EXTRACTION OF BENZODIAZEPINES FROM URINE BY CLEAN SCREEN XCEL® I COLUMN AND ANALYSIS BY LC-MS/MS OR GC-MS

Part #
CSXCE106 – CLEAN SCREEN XCEL® I 130 mg, 6 mL Tube
BETA-GLUC-10 – Selectrazyme® Beta-glucuronidase
SMTBSTFA-1-1 – SELECTRA-SIL® MTBSTFA w/ 1% TBDMCS
SLDA50ID21-5UM – Selectra® DA HPLC Column, 50 x 2.1 mm, 5 µm
SLPFPP100ID21-5UM - Selectra® PFPP HPLC Column, 100 x 2.1 mm, 5 µm

2. PREPARE SAMPLE FOR ENZYME HYDROLYSIS OF GLUCURONIDES

To 1-2 mL of urine sample, add 1 mL of acetate buffer (pH 5.0) containing 5,000 units/mL of Selectrazyme® β-glucuronidase.
Optionally, add 1 mL of acetate buffer and 25-50 µL of concentrated β-glucuronidase.
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.

2. APPLY SAMPLE

Load sample directly to column without any preconditioning.
Pull sample through at a rate of 1-2 mL/ minute.
Dry column thoroughly under full vacuum or positive pressure for 1 minute.

3. WASH

1 x 3 mL 100 mM phosphate buffer (pH 6.0).
1 x 3 mL CH_2Cl_2
Dry column thoroughly under full vacuum or positive pressure for a minimum of 5-10 minutes.

4. ELUTION

1 x 3 mL Ethyl Acetate:NH_4OH (98:2)
Collect eluate at 1 to 2 mL/minute.
NOTE: Prepare elution solvent daily.

5. DRY ELUTE

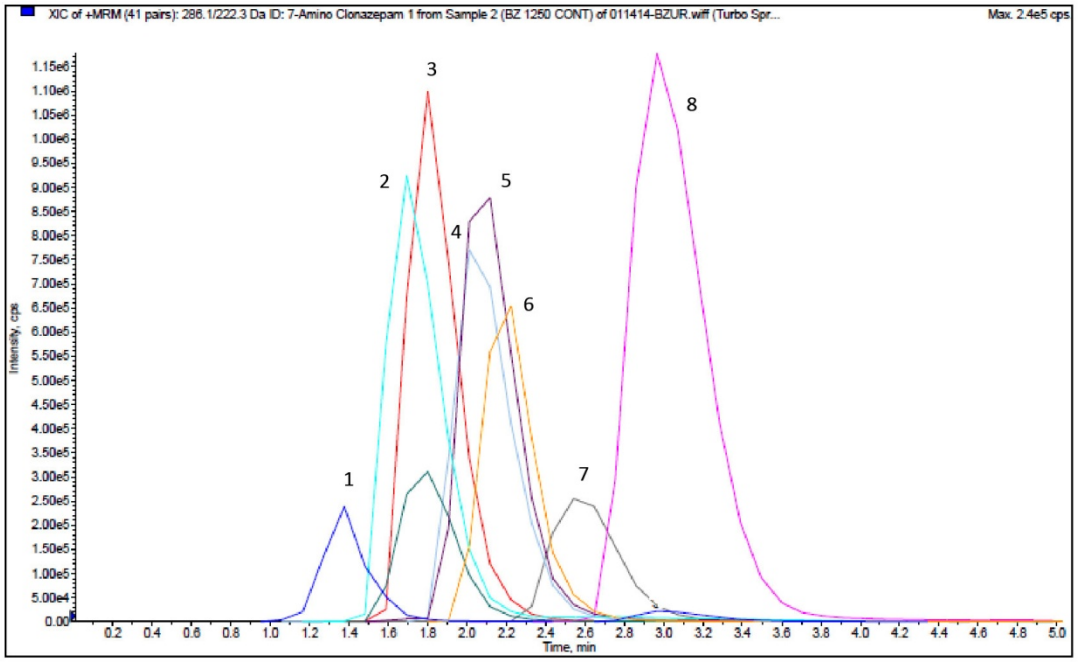
Evaporate fraction to complete dryness under stream of dry air or nitrogen at ~ 35 °C.

6. RECONSTITUTE / DERIVATIZE

- **LC-MS/MS:** Reconstitute sample in 100 µL of mobile phase
  Inject 10 µL.
- **GC-MS**: Dissolve residue in 50 µL of ACN and 50 µL MTBSTFA w/ 1% TBDMCS
  Overlay with N_2 and cap. Mix/vortex
  React 30 minutes at 70 °C; Cool and inject 1-2 µL

INSTRUMENT CONDITIONS (LC-MS/MS):

CHROMATOGRAM 1 SELECTRA® DA HPLC COLUMN



Analyte	MRM Transitions		Relative Retention Time (minutes)
	Q1	Q3	
1. 7-Amino Clonazepam	286.09	222.3	1.40
2. Oxazepam	287.09	241.3	1.70
3. Alpha- Hydroxy- Alprazolam	325.18	297.1	1.80
4. Clonazepam	316.13	270.2	2.10
5. Nordiazepam	271.09	140.1	2.10
6. Temazepam	301.12	255.2	2.20
7. Alprazolam	309.16	205.3	2.60
8. Diazepam	285.1	193.1	3.00

PARAMETERS

Mobile Phase A: 0.1% Formic Acid in D.I. H_2O

Mobile Phase B: 0.1% Formic Acid in Methanol

Flow Rate: 0.1 mL/minute

Polarity: Positive

Reconstitute: 100 µL

Injection Volume: 10 µL

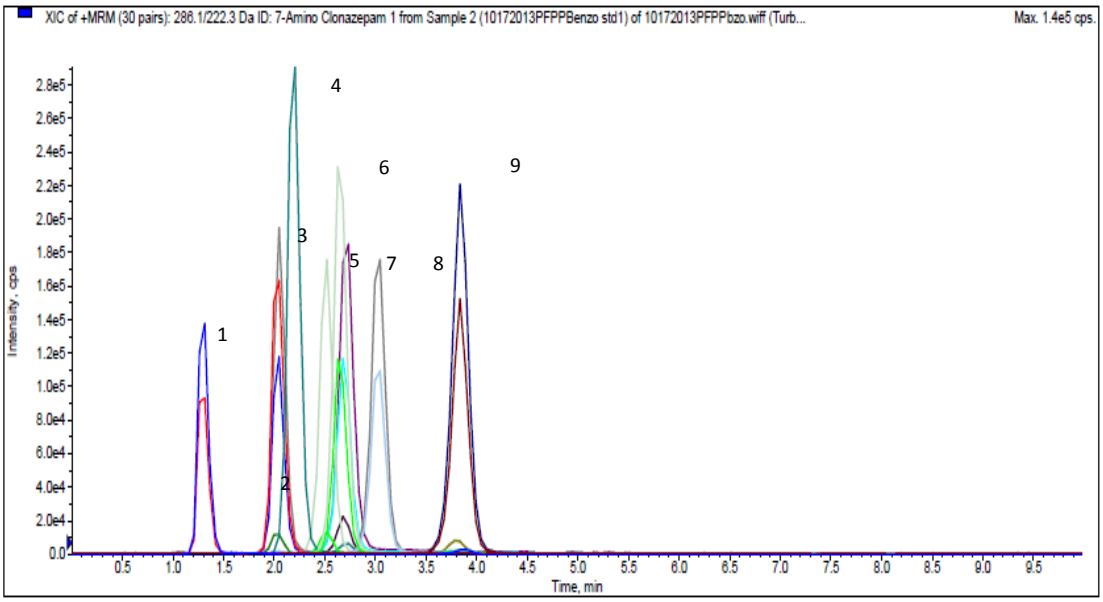
LC Column: Selectra® DA HPLC Column 50 x 2.1mm 5µm

Instrument: API 3200 Qtrap MS/MS with Shimadzu Prominence UFLC

Isocratic Flow:

Time	%A	%B
0.00	50	50
7.50	STOP	

CHROMATOGRAM 2 SELECTRA® PFPP HPLC COLUMN



Analyte	MRM Transitions		Relative Retention Time (minutes)
	Q1	Q3	
1. 7-Amino Clonazepam	286.09	222.3	1.30
2. Lorazepam	321.06	303.3	2.04
3. Alpha- Hydroxy- Alprazolam	325.18	297.1	2.05
4. Oxazepam	287.09	241.3	2.19
5. Clonazepam	316.13	270.2	2.51
6. Temazepam	301.12	255.2	2.65
7. Alprazolam	309.16	205.3	2.71
8. Nordiazepam	271.09	140.1	3.03
9. Diazepam	285.1	193.1	3.84

PARAMETERS

Mobile Phase A: 0.1% Formic Acid in D.I. H_2O

Mobile Phase B: 0.1% Formic Acid in Methanol

Flow Rate: 0.5 mL/minute

Polarity: Positive

Reconstitute: 100 µL

Injection Volume: 10 µL

LC Column: Selectra® PFPP HPLC Column 100 x 2.1 mm 5 µm

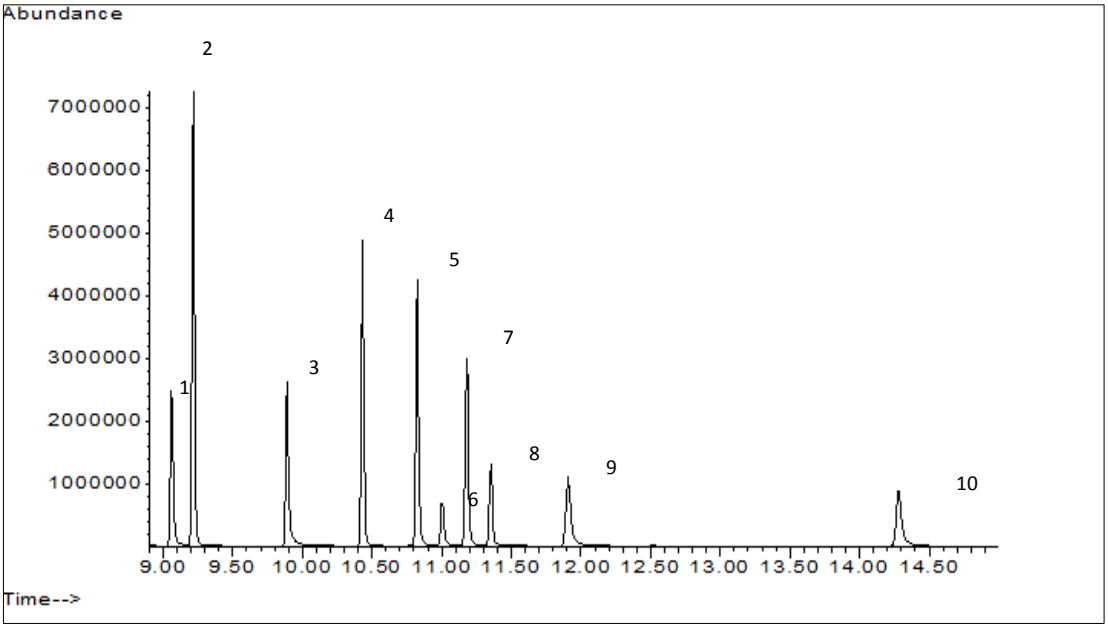
Instrument: API 4000 Qtrap MS/MS with Agilent 1200 Binary Pump SL

Isocratic Flow:

Time	%A	%B
0.00	40	60
10.0	STOP	

INSTRUMENT CONDITIONS (GC-MS):

CHROMATOGRAM



TBDMS IONS

Analyte	Quantify Ion	Qualifier Ion 1	Qualifier Ion 2	Relative Retention Time (min)
1. Diazepam	256.0	283.0	221.0	9.06
2. Nordiazepam TBDMS	327.0	383.1	369.0	9.22
3. Midiazolam	310.0	325.0	297.0	9.89
4. Oxazepam 2TBDMS	457.1	513.2	383.1	10.43
5. Temazepam TBDMS	357.0	283.0	385.1	10.82
6. 7-Amino Clonazepam TBDMS	342.0	399.1	328.0	11.00
7. Lorazepam 2TBDMS	491.1	513.2	533.1	11.18
8. Clonazepam TBDMS	372.0	326.0	429.0	11.36
9. Alprazolam	279.0	204.0	308.0	11.91
10. Alpha-Hydroxy Alprazolam TBDMS	381.0	423.1	346.0	14.28

PARAMETERS

GC/MS: Agilent - 5975C XL / 6890N GC/MS System with 7683B ALS System

GC capillary column: Rxi-5sil MS 30 m x 0.25 mm, 0.25 µm

Injector: 1 µL Splitless 250 °C

Oven temperature program: 160 °C for 0.5min; 15 °C/min to 310 °C for 4.50 minutes

Carrier gas: Helium

MSD condition: Aux temperature: 280 ºC, MS Source: 250 ºC, MS Quad: 150 ºC