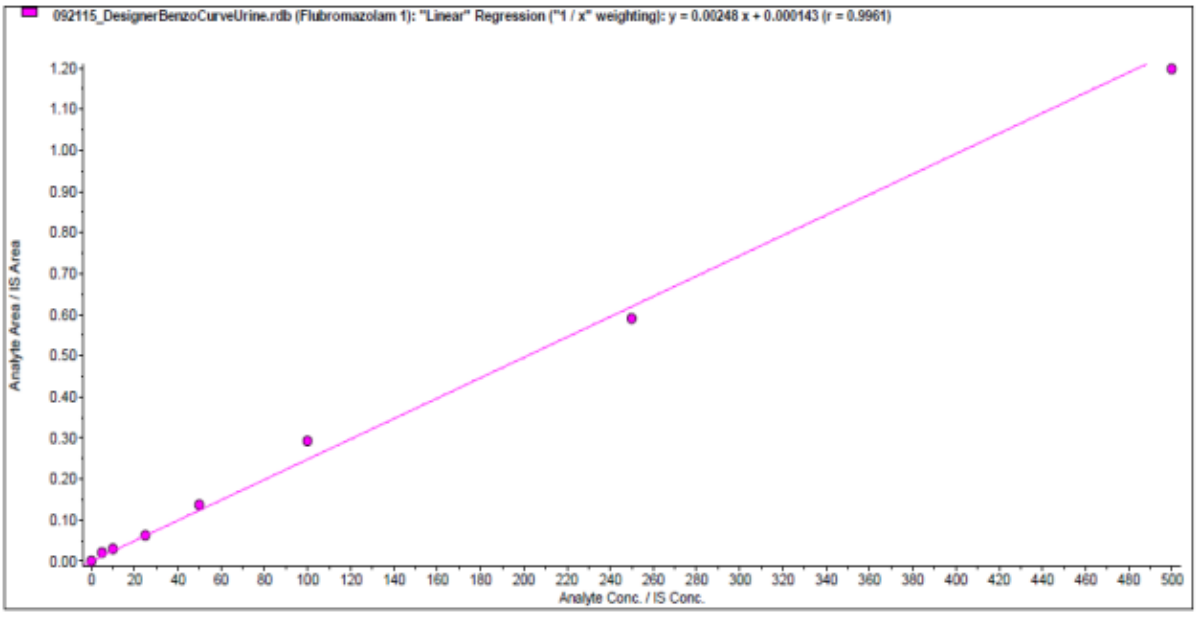

Figure 2: Designer Benzodiazepine (Flubromazolam)

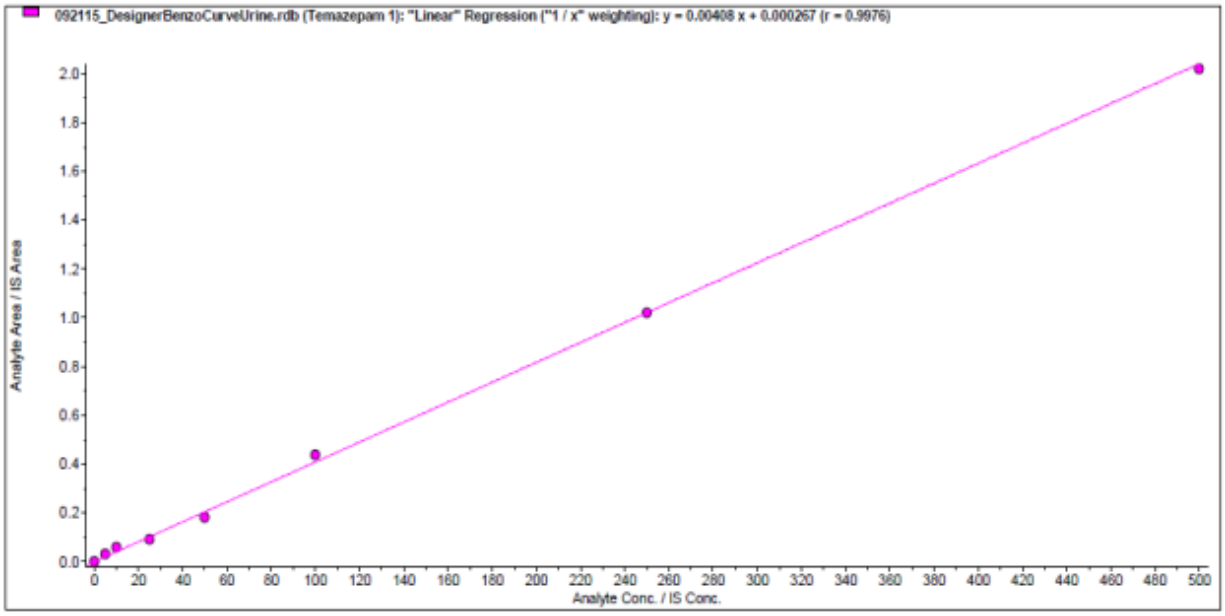

Figure 3: Prescription Benzodiazepine (Temazepam)

Results:

Analyte	Absolute Extraction Recovery (%, n=3)			Matrix Effect (%, n=3)			Overall Extraction Efficiency (%, n=3)		
	15 ng/mL	75 ng/mL	200 ng/mL	15 ng/mL	75 ng/mL	200 ng/mL	15 ng/mL	75 ng/mL	200 ng/mL
Diclazepam	78	66	71	28	19	19	56	54	57
Etizolam	89	79	91	5	2	7	84	77	85
Flubromazepam	92	86	79	0	-3	-9	93	88	86
Flubromazolam	90	76	87	-6	-8	-2	95	83	89
Phenazepam	98	77	85	24	17	24	74	64	64
Pyrazolam	81	72	86	21	18	18	64	59	71
7-Aminoclonazepam	90	67	85	36	19	11	58	54	76
Alpha-Hydroxy-Alprazolam	98	92	82	-43	-30	-29	140	119	107
Alprazolam	86	79	80	-58	-47	-42	137	116	114
Clonazepam	84	77	82	20	11	16	67	69	68
Diazepam	95	84	88	14	7	4	82	78	85
Lorazepam	106	78	92	24	15	23	81	66	71
Nordiazepam	114	91	103	41	29	27	68	64	75
Oxazepam	107	88	97	15	7	15	91	82	83
Temazepam	74	67	73	5	2	8	71	66	68
Midazolam	64	59	75	50	41	25	32	34	56

Discussion:

Traditional benzodiazepines primarily function as neutral analytes, however, there are some that have weakly basic functional groups allowing for ionization at a fixed pH. The Clean Screen® XCEL I column was chosen for this application due to its capability of simultaneously extracting neutral and basic compounds, while eliminating the need for time-consuming column conditioning and extensive solvent usage for sample cleanup.

The urine samples were adjusted to a pH of 5 for optimal enzyme hydrolysis. Following incubation, they were loaded directly onto the Clean Screen® XCEL I extraction columns. Although the majority of the analytes are primarily retained via hydrophobic interactions, use of this pH allows for any potential ionizable groups to be fully charged and retained via ion exchange. The percentage of organic in the wash was optimized in order to provide sufficient cleanup for the benzodiazepines without compromising on overall analyte recovery. After trying several various solvent combinations of hexane, methylene chloride and buffer containing a percentage of acetonitrile, it was determined that a buffer wash sequentially followed by a methylene chloride wash gave the cleanest final extract with the best overall recovery. An elution solvent of ethyl acetate with 2% ammonium hydroxide was chosen on account of its ability to not only displace any hydrophobic interactions, but also simultaneously disrupt ionic retention factors.

Conclusion:

1. By utilizing UCT's Clean Screen® XCEL I extraction columns in conjunction with the Selectra® DA HPLC column, prescription and synthetic benzodiazepine levels can be monitored simultaneously reducing both sample preparation and instrumental analysis time.
2. The universal nature of this extraction method makes it applicable to existing Benzodiazepines along with other newly emerging analogs.
3. It is strongly recommended to use matrix-matched calibration curves, which include isotopically labeled internal standards to compensate for any remaining matrix that is not removed via the extraction procedure.

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References:

[1] "Benzodiazepines Drug Class Information on RxList.com" RxList. N.p., 2 Apr. 2015. Web 28 Sept. 2015.

[2] https://pubmed.ncbi.nlm.nih.gov/24259203/

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