BENZODIAZEPINES IN URINE BY LC-MS/MS OR GC-MS CLEAN SCREEN® DAU EXTRACTION COLUMN

Part #
ZSDAU020 CLEAN SCREEN® DAU 200 mg, 10 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

1. **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. **CONDITION CLEAN SCREEN® EXTRACTION COLUMN:**
   1 x 3 mL CH_3OH.
   1 x 3 mL D.I. H_2O.
   1 x 3 mL 100 mM phosphate buffer (pH 6.0).
   NOTE: Aspirate at full vacuum or pressure

3. **APPLY SAMPLE:**
   Load at 1 to 2 mL/minute.

4. **WASH COLUMN:**
   1 x 3 mL D.I. H_2O.
   1 x 3 mL 5% Acetonitrile in 100 mM phosphate buffer (pH 6.0).
   Dry column (5 minutes at full vacuum or pressure).
   1 x 2 mL Hexane.

5. **ELUTE BENZODIAZEPINES:**
   1 x 3 mL Ethyl Acetate containing 2% NH_4OH
   collect eluate at 1 to 2 mL/minute.

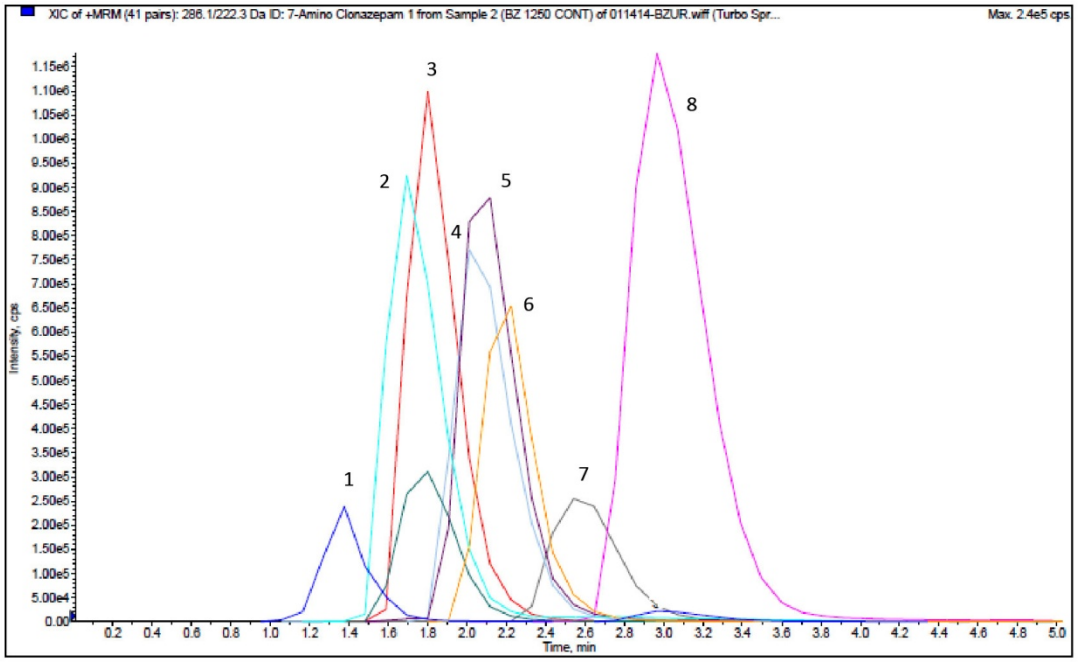
   NOTE: Prepare elution solvent daily.

6. **DRY ELUATE:**
   Evaporate to dryness at < 40 °C.

7. **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

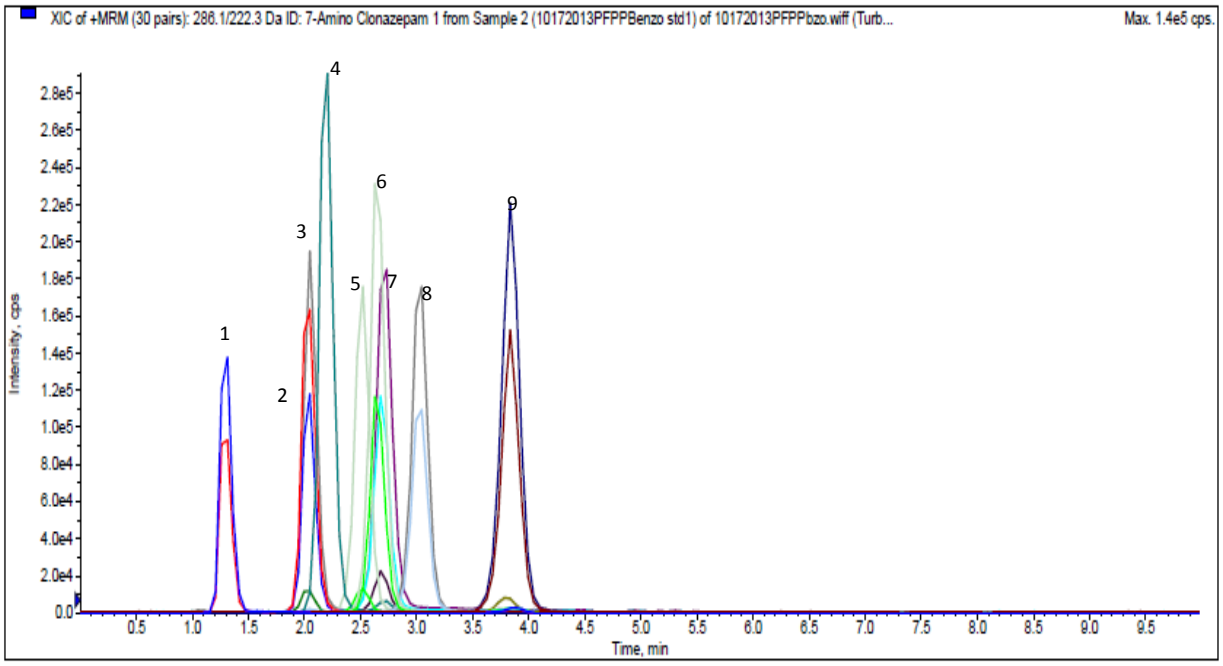
LC Column: Selectra® DA HPLC Column 50 x 2.1 mm 5 µm

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

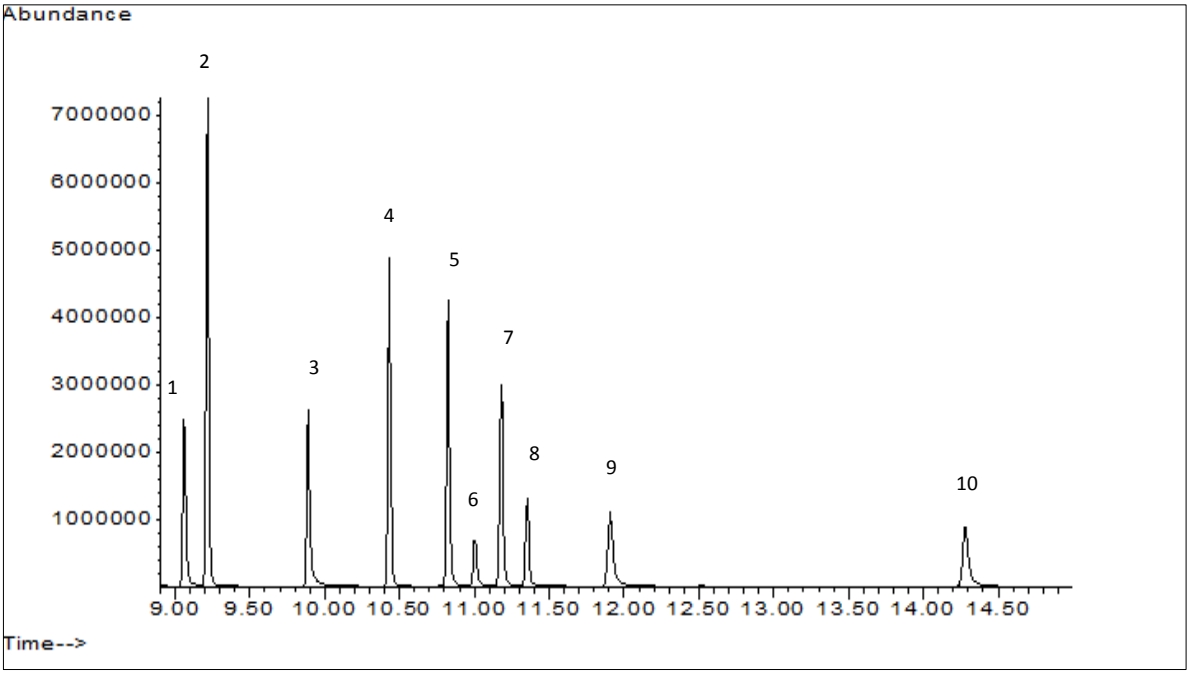
Instrument: API 4000 Qtrap MS/MS with Agilent 1200 Binary Pump SL

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 30m 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