HEALTH & ENVIRONMENTAL TESTING LABORATORY
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DETERMINATION OF BENZODIAZEPINES IN BLOOD BY LIQUID-LIQUID EXTRACTION AND LC-MS/MS ANALYSIS

Contents

Maine HETL- Forensic Toxicology

DETERMINATION OF BENZODIAZEPINES IN BLOOD BY LIQUID-LIQUID EXTRACTION AND LC-MS/MS ANALYSIS

Principle and Scope:

This method describes the procedures for the quantitative determination of Benzodiazepines and metabolites in whole blood. Deuterated internal standards for the compounds of interest are added to the whole blood samples, the compounds of interest and corresponding deuterated internal standards are then efficiently partitioned from the blood sample via a liquid/liquid extraction with organic solvent and separated on a Raptor Biphenyl column. The samples are then analyzed using a tandem mass spectrometer utilizing positive-ion electrospray.

Equipment and Supplies:

Volumetric Flasks various sizes

Volumetric cylinders various sizes

Disposable Glass vials 5-15 mL

Teflon lined caps

2mL microcentrifuge tubes

Autosampler vials with inserts

Autosampler caps

Vortex mixer

Disposable transfer pipettes

Pipettes- various

Evaporator

Specimen Requirements:

- Only whole blood samples shall be analyzed using this method.
- Whole blood samples are collected in tubes provided by HETL or by a qualified medical professional and upon receipt, stored under refrigeration at HETL (<10°C).

- If a case sample is found to be above the upper limit of quantitation then a re-extraction with dilution is necessary, sample volume permitting. If sample volume does not allow for a dilution or excessively high concentrations of the compound(s) of interest are still above the adjusted upper limit of quantitation, then the case sample shall be resulted out as "Compound detected >(Upper Limit of Quantitation)ng/mL". Validated dilutions include the following:

Dilution Factor	Volume of Case Sample	Volume of Water
1:2	100µL	100µL
1:4*	50µL	150µL

**Please note: 7-aminoflunitrazepam cannot be diluted 1:4.*

- If a case sample is found to have insufficient quantity to extract and analyze as a neat sample, then a dilution shall be performed to permit extraction and analysis to achieve quantitation. If the resulting raw data concentration is less than the compound of interests associated reporting level prior to the dilution factor calculation than the results shall be reported as negative.
- If a blood sample is received by the laboratory with volume not sufficient to perform standard OUI blood drug panel and only a portion of panel can be screened and confirmed for then the following comment shall be included in the COA: "Unable to perform standard OUI blood drug panel due to low sample volume." In addition, prior to starting testing the analyst shall reach out to the investigating officer notifying them that the sample is QNS to perform the standard OUI blood drug panel and discuss course of testing.
- Blood samples that were collected using non-DHHS certified blood collection tubes shall have the following comment included in the COA: "(Color) topped tube used for analysis."

Reagents and Reference Materials:

- Acetonitrile
- Methanol - High purity LC-MS quality
- Water - High purity LC-MS quality
- Formic Acid
- Ammonium Formate
- Water with 0.1% Formic Acid- High purity LC-MS quality, Fisher brand or equivalent
- **Stock Standard Solutions** – Individual and mixture solutions of target compounds and internal standards are purchased from traceable, approved vendors such as Cerilliant or Lipomed. All standards shall be stored at conditions recommended by the manufacturer.

- **7-amino flunitrazepam**
- **7-aminoclonazepam**
- **a-Hydroxyalprazolam**
- **Alprazolam**
- **Clonazepam**
- **Diazepam**
- **Etizolam**
- **Flunitrazepam**
- **Lorazepam**
- **Nordiazepam**
- **Oxazepam**
- **Temazepam**
- **Zolpidem**

- **7-amino flunitrazepam-d7**
- **Alprazolam-d5**
- **Clonazepam-d4**
- **Diazepam-d5**
- **Etizolam-d3**
- **Flunitrazepam-d7**
- **Lorazepam-d4**
- **Nordiazepam-d5**
- **Oxazepam-d5**
- **Temazepam-d5**
- **Zolpidem-d6**

- **Blank Blood matrix**- approved vendor supplied and checked for quality assurance prior to purchase. Store at <10°C.

Safety Precautions:

The solvents used are considered toxic. Repeated or prolonged exposure can produce targeted organ damage. Proper PPE shall be used when handling solvents.

Blood from unknown case samples shall be handled following Universal Precaution guidelines. Face shield in addition to PPE shall be utilized while pipetting blood.

Reagent Preparation:

All reagent preparations are to be recorded in the LCMS Reagent Preparation Log.

- **Mobile Phase A**: Water with 0.1% Formic Acid 5mM Ammonium Formate. This reagent is good for 1 month from preparation date. The Water with 0.1% Formic Acid can be purchased as a prepared reagent or prepared in the lab (1 mL formic acid QS to 1 L with LC-MS grade water. To 1L of Water with 0.1% Formic Acid add 0.315g of Ammonium Formate and mix by shaking until dissolved. This reagent is good for 1 month from the date of opening or until the earliest expiration date of any components used in the making.

- **Mobile Phase B**: Methanol. This reagent is good for 12 months from the date of opening or until the earliest expiration date of any components used in the making.

- **Acetonitrile**- This reagent is good for one year from date of open.

- **70:30 Water with 0.1% Formic Acid and 5mM Ammonium Formate:Methanol -**To a graduated cylinder or a 100mL volumetric flask add 30mL of LCMS grade Methanol and QS to 100mL with LCMS grade Water with 0.1% Formic Acid and 5mM Ammonium Formate. Different volumes may be prepared as long as the proportions are kept the same. This reagent is good for one month at room temperature or until the earliest expiration date of any components used in the making.

Calibration and Quality Control Stock Preparation:
All Benzodiazepines working stock preparations shall be recorded in the LCMS Reagent Preparation Log.

- **Benzodiazepines Parent 4µg/mL**: Add a portion of methanol to a 10 mL volumetric flask. Using only Cerilliant stock or equivalent add the following standards to or equivalents the volumetric flask to obtain a final concentration of 4µg/mL. QS to 10mL with LCMS grade Methanol. This parent stock is good for 12 months stored in the freezer or until the earliest expiration date of any components used in the making.

Analyte	Concentration (ug/mL)	Volume used (uL)
Benzodiazepine Multi Mix 8	250 ug/mL	160 uL
7-amino flunitrazepam	1 mg/mL	40 uL
7-aminoclonazepam	1 mg/mL	40 uL
Etizolam	1 mg/mL	40 uL
a-Hydroxyalprazolam	1 mg/mL	40 uL
Nordiazepam	1 mg/mL	40 uL
Zolpidem	1 mg/mL	40 uL

- **Benzodiazepines Stock A 400ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Benzodiazepines Parent Stock (4µg/mL) to the volumetric flask. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months stored in the freezer or until the earliest expiration date of any components used in the making.

- **Benzodiazepines Stock B 40ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Benzodiazepines Working Stock A (400ng/mL) to the volumetric flask. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months stored in the freezer or until the earliest expiration date of any components used in the making.

- **Benzodiazepines Stock C 4ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Benzodiazepines Working Stock B (40ng/mL) to the volumetric flask. QS to 10mL with LCMS

grade Methanol. This working stock is good for 12 months stored in the freezer or until the earliest expiration date of any components used in the making.

- **Benzodiazepines Internal Standard 800ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add the following or equivalents to the volumetric flask to obtain a final concentration of 800ng/mL. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 month stored in the freezer or until the earliest expiration date of any components used in the making.

Analyte	Concentration	Volume used
7-amino flunitrazepam-d7	100 µg/mL	80 µL
Alprazolam-d5	100 µg/mL	80 µL
Clonazepam-d4	100 µg/mL	80 µL
Diazepam-d5	100 µg/mL	80 µL
Etizolam-d3	100 µg/mL	80 µL
Flunitrazepam-d7	100 µg/mL	80 µL
Lorazepam-d4	100 µg/mL	80 µL
Nordiazepam-d5	100 µg/mL	80 µL
Oxazepam-d5	100 µg/mL	80 µL
Temazepam-d5	100 µg/mL	80 µL
Zolpidem-d6	100 µg/mL	80 µL

Quality Control Stock Preparation:
Blank matrix samples will be fortified respectfully at low, medium, and high levels using working stock created from stock standards from a separate vendor than the calibration source or if from the same vendor as the calibration source the standard is from a different lot number and a different dilution factor is utilized. A negative control will be created using an unfortified blank matrix. Internal standard shall be added and quality control samples will then be extracted and analyzed with each batch immediately following calibration. A full set of controls shall be run for each extraction batch of twenty samples. All quality control working stock preparations are recorded in the LCMS Reagent Preparation Log.

- **Benzodiazepines QC Parent 4µg/mL**: Add a portion of methanol to a 10 mL volumetric flask. Using only Lipomed stock or equivalent add the following or equivalents to the volumetric flask to obtain a final concentration of 4µg/mL. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months stored in the freezer or until the earliest expiration date of any components used in the making.

Analyte	Concentration	Volume used
7-amino flunitrazepam	1 mg/mL	40 uL
7-aminoclonazepam	1 mg/mL	40 uL
a-Hydroxyalprazolam	1 mg/mL	40 uL
Alprazolam	1 mg/mL	40 uL
Clonazepam	1 mg/mL	40 uL
Diazepam	1 mg/mL	40 uL
Etizolam	1 mg/mL	40 uL
Flunitrazepam	1 mg/mL	40 uL
Lorazepam	1 mg/mL	40 uL
Nordiazepam	1 mg/mL	40 uL
Oxazepam	1 mg/mL	40 uL
Temazepam	1 mg/mL	40 uL
Zolpidem	1 mg/mL	40 uL

- **Benzodiazepines QC Stock A 400ng/mL**: Add a portion of methanol to a 10 mL volumetric flask. Add 1mL of Quality Control Parent Stock (4µg/mL) to the volumetric flask. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months stored in the freezer or until the earliest expiration date of any components used in the making.

- **Benzodiazepines QC Stock B 40ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of the Quality Control Working Stock A (400ng/mL) to the volumetric flask. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months stored in the freezer or until the earliest expiration date of any components used in the making.

Table 1: Calibration Levels					
Calibration Level	Target Concentrations (ng/mL)	Blood	Vol Stock A (400 ng/mL)	Vol Stock B (40 ng/mL)	Vol Stock C (4 ng/mL)
		(uL)	(uL)	(uL)	(uL)
1	2	200			100
2	4	200		20	
3	20	200		100	
4	60	200	30		
5	100	200	50		
6	120	200	60		
7	160	200	80		
8	200	200	100		

Table 2: Quality Control Levels				
Quality Control Level	Target Concentration	Vol QC Stock A (400ng/mL)	Vol QC Stock B (40ng/mL)	Blank Whole Blood
	ng/mL	(µL)	(µL)	(µL)
Low (QCL)	6		30	200
Medium (QCM)	60	30		200
High (QCH)	160	80		200
Negative (Neg)	--			200

Extraction Procedure:

1. Case samples shall be removed from refrigeration storage, allowed to warm to room temperature and placed on a rocker for a minimum of ten minutes.
2. Calibrators are prepared as per Table 1: Calibration Levels
3. Quality Controls are prepared as per Table 2: Quality Control Levels
4. Transfer 200µL of each case sample into a micro centrifuge tube.
5. Pulse vortex
6. Add 25µL Internal Standard to each micro centrifuge tube (Note: the same lot of internal standard shall be used for all samples in an analytical batch.)
7. Pulse vortex
8. In a fume hood add 1mL of Acetonitrile to each micro centrifuge tube and cap.
9. Pulse Vortex 1 minute
10. Centrifuge at 8000 rpm for ten minutes.
11. In a fume hood remove the supernatant top layer and transfer into new labeled glass tubes
12. Dry down at room temperature with Nitrogen
13. Reconstitute in 100 µL 70:30 mobile phase Water w/0.1% Formic Acid 5mM Ammonium Formate: Methanol
14. Pulse vortex
15. Transfer to an autosampler vial with insert for analysis

Instrument Maintenance Procedure:
Refer to the HETL LC-MS/MS Instrument Maintenance Procedure a copy of which is located in the immediate area of the instrument.

Instrumentation and Data Acquisition Parameters:
The instrument method parameters will be printed out and placed in a method binder close to the instrument.

- Shimadzu 8030 Tandem Mass Spectrometer and LC system, Agilent 6470A Tandem Mass Spectrometer and LC system (or equivalent)
- LC Guard Cartridge – Restek Raptor Biphenyl
- LC Column- Restek Raptor Biphenyl 2.7µm 100x2.1mm
- Flow rate 0.6 mL/Min
- Mobile phase A: Water with 0.1% Formic Acid 5mM Ammonium Formate
- Mobile Phase B: Methanol
- Injection volume 20 uL
- LC oven 40ºC

Table 3: LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid 5mM Ammonium Formate	% B: Methanol
0.01	70	30
1.00	70	30
1.00	To MS	To MS
4.00	35	65
5.00	35	65
9.00	5	95
9.01	To Waste	To Waste
9.50	5	95
9.51	70	30
11.00	Stop	Stop

Table 4: Instrument Parameters for Target Analytes

Target Analyte	Precursor Ion (*m/z*)	Product Ion	
7-Aminoclonazepam	286	Quant	222
		Qualifier	121
Zolpidem	308	Quant	235
		Qualifier	263
7-Aminoflunitrazepam	284	Quant	135
		Qualifier	227
Lorazepam	321	Quant	275

		Qualifier	321
α-hydroxyalprazolam	325	Quant	297
		Qualifier	216
Clonazepam	316	Quant	270
		Qualifier	214
Oxazepam	287	Quant	241
		Qualifier	104
Alprazolam	309	Quant	281
		Qualifier	205
Flunitrazepam	314	Quant	268
		Qualifier	239
Nordiazepam	271	Quant	140
		Qualifier	165
Temazepam	301	Quant	255
		Qualifier	177
Diazepam	285	Quant	154
		Qualifier	193
Etizolam	343	Quant	314
		Qualifier	259

Table 5: Instrument Parameters for Internal Standards

Internal Standard	Precursor Ion (*m/z*)	Product Ion	
Zolpidem-d6	314	Quant	235
		Qualifier	314
7-Aminoflunitrazepam-d7	291	Quant	230
		Qualifier	291
Lorazepam-d4	325	Quant	279
		Qualifier	325
Clonazepam-d4	320	Quant	274
		Qualifier	320
Oxazepam-d5	292	Quant	246
		Qualifier	292
Alprazolam-d5	314	Quant	286
		Qualifier	314
Flunitrazepam-d7	322	Quant	276
		Qualifier	322
Nordiazepam-d5	276	Quant	140

		Qualifier	276
Temazepam-d5	306	Quant	260
		Qualifier	306
Diazepam-d5	290	Quant	154
		Qualifier	290
Etizolam-d3	346	Quant	317
		Qualifier	346

Identification & Quantitation

- For identification and quantitation criteria and guidelines please Refer to: LCMSMS Analysis, Acceptance, & Reporting Criteria Standard Operating Procedure.
- **Limit of Detection, Limit of Quantification & Reporting Levels:** the limits of detection and quantification have been determined as illustrated in Table 6: Limits of Detection and Quantification and are linear within the range indicated. If the lowest or highest calibrator has been dropped then the quantification window shall be adjusted to reflect the change as needed.

Table 6: Limit of Detection, Limit of Quantification & Reporting Levels

Analyte	RL/LLOQ/LLOQ	ULOQ
7-amino flunitrazepam	4ng/mL	200ng/mL
7-aminoclonazepam	4ng/mL	200ng/mL
a-Hydroxyalprazolam	4ng/mL	200ng/mL
Alprazolam	4ng/mL	200ng/mL
Clonazepam	4ng/mL	200ng/mL
Diazepam	4ng/mL	200ng/mL
Etizolam	4ng/mL	200ng/mL
Flunitrazepam	4ng/mL	200ng/mL
Lorazepam	4ng/mL	200ng/mL
Nordiazepam	4ng/mL	200ng/mL
Oxazepam	4ng/mL	200ng/mL
Temazepam	4ng/mL	200ng/mL
Zolpidem	4ng/mL	200ng/mL

Quality Control Requirements:

- For quality control requirements, acceptance criteria, and guidelines please Refer to: LCMSMS Analysis, Acceptance, & Reporting Criteria Standard Operating Procedure.

Uncertainty of Measurement:

- For estimation of uncertainty of measurement please Refer to: LCMSMS Analysis, Acceptance, & Reporting Criteria Standard Operating Procedure.

Documentation:

Each batch (example: BENZ082819EAF) folder shall contain:

- Raw/Summary data from the instrument for all calibrators and quality controls
- Calibration Report from the instrument
- LC-MSMS Batch Review Form
- Internal Standard Area Report
- Qualifier Ion Ratio Report (when applicable)
- Instrument Sequence Table
- Benzodiazepines in Blood Bench Sheet Form

Each Sample folder shall contain:

- Laboratory Blood Analysis Request Form
- Receipt/Contract for Examination Form/Chain of Custody
- Blood Kit Inventory Worksheet
- Blood Drug Screen Worksheet
- Raw/Summary data from the instrument including reinjections if applicable
- LC-MSMS Sample Review Form
- Blood Drug Results Worksheet
- Case Review Form

Waste Management:

Residual organic solvent, standards and instrument waste must be disposed in accordance with Federal and Maine law.

Any lab ware exposed to blood shall be disposed of in hazardous waste containers for proper disposal.

References:

Fraser, E. and Ingalls, N., in-house development and validation for detection and quantitation of cannabinoids in blood.

Fraser, E. and Ingalls, N., in-house development and validation for detection and quantitation of narcotics in blood.

Analysis of Benzodiazepines in Blood by LC/MS/MS Application Note. Rivera, Walker, Stockham, Sims, and Hughes. Agilent Technologies 2006.

Revision:

REVISED BY	REV#	DATE	Revisions
LN	1	10-29-2019	Table 6: LLOQ increased from 2ng/mL to 4ng/mL for all compounds.
EAF	2	12/17/19	Updated dilution section, removed zopiclone from table 4, 5, & 6 see initial validation, added qualifier ion ratio report to batch documentation section.
EAF	3	10/9/2020	Annual Review: Table 6 removed LLOD column, combined RL/LLOD/LLOQ. Instrument and data parameters: Removed Nebulizing gas flow, DL temperature, Heat block, Drying gas flow, CID gas, all CE, and all Dwell times. All of these parameters are instrument specific and cannot be used for method replications. All of these parameters are still located in the printed instrument data acquisition methods located by the instrument. Documentation: qualifier ion ratio report added “when applicable” and removed STARLIMs batch sequences.
EAF	4	03/15/2021	Added Agilent 6470A information/reworded to include Agilent 6470A. Reworded reporting limit wording regarding removing calibration points. Added QNS sample and non-DHHS information to Specimen Requirement section.