SOP 402 - Benzodiazepine Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

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Benzodiazepine Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)		**402**
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	**Revision:**	**Revision Date/Initials:**
	10.1.1.4 – Updated RT acceptance range 10.1.2.1 – Updated IRC acceptance range 10.1.3.2 – Updated Calibrator acceptance range 10.1.4.1 – Updated QC acceptance range 10.1.4.2 - Deleted	MSF – 05/08/2015
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	9.1.1 – Added reference to SOP-055 10.2.4.1 – Added reference to QA Manual (Cal point exclusion)	MSF – 04/04/2016
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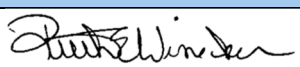
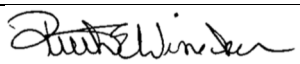
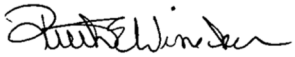
Approving Authority Name	**Approving Authority Signature**	**Approval Date**
Ruth E. Winecker, Ph.D.		04/17/2015
Ruth E. Winecker, Ph.D.		06/19/2016
Ruth E. Winecker, Ph.D.		12/07/2017

SOP 402 - Benzodiazepine Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

1. **Principle of Assay**
   1.1. This method is designed to confirm and quantitate commonly prescribed and abused benzodiazepines, and select metabolites, in biological specimens by Liquid Chromatography tandem-electrospray Mass Spectrometry (LC/MS/MS). The method is divided into two assays, based upon the frequency encountered in this laboratory, "Benzo I": alprazolam, clonazepam, 7-aminoclonazepam, lorazepam, diazepam, nordiazepam, and "Benzo II": temazepam, oxazepam, midazolam, triazolam. The assays only differ in the benzodiazepines detected, and are extracted from their biological matrix by protein precipitation and identified by the retention time and ion ratio of product ions. The target benzodiazepines are subject to matrix effects, thus stable isotopically labeled internal standards are used (1).

   Benzodiazepines are widely used drugs prescribed for anxiety, panic, and seizure disorders. The various benzodiazepines differ by FDA approved indications, as well as onset of action, *i.e.* short, intermediate, and long acting. Pharmacologically, benzodiazepines are positive allosteric modulators on the gamma amino butyric acid (GABA)-A receptor. Benzodiazepines bind to the GABA-A receptor at a location other than the binding site, resulting in a conformational change that enhances the receptors activity. Endogenous GABA is inhibitory, and reduces the excitability of neurons, creating a net calming effect that is potentiated by benzodiazepines. (2)

   Adverse central nervous system (CNS) effects of benzodiazepines include anterograde amnesia, lethargy, motor impairment, disinhibition and even delirium (2). Like other CNS depressants, benzodiazepines can have additive or potentiating effects when combined with alcohol, or opioids, such as methadone (3) and buprenorphine (4), necessitating the confirmation of those classes of drugs as well.

   In this laboratory, targeted screening for the before mentioned benzodiazepines is typically done in central blood specimens (*e.g.* aorta, inferior vena cava) via the multi-drug LC/MS screen (SOP 120). These target drugs have low volumes of distribution (Vd <5 L/kg), and while not thought to exhibit classic postmortem redistribution, can be subject to pre-analytical issues or site dependence, especially in circumstances involving aspiration or leakage from the GI. Regardless, confirmation and quantitation is typically done in peripheral blood specimens (*e.g.* femoral, iliac) to more accurately reflect drug concentration at the time of death (5).

2. **Specimens**
   2.1. This procedure is applicable to urine, blood, serum, *bile, properly prepared tissue specimens (typically 1:4 homogenates), and *gastric contents. A 0.5 mL (g) sample (in duplicate) size is generally employed unless a dilution is

required so that the calibration curve encompasses the expected range of unknown specimens.

2.1.1. *For non-typical matrices, an additional 0.5mL aliquot shall be taken (volume permitting), spiked with appropriate QC, and analyzed to help identify any matrix effects. (See Non-Matched Matrix Protocol section of the QA/QC manual).

3. **Reagents and Materials**
   - 3.1. DI water, HPLC grade
   - 3.2. Methanol, HPLC grade
   - 3.3. Acetone, HPLC grade
   - 3.4. Acetonitrile, HPLC grade
   - 3.5. Deuterated Benzodiazepine Internal Standard Mix
   - 3.6. Benzodiazepine Standard Mix
   - 3.7. Benzodiazepine QC Standard Mix
   - 3.8. Blank blood, urine, liver homogenate(drug-free)

4. **Standards, Controls, and Solutions**

4.1. **Benzodiazepine Internal Standard Stock Solution (10µg/mL)**

4.1.1. Into a 10mL volumetric flask, add the following:

4.1.1.1. 1 ampule (~1mL) of 7-aminoclonazepam-d4 (Cerilliant - 100µg/mL)

4.1.1.2. 1 ampule (~1mL) of Alprazolam-d5 (Cerilliant - 100µg/mL)

4.1.1.3. 1 ampule (~1mL) of Diazepam-d5 (Cerilliant - 100µg/mL)

4.1.1.4. 1 ampule (~1mL) of Nordiazepam-d5 (Cerilliant - 100µg/mL)

4.1.1.5. Fill to the line with methanol, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601). See SOP-010

4.2. **Benzodiazepine Internal Standard Working Solution (1000ng/mL)**

4.2.1. Into a 10mL volumetric flask, add 1mL of Benzodiazepine Internal Standard Stock Solution (10µg/mL) with a micropipette.

4.2.2. Fill to the line with methanol, insert stopper and invert three times to mix. Transfer to properly labeled 16x100mm screw topped test tubes and cap. Store in laboratory refrigerator (R1-2601). See SOP-010

4.3. **Benzodiazepine Calibrators and Positive Controls –** these standards are to be prepared by the QA/QC Chemist or appointee. Inform the QA/QC Chemist if calibration/control standards need to be made.

4.4. **Water with 0.1% formic acid.**

4.4.1. To a 4L bottle of HPLC grade water, add 4 mL of formic acid.

4.4.2. Label bottle as "LC/MS - Water with 0.1% formic acid".

4.5. **Acetonitrile with 0.1% formic acid**

4.5.1. To a 4L bottle of HPLC grade acetonitrile, add 4 mL of formic acid.

4.5.2. Label bottle as "LC/MS – Acetonitrile with 0.1% formic acid".

4.6. **Methanol with 0.1% formic acid**

4.6.1. To a 4L bottle of HPLC grade methanol, add 4 mL of formic acid

4.6.2. Label bottle as "LC/MS - Methanol with 0.1% formic acid".

5. **Equipment and Special Supplies**

5.1. 16 x 125, or 16 x 100 disposable test tubes*
5.2. Centrifuge 2000 x g
5.3. Vortex mixer
5.4. Nitrogen evaporator
5.5. Micro Pipette
5.6. LC autosampler vials, 12 x 32mm
5.7. Polyspring inserts, 5mm O.D.

5.7.1. *These test tube sizes are recommended for best extraction efficiency. Other test tube sizes may be used if needed.

6. **Instrumentation and Parameters**

6.1. Windows PC with Thermo LCQuan and Xcaliber software

6.1.1. Instrument method (TSQ02): "Benzodiazepines_EZ-Method" & "Benzo-II_EZ-Method"

6.1.2. Click here for instrument parameters.

6.2. Thermo Surveyor LC autosampler, or equivalent

6.3. Thermo Surveyor LC pump, or equivalent

6.4. Thermo TSQ triple quadrupole mass spectrometer

7. **Target Ions** (± 1 nominal mass)

Benzo-I:

7.1.	7-aminoclonazepam	(**286** 222 250)
7.2.	7-aminoclonazepam-d4	(**290** 226 254)
7.3.	Nordiazepam	(**271** 140 208)
7.4.	Nordiazepam-d5	(**276** 213 140)
7.5.	Lorazepam	(**321** 275 303)
7.6.	Alprazolam	(**309** 281 205)
7.7.	Alprazolam-d5	(**314** 286 210)
7.8.	Clonazepam	(**316** 270 214)
7.9.	Diazepam	(**285** 193 154)
7.10.	Diazepam-d5	(**290** 198 227)

Benzo-II:

7.11.	Midazolam	(**326** 291 249)
7.12.	Oxazepam	(**287** 241 269)
7.13.	Oxazepam-d5	(**292** 246 274)
7.14.	Nordiazepam-d5	(**276** 213 140)
7.15.	Triazolam	(**343** 308 239)
7.16.	Triazolam-d4	(**347** 312 243)
7.17.	Temazepam	(**301** 255 283)
7.18.	Temazepam-d5	(**306** 260 288)

7.18.1. Note: The precursor ion of each analyte is listed first and bolded, the first product ion- used for quantification-is second, followed by the second product ion-used for confirmation.

8. **Procedure**

8.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 16x125mm test tubes.

8.2. Add the appropriate quantity (according to the Standard and Control Worksheet) of the Deuterated Benzodiazepine Internal Standard Mix to all tubes.

8.3. Add the appropriate quantity (according to the Standard and Control Worksheet) of the Benzodiazepine Calibration Standard and the Benzodiazepine Control Standard to the tubes labeled as standards and controls (QC), respectively, labeling test tubes as you go. Only internal standard should be present in the test tube(s) labeled “Blank”.

8.4. Add 0.5 mL blank blood to each of the standards and controls. Include a urine blank/QC (0.5 mL) and/or a liver homogenate blank/QC (0.5 g) if there are urine/liver specimens in load.

8.5. Add the appropriate amount of predetermined unknown specimen to appropriate test tubes, labeling test tubes as you go. (See Specimens section)

8.6. Vortex all test tubes for 10 seconds.

8.7. Add 3.5mL acetone to each tube and vortex for 20 seconds.

8.8. Centrifuge at 2000 x g for 10 minutes.

8.9. Decant the top acetone layer into clean and labeled 16 x 100 test tubes.

8.10. Place in nitrogen evaporator, and evaporate at 55° C to dryness.

8.11. Remove dried specimens from nitrogen evaporator and reconstitute with 200µL of methanol.

8.12. Vortex for 10 seconds.

8.13. Transfer ~150 µL of each extract to appropriately labeled autosampler vials fitted with 200 µL polyspring insert and place in the autosampler tray of the Thermo TSQ triple-quadrupole LC/MS/MS.

8.14. Build and initiate sequence as directed in SOP-053.

9. **Calculations**

9.1. Quantification

9.1.1. The method for processing the data using the Thermo LCQuan software is "Benzodiazepines_EZ_Method". It is used to calculate the internal standard response ratios, raw amounts, concentration, and ion ratios (SOP-055).

9.1.2. These calculations are computed as follows:

9.1.2.1. Internal Standard Response Ratio:

9.1.2.1.1. Response Ratio = QN_a / QN_{istd}

9.1.2.1.2. QN_a = response of the quantitative ion of the analyte

9.1.2.1.3. QN_{istd} = response of the quantitative ion of the internal standard amount

9.2. Calibration

9.2.1. A linear regression resulting from the 5 standards is used to quantitate the analytes in the case. The area of the analyte divided by the area of the internal standard is used in the resulting formula of the calibration curve.

9.3. Dilution Factor

9.3.1. D = Total volume/Sample volume

9.4. Multiplier for homogenates, dilutions, and non-standard volumes

9.4.1. $M = (V_{curve} / V_{samp}) \times D$
- 9.4.1.1. M = Multiplier
- 9.4.1.2. D = Dilution Factor
- 9.4.1.3. V_{curve} = matrix volume of calibration curve
- 9.4.1.4. V_{samp} = matrix volume of specimen

9.5. Concentration

9.5.1. C = (A / V) * M

- 9.5.1.1. C = Concentration (ng/mL) of the analyte in the unknown case.
- 9.5.1.2. A = Amount of drug in sample
- 9.5.1.3. V = Volume of sample
- 9.5.1.4. M = Multiplier

9.6. Max/Min

9.6.1. Percent Difference = $((R_h / R_l)-1) \times 100$
- 9.6.1.1. R_h = high result
- 9.6.1.2. R_l = low result

9.7. Average

9.7.1. Average = $(R_1 + R_2) / 2$
- 9.7.1.1. R_1 = first result
- 9.7.1.2. R_2 = second result

9.8. Qualifier Ion Ratios

9.8.1. Ratio 1 = $(QL_1/QN) \times 100$
- 9.8.1.1. QL_1 = response of the quantifying product ion
- 9.8.1.2. QN = response of the qualifying product ion

10. Quality Control

10.1. Internal Standards

Analyte	Associated Internal Standard
7-Aminoclonazepam	**7-aminoclonazepam-d4**
Alprazolam	**Alprazolam-d5**
Diazepam	**Diazepam-d5**
Nordiazepam	**Nordiazepam-d5**
Clonazepam	
Lorazepam	

10.1.1.

10.2. Acceptance criteria

10.2.1. Chromatogram

10.2.1.1. Peaks must be Gaussian shaped (symmetrical).

10.2.1.2. Peaks must not exhibit extreme fronting or tailing.

10.2.1.3. Peaks sharing parent/product ions must have baseline resolution.

10.2.1.4. Retention time must not deviate outside ± 3% (minimum window 7.2 seconds) of target, based upon the retention time of the calibrators and controls.

10.2.2. Mass spectroscopy

10.2.2.1. The ion ratio of all samples must not be greater than ± 20% of the target ratio as determined by a mid-level calibrator (CAL 4).

10.2.2.2. Coelution of quantifying and qualifying ions must not be greater than 0.025 minutes.

10.2.3. Calibrators

10.2.3.1. Analytical curves must have a coefficient of determination (R^2) of 0.992 or greater.

10.2.3.2. Each calibrator, when calculated against the calibration curve, must not deviate outside ± 20% of the target value (25% at LOQ).

10.2.3.3. Refer to "Calibration curve point exclusion guidelines" section of the QA/QC Manual

10.2.4. Controls

10.2.4.1. Controls must calculate within ± 20% of the target value.

10.2.5. Blanks

10.2.5.1. Blanks should not contain any target analyte signal with an internal standard response ratio greater than 10% that of the lowest calibrator for the same analyte.

10.2.6. Any deviation from the above will result in repeated analysis.

11. **Validation of Method**

Method Validation: Benzodiazepines Updated 9/29/2015

Parameter	Desired Limit	Result
Bias	Bias at Low and High QC ≤ ±20% *Updated Values	**7-aminoclonazepam** Low: -15.86% High: -14.40%
		Alprazolam Low: -2.67% High: -2.70%
		***Clonazepam - (Nordiazepam-d5)** Low: -1.29% High: 1.27%
		Diazepam Low: -0.84% High: -4.17%
		***Lorazepam - (Nordiazepam-d5)** Low: 2.41% High: 4.64%
		Nordiazepam Low: -5.27% High: -3.40%
Precision	Precision (%CV) at Low and High QC ≤ 20% *Updated Values	**7-aminoclonazepam** Low: 3.64% High: 3.57%

		Alprazolam Low: 3.61% High: 1.57%
		***Clonazepam - (Nordiazepam-d5)** Low: 6.18% High: 3.69%
		Diazepam Low: 4.95% High: 5.34%
		***Lorazepam - (Nordiazepam-d5)** Low: 7.8% High: 5.14%
		Nordiazepam Low: 4.68% High: 4.15%
Calibration model	Linear (weighting = "best fit" based on %RSD of 5 calibration curves) *Updated Values	**7-aminoclonazepam -** Linear 1/x weighting
		Alprazolam - Linear 1/x weighting
		***Clonazepam** - Linear 1/x weighting
		Diazepam - Linear 1/x weighting
		Lorazepam - Linear 1/x weighting
		Nordiazepam - Linear 1/x weighting
Carryover	Carryover after highest calibrator does not exceed 10% of signal of lowest calibrator	No carryover detected in blank following specimen with concentration of 2X high calibrator.
Interference Studies	No interfering signal from matrix, internal standard, common drugs of abuse (including metabolites), OTC drugs, and Prescription medications.	No significant interference from common drugs of abuse (including metabolites), OTC drugs, and prescription medications was observed.
Ionization/Suppression: (Not needed if IS coelutes within 0.05 min.)	< 25% suppression or enhancement and <25% RSD due to matrix (if so, evaluate impact on LOD, LOQ, and Bias)	Not evaluated - IS coelute within 0.05 minutes of associated analytes.
LOD (Calculate: 3.3xSD Y-intercept/Mean of Slope) Exception: Clonazepam - determined experimentally	**7-aminoclonazepam: <20ng/mL**	**7-aminoclonazepam: 4.7 ng/mL**
	Alprazolam: <5ng/mL	**Alprazolam: 0.906 ng/mL**
	Clonazepam: <5ng/mL ***Updated Values**	***Clonazepam: 0.7ng/mL**

	Diazepam: <20ng/mL	**Diazepam: 4.05 ng/mL**
	Lorazepam: <10ng/mL	**Lorazepam: 2.76 ng/mL**
	Nordiazepam: <20ng/mL	**Nordiazepam: 5.53 ng/mL**
LOQ (Set to lowest calibrator with acceptable Bias/Precision).	**7-aminoclonazepam: =20ng/mL**	**7-aminoclonazepam: =20ng/mL**
	Alprazolam: =5ng/mL	**Alprazolam: =5ng/mL**
	Clonazepam: =5ng/mL	**Clonazepam: =5ng/mL**
	Diazepam: =20ng/mL	**Diazepam: =20ng/mL**
	Lorazepam: =10ng/mL	**Lorazepam: =10ng/mL**
	Nordiazepam: =20ng/mL	**Nordiazepam: =20ng/mL**

12. **Reporting**

12.1. The percent difference of duplicate analysis for an analyte must be less than or equal to 25% (see Max/Min in Calculations section (9.6)).

12.2. Reporting of duplicate analysis should be done according to the table below:

Reporting Duplicates

- Dilution factors of 1 and 1

Dil / Scenario	1	1	REPORT
A	In curve	In curve	Average
B	In curve	AQL or BQL	"In" value
C	In curve	ND *	Repeat
D	AQL/BQL	AQL/BQL	Less than/ Greater than
E	BQL	ND	ND

* ND = None Detected, due to IRC, S/N threshold, r.t., or other

12.2.1.1. In Curve = Measured concentration (pre-multiplier) falls within the calibration range

12.2.1.2. AQL = Measured concentration (pre-multiplier) falls Above Quantitation Limit

12.2.1.3. BQL = Measured concentration (pre-multiplier) falls Below Quantitation Limit

12.2.1.4. ND = None Detected

12.3. Averaging reportable values

12.3.1. Results for duplicate analysis (both falling within calibration curve) shall be truncated prior to averaging.

12.3.2. Enter calculated concentration for each specimen into toxlog.

12.4. Significant figures

12.4.1. Concentrations are truncated and reported with two significant figures in mg/L.

13. **Preparation of Load**

13.1. The load paperwork and data is to be arranged in the following order:

13.1.1. Assignment cover sheet

13.1.2. Comments or note to file if applicable

13.1.3. Load summary

13.1.4. Specimen worklist

13.1.5. Chain of custody (Specimen)

13.1.6. Aliquot chain of custody

13.1.7. Standard and control worksheet

13.1.8. Sequence summaries/calibration reports – paper clipped

13.1.9. Calibrator data - paper clipped

13.1.10. Blank matrix data - paper clipped

13.1.11. Control data - paper clipped

13.1.12. Specimen data – stapled

14. **References**

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