



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

## UCT Part Numbers

### CSXCE106

Clean Screen® XCEL I  
130mg / 6mL SPE Cartridge

### SPHACE5001-5

Select pH Buffer  
100 mM Acetate pH 5.0

### BETA-GLUC-50

Selctrazyme®  $\beta$  - glucuronidase  
50mL liquid form

### SLDA100ID21-3UM

Selectra® DA LC column  
100 x 2.1 mm, 3  $\mu$ m

### SLDAGDC21-3UM

Selectra® DA guard column  
10 x 2.1 mm, 3  $\mu$ m

### SLGRDHLDR

Guard Column 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.



CLINICAL



FORENSICS

## Sample Pretreatment:

To 1 mL of urine sample, add 1 mL of 100 mM Acetate Buffer (pH=5) and 25-50  $\mu$ L of concentrated Selectrazyme<sup>®</sup>  $\beta$ -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 Procedure:

### 1. Sample Extraction

- a) Apply the sample to the SPE cartridge (if required, use a low vacuum to draw the sample through at  $\leq 3$  mL/min).

### 2. Wash cartridge

- a) 1  $\times$  3 mL Acetate Buffer pH 5
- b) 1  $\times$  3 mL Methylene Chloride
- c) Dry cartridges for  $\sim 10$  minutes under a high vacuum.

### 3. Elution

- a) Elute with 1 $\times$ 3 mL Ethyl Acetate containing 2% Ammonium Hydroxide (EA:NH<sub>4</sub>OH, 98:2 v/v).
- b) Evaporate the sample to dryness under a gentle stream of nitrogen.
- c) Reconstitute in 100  $\mu$ L of 50:50 Methanol: D.I. H<sub>2</sub>O and vortex for 1 minute.
- d) Transfer sample to an autosampler vial containing a low volume insert.



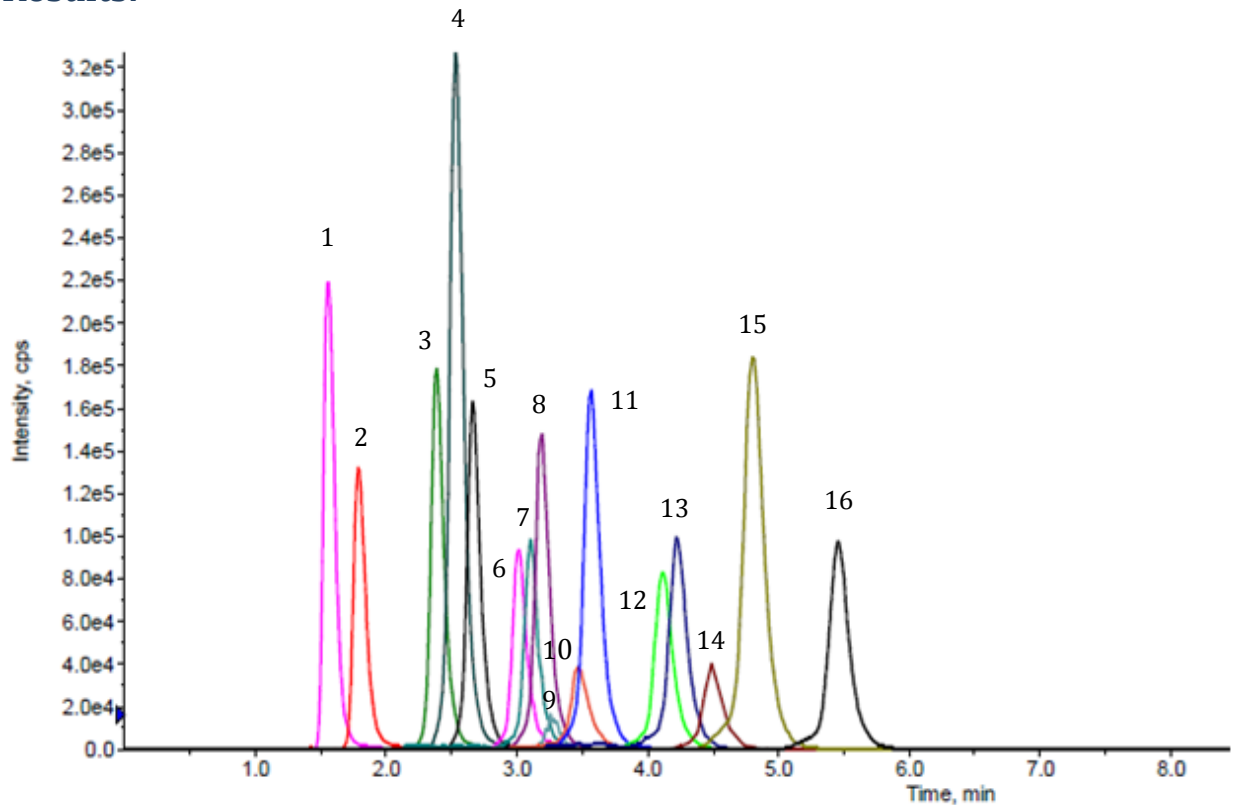
## LC-MS/MS Parameters:

Instrumentation	
HPLC system	Agilent 1200 Binary Pump SL
MS system	API 4000 QTRAP (MS/MS)
HPLC column	UCT Selectra® DA, 100 × 2.1 mm, 3 μm (p/n: SLDA100ID21-3UM)
Guard column	UCT Selectra® DA, 10 × 2.1 mm, 3 μm (p/n: SLDAGDC21-3UM)
Guard column holder	p/n: SLGRDHLDR
Column temperature	40°C
Flow rate	300 μL/min
Injection volume	10 μL

Mobile Phase Gradient		
Time (min)	% Mobile Phase A (0.1% Formic Acid in Water)	% Mobile Phase B (0.1% Formic Acid in MEOH)
0.0	30	70
1.0	30	70
6.0	0	100
6.5	0	100
7.0	30	70
10.0	30	70

MRM transitions (ESI <sup>+</sup> )					
	Compound	t <sub>R</sub> (min)	Precursor ion	Product ion 1	Product ion 2
1	7-amino Clonazepam	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	Flurbromazepam	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	18539	206.0
10	Pyrazolam	3.45	356.0	206.1	167.2
11	Temazepam	3.57	301.1	255.2	177.2
12	Flubromazepam	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	345.0	316.1	291.1

## Results:



Urine 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
Diclozepam	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-Amino Clonazepam	90	67	85	36	19	11	58	54	76
∞-OH-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

## Blood Results

Analyte	Absolute Extraction Recovery (%, n=3)			Matrix Effect (%, n=3)			Overall Extraction Efficiency (%, n=3)		
	1.5 ng/mL	3.0 ng/mL	20.0 ng/mL	1.5 ng/mL	3.0 ng/mL	20.0 ng/mL	1.5 ng/mL	3.0 ng/mL	20.0 ng/mL
Diclozepam	81	81	84	37	39	40	51	49	51
Etizolam	81	90	91	6	7	11	89	84	81
Flubromazepam	91	82	82	26	25	21	68	62	65
Flubromazolam	92	86	86	5	4	3	87	83	84
Phenazepam	79	70	78	28	34	36	57	47	50
Pyrazolam	81	98	104	-30	1	18	106	98	85
7-Amino Clonazepam	62	73	58	42	56	51	36	32	28
∞-OH-Alprazolam	104	106	95	-31	-10	-13	136	117	104
Alprazolam	93	78	94	13	-14	5	81	88	90
Clonazepam	86	59	74	13	-1	7	75	60	69
Diazepam	79	79	82	10	11	11	72	70	73
Lorazepam	89	63	89	-43	-58	4	162	100	86
Nordiazepam	84	85	90	13	21	18	73	68	74
Oxazepam	82	81	76	8	13	9	76	71	69
Temazepam	75	72	74	0	-4	-1	75	76	74
Midazolam	89	88	86	24	31	21	68	61	68



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