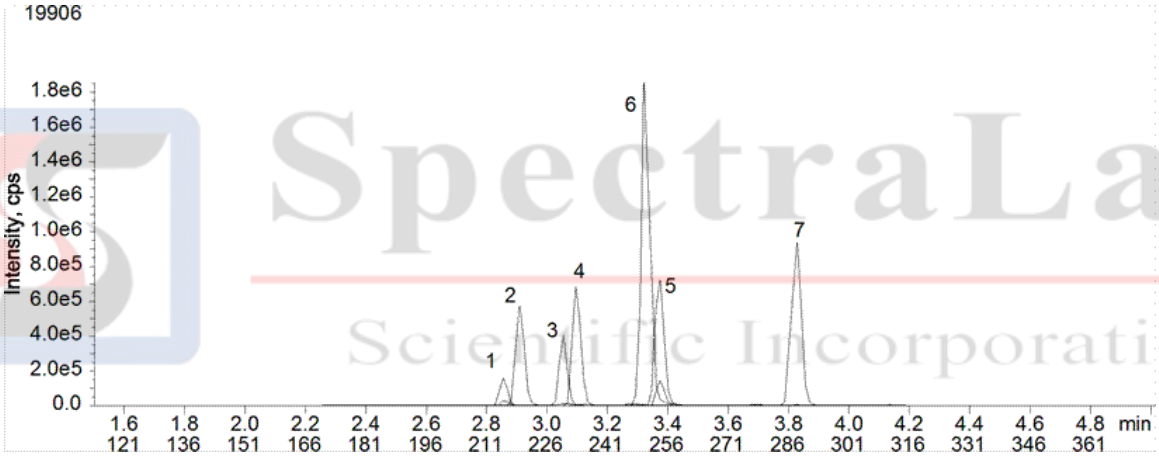
Analysis of Benzodiazepines by LC-MS-MS

Method 1: Benzodiazepines

Instrumentation: Waters ACQUITY UPLC system **with** AB Sciex LC/MS/MS System
Column: Waters XBridge C18 2.5µm 2.1 X 50mm Column (Ctrl + Click to follow link)
Elution Type: Gradient
Elution A: 10mM Ammonium Formate
Elution B: Acetonitrile
Gradient Profile:

Step No.	Time (min)	Pct A	Pct B
1	0	85	15
2	4	45	55

Flow Rate: 0.5 mL/min
Col. Temp: Ambient
Detection: Tandem Mass Spec (MS-MS) **@ amu (22°C)** (Ctrl + Click to follow link)
Detector Info: AB Sciex API 4000 (Ctrl + Click to follow link)



1: alpha-Hydroxyalprazolam
2: Oxazepam
3: Lorazepam
4: Clonazepam
5: Nordiazepam
6: Temazepam
7: Diazepam

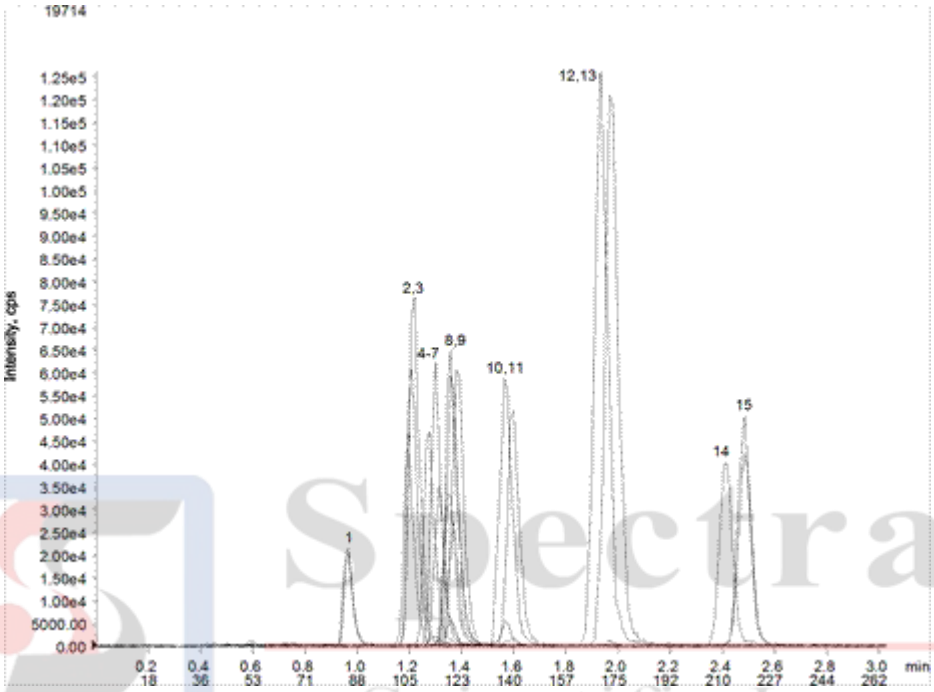
Method 2: Benzodiazepines from Urine

Instrumentation: Waters ACQUITY UPLC system **with** AB Sciex LC/MS/MS System
Column: Waters XBridge C18 2.5µm 2.1 X 50mm Column (Ctrl + Click to follow link)
Elution Type: Gradient
Mobile Phase A: Water with 0.1% Formic Acid
Mobile Phase B: Methanol with 0.1% Formic Acid
Gradient Profile:

Step No.	Time (min)	Pct A	Pct B
1	0	65	35
2	4	5	95
3	4.01	65	35
4	7	65	35

Flow Rate: 0.3 mL/min
Col. Temp: Ambient
Detection: Tandem Mass Spec (MS-MS) **@ amu (22°C)** (Ctrl + Click to follow link)
Detector Info: AB Sciex API 3000 (Ctrl + Click to follow link)
MS Conditions
Source: Cur-11, CAD-12, IS-4500, Tem-450, NEB-12
Dwell: 25ms
Polarity: Positive

Sample Preparation:
Solid Phase Extraction (SPE) procedure: Note: The solvent volumes shown below are for a 100 mg bed mass. The solvent volumes will need to be adjusted for a smaller or larger bed mass.
Load: Pre-Treatment: To 2mL urine, add 1mL beta-glucuronidase (contains 5,000 F units/mL Patella vulgata in 100mM acetate buffer(pH 5.0).
Mix/vortex.
Hydrolyze for 3 hours at 60C.
Let cool and add 1mL 100mM Phosphate buffer (pH 6.0).
Centrifuge for 5 minutes at 5000 rpm and discard pellet. Load directly onto SPE cartridge - NO CONDITIONING REQUIRED (Extraction automated using a Gilson GX-274 ASPEC liquid handler)
Wash: Acetonitrile:Water (20:80) 2 mL @ 2 mL/min
Dry: 10 minutes @ full vacuum
Elute: Ethyl acetate:Isopropanol (85:15) 2 mL @ 2 mL/min
Final Prep and Analysis: Evaporate eluant under a stream of N_2 gas at 50ºC. Reconstitute with 1mL of 35% methanol. Inject: 5 µL on HPLC / Mass Spectrometer (MS) @ amu (ambient)



1 alpha-Hydroxyalprazolam
2 Oxazepam-d5
3 Oxazepam
4 Alprazolam-d5
5 Alprazolam
6 Nordiazepam-d5
7 Nordiazepam
8 Lorazepam-d4
9 Lorazepam
10 Clonazepam-d4
11 Clonazepam
12 Temazepam-d5
13 Temazepam
14 Diazepam-d5
15 Diazepam

Method 3: Benzodiazepines from Blood

Instrumentation: Agilent 1100-series vacuum degasser, binary pump, autosampler, thermostatted column compartment, diode array detector (DAD) with micro-flow cell **and** Applied Biosystem 3200 Q TRAP LC/MS System
Column: ZORBAX Eclipse XDB-C8, 150 × 4.6 mm, 5 µm (Ctrl + Click to follow link)
Elution Type: Gradient
Mobile Phase A: 20 mM Ammonium formate, pH 9 in water
Mobile Phase B: Methanol
Gradient Profile:

Step No.	Time (min)	Pct A	Pct B
1	0	40	60
2	15	20	80
3	16	0	100
4	21	0	100

Flow Rate: 0.7 mL/min
Col. Temp: Ambient
Detection: Tandem Mass Spec (MS-MS) LC/MSD Trap **@ amu (22°C)** (Ctrl + Click to follow link)
Detector Info: Applied Biosystem 3200 Q TRAP LC/MS System (Ctrl + Click to follow link)
MS Conditions
Source: Positive APCI
Nebulizer: 60 psig
Vaporizer: 400 °C
Drying gas flow: 5 L/min
Drying gas temp: 350 °C
Vcap: 3000 V
Corona: 4 µA
Scan: m/z 150–400
Averages: 2
SPS settings: Target mass m/z 300
Compound stability 60% (Skim 1: 24 V, Cap exit offset: 69 V)
Trap drive 100% (resulting value 27)
Precursor isolation width: 2.5 amu
Cutoff: 45% (113–175 m/zfor these compounds)
MRM: Eight time segments as shown in the table below:

Group number [min]	Benzodiazepine/ metabolite	RT (min)	Precursor ion [M+H]+	Major ion (m/z)	Fragmentation amplitude (V)	Fragmentation width (m/z)
1	7-aminonitrazepam	2.6	252	224	2.00	10
[1.00–4.00]	7-aminoclonazepam	2.8	286	250	2.50	10
	7-aminoflunitrazepam	3.1	284	264	1.88	10
2	Bromazepam	5.3	316	288	1.92	10
[4.00–5.70]						
3	Clonazepam	6.1	316	270	2.00	10
[5.70–6.70]	Nitrazepam	6.2	282	236	1.86	10
	Flunitrazepam	6.3	314	268	1.90	10
4	Clobazam	7.3	301	259	3.37	40
[6.70–8.80]	Flurazepam	7.6	388	315	2.60	40
	Triazolam	7.8	343	308	3.57	40
	Alprazolam	8.3	309	281	4.67	40
	Lorazepam	8.3	321	275	2.98	40
	Oxazepam	8.6	287	241	3.32	40
5	N-desalkylflurazepam	9.2	289	261	4.57	40
[8.80–11.10]	Temazepam	9.6	301	255	3.72	40
6	Nordiazepam	12.1	271	243	1.88	10
[11.10–13.00]						
7	Diazepam	13.9	285	257	1.90	10
[13.00–17.00]	Midazolam	14.9	326	291	2.05	10
8	Prazepam	19.4	325	271	1.90	10
[17.00–21.00]						

Sample Preparation:

To 0.5-mL blood in a glass screw-top tube was added 50 µL of freshly prepared internal standard (Prazepam) working solution (5 µg/mL in water). To this tube was added 1.75 mL of 4.5% ammonia solution and 10 mL of 1-chlorobutane, and the contents rolled on a mechanical mixer for 10 minutes. After centrifuging the solvent was drawn off, transferred to a clean glass tube and evaporated to dryness in a Jouan centrifugal evaporator. The residue was dissolved in 100 µL of the initial mobile phase.

Results:

This method can easily detect, confirm and quantify multiple benzodiazepines and metabolites in a single analysis. The technique is suitable for screening analyses and confirmation of identity of benzodiazepines at their lowest reported therapeutic and sub-therapeutic concentrations using only 500 µL of blood. It is possible to detect down to 0.006 mg/L (6 ng/mL) of alprazolam while still obtaining a clear identification with a full-scan MS/MS spectrum.

References:

http://www.phenomenex.com/Application/Detail/19906
http://www.phenomenex.com/Application/Detail/19714
http://www.chem.agilent.com/Library/applications/5989-4737EN.pdf