Section 6 – Analytical Methods, Specific

Part 5 – Benzodiazepines/Z-drugs

Purpose and Scope

This document presents methods to analyze specimens suspected of containing benzodiazepines, z-drugs and various metabolites. These drugs are extracted from biological samples using solid phase extraction (SPE) technology and then analyzed by GC/MS or LC/MS/MS. If samples are analyzed by GC/MS, derivatization is required for some analytes. Urine samples are hydrolyzed prior to extraction and analysis. A specialized extraction procedure for clonazepam is also included.

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6.5.1 Definitions

Hydrolysis – a process in which the conjugate bond is broken to allow the freed moiety to be detected.
Total drug concentration – The sum of the unbound/free and bound/conjugated drug.

6.5.2 Chemicals and Reagents

6.5.2.1 Chemicals

Abalonase ultra
Acetonitrile
BGTurbo enzyme (β-Glucuronidase)

BSTFA+
Ethyl acetate
β-Glucuronidase solution (IMCSzyme 3S)
β-Glucuronidase G7017 (from *Helix pomatia)*
β-Glucuronidase solution, from abalone
MTBSTFA+
Hexanes
Instant buffer I
Rapid hydrolysis buffer
Methanol
Methanol (LC/MS grade)
Acetonitrile (LC/MS grade)
Isopropanol (LC/MS grade)
Methylene chloride
Formic acid (LC/MS grade)
LC/MS/MS water

6.5.2.2 Reagents

Abalonase enzyme solution
0.1M Acetate buffer, pH 5.0
0.1M Acetate buffer, pH 4.5
1M Acetic acid, pH 2.4
1 M Acetic acid: acetonitrile
Alprazolam extraction solvent
EA with 2% Ammonium Hydroxide
Basic elution solvent
Benzo extraction solvent
Borate buffer, saturated, pH 9.0
Clonazepam extraction solvent
Enzyme hydrolysis reagent (from limpets or from *Helix pomatia*)
0.1M Phosphate buffer, pH 6.0
0.1M Phosphate buffer, pH 6.0 (LC/MS/MS)
LC/MS/MS mobile phase - Methanol with 0.1% formic acid
LC/MS/MS mobile phase - Water with 0.1% formic acid
LC/MS/MS mobile phase - Water with 2% formic acid
LC/MS/MS needle wash
LC/MS/MS seal wash

See TXPM 2.1 Chemicals and Reagents for necessary chemicals and preparation instructions.

6.5.3 Equipment and Supplies

Centrifuge, capable of ≥4,000 rpm
Evaporator/Concentrator
GC/MS Instrument, (Agilent or similar) with applicable software, a compatible computer, and printer
Heating Block with NIST traceable thermometer
LC/MS/MS Instrument, (Waters Inc. or similar) with applicable software, a compatible computer, and printer
PTFE septa and caps, pre-slit and in-house assembled
Pipets – Pasteur, disposable
Pipets – Transfer, disposable
Pipetters – air displacement with disposable tips

Pipetters – positive displacement (QP specifications) with disposable tips
Repeater pipette
Screw Caps – Teflon® lined
Solid Phase Extraction Manifold
SPE Columns, Clean Screen® Extraction Column (ZSDAU020) from UCT (200 mg)
Test Tube Rocker or Rotator
Tubes – round/conical bottom borosilicate glass with screw tops or disposable culture tubes
Vials for LC/MS/MS Autosampler with inserts and pre-slit caps
Vials for GC Autosampler with inserts and caps
Vortex Mixer

6.5.4 Procedure

6.5.4.1 Specimen Criteria

- 1 mL whole blood, biological fluids, or tissue homogenates with preservatives, if applicable, for GC/MS analysis.
- 500 µL whole blood or urine with preservatives, if applicable for LC/MS/MS analysis.
- Samples will be removed from storage and brought to room temperature before analysis.

6.5.4.2 Certified Reference Materials

Spiking solutions will be prepared from certified reference materials. Spiking solutions for qualitative analytes may be prepared from reference materials (see TXPM 2.4 Reference Materials and Certified Reference Materials).

Internal standard solution may be deuterated versions of the analytes.

6.5.4.3 Calibrators

Calibrators are prepared utilizing the spiking solutions. See Table 1 for an example of calibrator concentrations for GC/MS analysis. See Table 2 for an example of calibrator concentrations for LC/MS/MS analysis.

Table 1 – An example of calibrator concentrations for GC/MS.

Drug or Metabolite	Cal A ug/L	Cal B ug/L	Cal C ug/L	Cal D ug/L
Diazepam	500	250	100	50
7-Aminoflunitrazepam	100	50	20	10
Midazolam	100	50	20	10
Estazolam	100	50	20	10
Alprazolam	100	50	20	10
Triazolam	100	50	20	10
7-Aminoclonazepam	100	50	20	10
α-OH-alprazolam	100	50	20	10
α-OH-triazolam	100	50	20	10
Nordiazepam	500	250	100	50
Oxazepam	200	100	50	25
Lorazepam	100	50	20	10
Temazepam	100	50	20	10
2-OH-ethylflurazepam	100	50	20	10

Table 2 – An example of calibrator concentrations for LC/MS/MS.

Drug or Metabolite	Cal 7 ug/L	Cal 6 ug/L	Cal 5 ug/L	Cal 4 ug/L	Cal 3 ug/L	Cal 2 ug/L	Cal 1 ug/L
7-aminoclonazepam	240	160	80	40	20	10	5
Alprazolam	240	160	80	40	20	10	5
Clonazepam	240	160	80	40	20	10	5
Diazepam	1000	500	200	100	40	20	10
Etizolam	240	160	80	40	20	10	5
Lorazepam	240	160	80	40	20	10	5
Nordiazepam	1000	500	200	100	40	20	10
Oxazepam	1000	500	200	100	40	20	10
Temazepam	1000	500	200	100	40	20	10
Zolpidem	240	160	80	40	20	10	5
α-OH-alprazolam	240	160	80	40	20	10	5

6.5.4.4 Controls

Negative and positive controls must meet requirements defined in TXPM 4.4 Quantitative and Qualitative Quality Controls or TXPM 4.9 Liquid Chromatographic and Tandem Mass Spectral Quality Control.

6.5.4.5 Hydrolysis Procedures

Urine specimens should be hydrolyzed to determine the total drug concentration prior to extraction using one of the following hydrolysis procedures. Blood specimens may also be hydrolyzed for GC/MS. A glucuronide positive control shall be used to ensure that the hydrolysis was effective, if available.

6.5.4.5.1 Hydrolysis Procedure – Option 1- GC/MS

1) Into appropriately labeled tubes add:
    - Hydrolysis reagent (per Table 3).
    - Spike internal standard and controls, as necessary.
    - 1 mL unknown or blank matrix.
2) Gently mix the tubes.
    - For bloods, allow to equilibrate for 10 minutes prior to hydrolysis.
3) Cap and if applicable, incubate (per Table 3).
4) If applicable, cool the tubes.
5) To each tube add:
    - 7 mL 0.1M acetate buffer, pH 5.0
6) Continue with step 7 in TXPM 6.5.4.6 below.

Table 3 – Hydrolysis Methods

Hydrolysis Method	Hydrolysis Reagent	Minimum Time	Temperature (+/- 5°C)
Enzyme hydrolysis reagent (from Helix pomatia or limpets)	1mL enzyme hydrolysis reagent	2 hours	60°C
Campbell Science β-glucuronidase solution, from abalone	≥60µL β-glucuronidase, from abalone 200µL acetate buffer, pH 5.0	30 minutes	65°C
Kura Biotech BGTurbo High Efficiency Recombinant β-Glucuronidase	400µL Instant Buffer I 200µL BGTurbo Enzyme 1100 µL H_2O	No wait	Room temperature
United Chemical Technologies (UCT) Abalonase Ultra β-glucuronidase enzyme	1mL Abalonase Enzyme Solution	30 minutes	65°C
Integrated Micro-Chromatography Systems (IMCS) 3S genetically modified β-glucuronidase	400µL Rapid Hydrolysis Buffer 20µL β-glucuronidase	30 minutes	55°C

6.5.4.5.2 Hydrolysis Procedure – Option 2 – LC/MS/MS

1) Into appropriately labeled tubes add:
   - 200 µL Instant Buffer I
   - 100 µL BGTurbo
   - 550 µL H_2O
   - Spike internal standard and controls, as necessary
   - 500 µL unknown or blank matrix
2) Gently mix the tubes.
3) Incubate at room temperature for zero minutes (i.e. no wait hydrolysis)
4) Add 2 mL 0.1M phosphate buffer, pH 6.0 (LC/MS/MS).
5) Continue with step 5 in TXPM 6.5.4.8 below.

6.5.4.6 Benzodiazepine Procedure Option 1 - SPE with TMS Derivatization and GC/MS Analysis

1) Label tubes accordingly.
2) Spike internal standard, calibrators and controls as necessary.
3) May add 200-500 uL H_2O to each tube.
4) Pipet 1 mL blank matrix into calibrators and controls and 1 mL unknown into appropriately labeled tubes.
5) Vortex for 5-10 seconds and equilibrate for ≥10 minutes.
6) To each tube add:
   - 8 mL 0.1M acetate buffer, pH 5.0
7) Cap and rock for ≥10 minutes.
8) Centrifuge for ≥10 minutes.
9) Set up the SPE manifold with SPE columns, Clean Screen® Extraction Column (ZSDAU020).
10) Prep the SPE columns with:
    - 1 mL methylene chloride
    - 3 mL methanol
    - 2 mL H_2O
    - 1 mL 0.1M acetate buffer, pH 5.0
11) Transfer the supernatant from the tubes into the appropriate SPE column. Avoid transferring any of the pellet from the bottom of the tube.
12) Sequentially wash the columns with:
    - 1 mL H_2O
    - 3 mL 1M acetic acid, pH 2.4
13) Dry the columns high pressure for ≥12 minutes.
14) Turn off the high pressure and wash the columns with:
    - 1 mL hexanes
15) Re-dry the SPE columns under high pressure for ≥5 minutes.
16) Wash the columns with:
    - 4 mL ethyl acetate
17) Briefly turn on the high pressure to draw out the last of the solvent from the columns.
18) Re-dry the SPE columns under high pressure for ≥3 minutes.
19) Turn off the high pressure. Put the labeled tubes into the manifold and elute with:
    - 3 mL basic elution solvent.
20) Briefly turn on the high pressure to draw out the last of the solvent out of the columns.
21) Evaporate to dryness at ≤50°C under nitrogen.
22) To each tube add:
    - 50 uL acetonitrile
23) Vortex and transfer to appropriately labeled autosampler vials with inserts. Reserve tubes for step 27. Cap the vials with in-house assembled/pre-slit **PTFE** septa caps.
24) The samples are now ready for <u>underivatized</u> GC/MS analysis.
    Note: Underivatized benzodiazepines may include diazepam, 7-aminoflunitrazepam, alprazolam, estazolam, midazolam, and triazolam.
25) After underivatized GC/MS analysis: to each autosampler vial perform a quantitative solvent rinse by doing the follow steps **3** times:
    - Add 100 uL acetonitrile
    - Vortex
    - Transfer to the appropriate tube
26) Evaporate to dryness at ≤50°C under nitrogen.
27) To each tube add:
    - Blood samples with 25 uL acetonitrile and 25 uL BSTFA+
    - Urine samples with 25 uL acetonitrile and 75 uL BSTFA+
28) Cap and incubate at 90±5°C for ≥ one hour.
29) Centrifuge for ≥3 minutes.
30) Transfer the solution into the appropriately labeled autosampler vials with inserts. Cap the vials with new in-house assembled/pre-slit **PTFE** septa caps.

The samples are now ready for analysis by GC/MS.

Note: Derivatized benzodiazepines may include 7-aminoclonazepam, 7-aminoflunitrazepam, alpha-hydroxyalprazolam, alpha-hydroxytriazolam, nordiazepam, oxazepam, lorazepam, temazepam and 2-hydroxyethylflurazepam.

6.5.4.7 Clonazepam Procedure – SPE with TBDMS Derivatization

1) Label tubes appropriately.
2) Spike internal standard, calibrators and controls as necessary.
3) May add 200-500 uL H_2O to each tube.
4) Pipet 1 mL blank matrix into calibrators and controls and 1 mL unknown into appropriately labeled tubes.
5) Vortex for 5-10 seconds and equilibrate for ≥10 minutes.
6) To each tube add:
   - 8 mL 0.1M phosphate buffer, pH 6.0
7) Cap and rock for ≥10 minutes.
8) Centrifuge for ≥10 minutes.
9) Set up the SPE manifold with SPE columns, Clean Screen® Extraction Column (ZSDAU020).
10) Prep the SPE columns with:
    - 3 mL methanol
    - 2 mL H_2O
    - 1 mL 0.1M phosphate buffer, pH 6.0
11) Transfer the supernatant from the tubes into the appropriate SPE column. Avoid transferring any of the pellet from the bottom of tube.
12) Wash the columns with:
    - 3mL clonazepam extraction solvent
13) Dry the columns under high pressure for ≥12 minutes.
14) Wash the columns with:
    - 3 mL hexanes
15) Dry the columns under high pressure for ≥12 minutes.
16) Turn off the high pressure. Put labeled tubes into the manifold and elute with:
    - 2 aliquots of 3 mL ethyl acetate.
17) Briefly turn on the high pressure to draw out the last of the solvent from the columns.
18) Evaporate to dryness at ≤50°C under nitrogen.
19) To each tube add:
    - 25 uL ethyl acetate
    - 25 uL MTBSTFA+
20) Vortex, and transfer into appropriately labeled autosampler vials with inserts. Cap the vials.
21) Derivatize at room temperature for at least 20 minutes.

The samples are now ready for analysis by GC/MS.

6.5.4.8 Benzodiazepine Procedure Option 2 – SPE with LC/MS/MS Analysis

1) Label tubes accordingly.
2) Spike internal standard, calibrators and controls as necessary.
3) Pipet 500 µL blank matrix into calibrators and controls and 500 µL unknown into appropriately labeled tubes.
4) Add 3 mL 0.1M phosphate buffer, pH 6.0 (LC/MS/MS) to each tube.
5) Vortex for 5-10 seconds and equilibrate for ≥5 minutes.
6) Centrifuge for ≥10 minutes.
7) Set up the SPE manifold with SPE columns, Clean Screen® Extraction Column (ZSDAU020).

8) Prepare the SPE columns with:
   - 3 mL methanol
   - 3 mL H_2O (LC/MS/MS grade)
   - 1 mL 0.1M phosphate buffer, pH 6.0 (LC/MS/MS)
9) Transfer the supernatant from the tubes into the appropriate SPE column without transferring the pellet. Load at 0-1 mL/min (≤ 5 psi on PPM), no faster to avoid analyte breakthrough.
10) Sequentially wash the columns with:
    - 3 mL H_2O (LC/MS/MS grade)
      - Ensure no blood is on the walls of the column. Options include using a kimwipe, cotton tip applicator, or using the water in this step to rinse the walls.
    - 2 mL 1M acetic acid:acetonitrile
      - Ensure minimal to no aqueous is on the walls of the column
    - 2 mL hexanes
11) Dry the columns under high pressure for ≥20 minutes.
12) Put the labeled tubes into the manifold and elute with:
    - 3 mL EA with 2% Ammonium Hydroxide
      - Collect eluent with minimal airflow (1 drop per second approximately).
13) Evaporate to dryness at ≤40°C under nitrogen.
14) Return the labeled tubes into the manifold and elute with:
    - 3 mL EA with 2% Ammonium Hydroxide
      - Collect eluent with minimal airflow (1 drop per second approximately).
15) Briefly turn on the high pressure to draw out the last of the solvent out of the columns.
16) Evaporate to dryness at ≤40°C under nitrogen.
17) To each tube:
    - add 40 µL methanol (LC/MS grade)
    - Vortex
    - Transfer the solution into appropriately labeled autosampler vials
18) Repeat step 17.
19) To each autosampler vial:
    - Add 120 µL H_2O (LC/MS/MS grade)
20) Cap the vials with pre-slit PFTE septa caps.

The vials are ready for LC/MS/MS analysis

6.5.4.9 LC/MS/MS Performance Characteristics

Table 4 - Quantitative analytes

Drug or Metabolite	LOD (µg/L)	LLOQ (µg/L)	Working range (µg/L)	Dilution* (1:1) 250 µL	Dilution* (1:4) 100 µL	Dilution* (1:9) 50 µL
7-Amino clonazepam	5	5	5-240	Yes	Yes	Yes
Alprazolam	5	5	5-240	Yes	Yes	Yes
a-OH alprazolam	5	5	5-240	Yes	Yes	Yes
Clonazepam	5	5	5-240	Yes	Yes	Yes
Diazepam	10	10	10-1000	Yes	Yes	Yes
Etizolam	5	5	5-240	No	No	No
Lorazepam	5	5	5-240	Yes	Yes	Yes
Nordiazepam	10	10	10-1000	Yes	Yes	Yes
Oxazepam	10	10	10-1000	Yes	Yes	Yes
Temazepam	10	10	10-1000	Yes	Yes	Yes
Zolpidem	5	5	5-240	Yes	Yes	Yes

*When an unknown requires dilution to report quantitative results, a dilution QPC must be prepared the same as the unknown and run with the same dilution factor as the unknown.

Table 5 - Qualitative analytes
Limit of detection is 5 µg/L for the below analytes

Drug/Metabolite	Drug/Metabolite
2-OH-ethylflurazepam	Estazolam
7-Aminoflunitrazepam	Flualprazolam
8-Aminoclonazolam	Flubromazepam
Adinazolam	Flubromazolam
a-OH-clonazolam	Flunitrazepam
a-OH-etizolam	Flurazepam
a-OH-flubromazolam	Lormetazepam
a-OH-triazolam	Midazolam
Bromazepam	Norchlordiazepoxide
Bromazolam	Nimetazepam
Chlordiazepoxide	Nitrazepam
Clobazam	Phenazepam
Clonazolam	Pyrazolam
Delorazepam	Triazolam
Demoxepam	Zaleplon
Diclazepam	Zopiclone

6.5.4.10 LC/MS/MS Instrument Parameters

Column:
Waters Acquity UPLC BEH C18 1.7 µm 2.1 x 100 mm
Optional: Pre-column or inline filter unit

LC Parameters:
Sample Manager Temperature Setpoint: 4°C ±12.0 °C
Column Temperature Setpoint: 50 °C ±5.0 °C
Injection Volume: 1 µL
Flow rate: 0.400 mL/min

Example mobile phases:
Mobile phase A: LC/MS/MS water
Mobile phase B: Methanol (LC/MS grade)
Mobile phase C: Water with 2% formic acid

Table 6 – Quaternary Solvent Manager Gradient Example

Time (minutes)	% A	%B	%C	Curve
Initial	65	30	5	6
2.50	30	65	5	6
3.25	25	70	5	6
4.50	18	77	5	6
4.51	5	90	5	6
4.80	5	90	5	6
5.91	65	30	5	6
9.00	65	30	5	6

Note: Variations in gradient may exist due to instrument capabilities, column properties, etc

Example mobile phases:
Mobile phase A: Water with 0.1% formic acid
Mobile phase B: Methanol with 0.1% formic acid

Table 7 – Binary Solvent Manager Gradient Example

Time (minutes)	% A	%B	Curve
Initial	65	35	6
0.76	65	35	6
3.26	30	70	6
4.01	25	75	6
5.26	18	82	6
5.27	5	95	6
6.56	5	95	6
6.67	65	35	6
9.76	65	35	6

Note: Variations in gradient may exist due to instrument capabilities, column properties, etc

MS/MS Parameters:
Capillary Voltage Setpoint: 0.7 kV
Ionization: ESI, positive
Source Temperature Setpoint: 150 °C
Desolvation temperature Setpoint: 500 °C
Cone Gas Flow Setpoint: 20 L/hr
Desolvation Gas Flow Setpoint: 800 L/hr
Note: Variations in capillary voltage may exist between different instruments

Table 8 - Ion Transitions (**Quantitation ions in bold)**

Compound	Precursor ion	Product Ions	Cone (V)	Collision (eV)
Nordiazepam	271	**140**	18	28
		165		
D5-Nordiazepam	276	**140**	59	30
		165		26
Nitrazepam	282	180	45	36
		207		34
7-Aminoflunitrazepam	284	135	2	28
		227		24
Diazepam	285	**154**	2	26
		193		32
7-Aminoclonazepam	286	**121**	14	30
		222		24
Norchlordiazepoxide	286	227	34	22
		232		34
Oxazepam	287	104	48	40
		241		24
Demoxepam	287	104	52	22
		180		20
D4-7 Aminoclonazepam	290	**121**	28	32
		226		24
D5-Diazepam	290	154	4	28
		198		32
D7-7 Aminoflunitrazepam	291	230	92	30
		138	90	26
D5-Oxazepam	292	**246**	38	18
		274		14
Estazolam	295	205	6	38
		267		24
Nimetazepam	296	221	40	34
		268		22
Chlordiazepoxide	300	192	25	30
		227		24
D5-Estazolam	300	210	14	42
		272		24

Temazepam	301	177	24	40
		255	14	16
Clobazam	301	224	40	34
		259		20
Delorazepam	305	140	59	30
		206		34
D5-Temazepam	306	**177**	38	40
		198		34
Zaleplon	306	236	50	26
		264		20
Zolpidem	308	**235**	58	40
		263		25
Alprazolam	309	**205**	45	42
		274		24
D5-Alprazolam	314	210	64	40
		286		28
Flunitrazepam	314	183	45	50
		239		34
D7-Zolpidem	315	**242**	58	40
		270		25
Clonazepam	316	214	52	40
		270		24
Bromazepam	316	182	24	32
		209		26
Diclazepam	319	154	40	30
		227		30
D4-Clonazepam	320	218	52	36
		274		22
Lorazepam	321	229	40	30
		275		20
8-Aminoclonazolam	324	146	54	30
		220	79	38
α-OH-alprazolam	325	216	42	38
		297		26
Midazolam	326	223	4	36
		291		26
Flualprazolam	327	223	62	40
		292		26
D5-α-OH-alprazolam	330	221	62	38
		302		24
OH-ethyl-flurazepam	333	109	52	26
		211		36
Flubromazepam	333	184	12	28
		226		32
Lormetazepam	335	177	39	42
		227		36

Triazolam	343	239	45	40
		308		26
Etizolam	343	138	6	36
		314		24
Phenazepam	348	179	45	50
		206		34
Adinazolam	352	205	46	50
		295		22
Bromazolam	353	325	40	24
		205		44
Pyrazolam	353	167	45	34
		206		30
Clonazolam	354	280	10	34
		326		26
α-OH-etizolam	359	315	2	20
		282		24
α-OH-triazolam	359	277	12	34
		331		28
α-OH-clonazolam	370	296	36	36
		342		26
Flubromazolam	371	223	12	44
		292		26
α-OH-flubromazolam	387	223	50	48
		359		28
Flurazepam	388	288	40	24
		315		24
Zopiclone	389	217	22	36
		245		14

Notes: Precursor ions, product ions, cone voltages and collision energies may vary

Table 9 - Quantitative analytes calibration models and weighting

Drug or Metabolite	Calibration	Weighting	Origin
7-aminoclonazepam	quadratic	1/x	exclude
Alprazolam	linear	1/x^2	exclude
Clonazepam	quadratic	1/x	exclude
Diazepam	quadratic	1/x	exclude
Etizolam	linear	1/x^2	exclude
Lorazepam	quadratic	1/x	exclude
Nordiazepam	linear	1/x	exclude
Oxazepam	quadratic	1/x^2	exclude
Temazepam	quadratic	1/x^2	exclude
Zolpidem	linear	1/x	exclude
a-OH-alprazolam	linear	1/x	exclude

6.5.4.11 Limitations of LC/MS/MS Analysis

- No known matrix interferences were detected during validation.
- No drug interferences were detected during validation. Post-validation discoveries:
  - 4-chloro-deschloroalprazolam can be a potential interference of alprazolam.
  - Use of the precursor ion 309 and daughter 165 for alprazolam will help differentiate these analytes.
  - Further work will be conducted to add 4-chloro-deschloroalprazolam to the scope of testing, if needed.
- Quantitative results may be reported for 7-aminoclonazepam, alprazolam, clonazepam, diazepam, lorazepam, nordiazepam, oxazepam, temazepam, zolpidem, and alpha-hydroxy alprazolam at a dilution.
- Quantitative results may not be reported for etizolam in samples that are analyzed at a dilution (i.e., less than 500 µL sample volume).
- Zopiclone and eszopiclone are stereoisomers and are not differentiated via LC/MS/MS.

6.5.4.12 LC/MS/MS Stability

- Validation showed that the analytes listed in table 8 are stable once extracted and stored in the cooled autosampler for **two** days in blood.

  - Bromazolam, a-OH-clonazolam, diclazepam, flualprazolam, flurazepam, phenazepam, and adinazolam are stable for **one** day in blood.

- Validation showed that the analytes listed in table 8 are stable once extracted and stored in the cooled autosampler for **two** days in urine.
  - 7-aminoclonazepam, 8-aminoclonazolam, pyrazolam, zopiclone, and 7-aminoflunitrazepam are stable for **one** day in urine.

6.5.5 Results, Conclusions, and Data Analysis

These procedures are a means of extracting the substance of interest from a sample matrix in preparation for confirmation and/or quantitation by GC/MS or LC/MS/MS. See TXPM 4.5 Gas Chromatographic and Mass Spectral Quality Control or TXPM 4.9 Liquid Chromatographic and Tandem Mass Spectral Quality Control for information about the types of results and conclusions that can be made following these analyses.

- If zopiclone is confirmed, it will be reported as zopliclone/eszopiclone.
- Blood and urine samples may be analyzed by LC/MS/MS after their stability day and must be evaluated qualitatively.
  - If the analyte is acceptable, then report qualitatively. If the analyte is unacceptable, then report INC. Remediation includes reanalysis.

6.5.6 Reporting

Reporting will be done after GC/MS or LC/MS/MS analysis according to the guidelines in TXPM 3.6 Gas Chromatograph with Mass Spectrometry or TXPM 3.9 Liquid Chromatography with Tandem Mass Spectrometry and TXPM 4 – Quality Guidelines.

6.5.7 References

1) Oehldrich J & Collins CD, unpublished work, Wisconsin State Crime Laboratories
2) Jochemsen R & Breimer D, Journal of Chromatography, 223, p.438, 1981
3) Benzodiazepines and GHB Detection and Pharmacology ed. Salvatore J. Salamone: p.38 Flunitrazepam extraction pH 9-9.5 borate buffer with Diethyl Ether:Methylene Chloride (2:1) or Diethyl Ether:Chloroform (4:1); p. 58
4) United Chemical Technologies, Inc., Clonazepam & 7-Aminoclonazepam in Urine for GC/MS Confirmations using 200 mg Clean Screen Extraction Column
5) United Chemical Technologies, Inc., Benzodiazepines in Blood, plasma/serum, and tissue for LC/MS/MS Confirmations using 200 mg Clean Screen column
6) Colorado Bureau of Investigation TOX 10-19 Benzodiazepines and Z-Drugs quantitation by LC/MS/MS (Document #15967, Revision 8, Issue date 12/15/2022)
7) Milwaukee County Medical Examiner's Benzodiazepine Confirmation/Quantitation method
8) Wisconsin State Lab of Hygiene: Environmental Health Division, Forensic Toxicology Program, Benzodiazepines and Z-drugs Quantitation and Confirmation (revision 2.0, effective 7/12/2021)
9) Virginia Department of Forensic Science Benzodiazepines, Zolpidem, Zopiclone, and Zaleplon Quantification and Confirmation by LC/MS/MS (Qualtrax ID 2816, Revision 19, Issue date 6/22/2021)
10) Waters Application Note: Quantitative Analysis of 21 Benzodiazepine Drugs, Zolpidem and Zopiclone in Serum Using UPLC-MS/MS
11) Procedure For the Selection and Validation of a Calibration Model I-Description and Application, *Journal of Analytical Toxicology*, 2017;41:261–268 doi: 10.1093/jat/bkx001

6.5.8 Safety

Universal safety precautions should be taken for biological specimens and organic waste. No novel safety concerns.

6.5.9 Method Validation

The GC/MS method was established prior to the Wisconsin Crime Laboratories ISO 17025 accreditation in 2012. Use of this method throughout the years has demonstrated fitness for its intended purpose.

LC/MS/MS method was approved. Files are stored in the Unit Quality Records. LC/MS/MS method validated to ANSI/ASB Standard 036, First edition, 2019 Standard Practices for Method Validation in Forensic Toxicology.