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	HEALTH & ENVIRONMENTAL TESTING LABORATORY **Forensic Toxicology** **221 State Street** **Augusta, ME 04333**

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

Contents

Maine HETL- Forensic Toxicology

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

Principle and Scope:

This method describes the procedures for the quantitative determination of Stimulants 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
1:10	20µL	180µL
1:20*	10µL	190µL

**Please note: Methamphetamine cannot be diluted 1:20.*

- 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.

  - **Amphetamine**
  - **Benzoylecgonine**
  - **Cocaethylene**
  - **Cocaine**
  - **Ketamine**
  - **MDA**
  - **MDMA**
  - **Methamphetamine**
  - **Methylphenidate**
  - **Norketamine**
  - **PCP**
  - **Amphetamine-d8**
  - **Benzoylecgonine-d8**
  - **Cocaine-d3**
  - **Ketamine-d4**
  - **MDA-d5**
  - **MDMA-d5**
  - **Methamphetamine-d5**
  - **Methylphenidate-d9**
  - **Norketamine-d4**
  - **PCP-d5**

- **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 or the manufacturer's expiration date, if earlier.

- **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 Stimulant working stock preparations shall be recorded in the LCMS Reagent Preparation Log.

- **Stimulants Parent 20µg/mL**: Add a portion of methanol to a 5.0 mL volumetric flask. Using only Cerilliant stock or equivalent add the following standards or equivalents to the volumetric flask to obtain a final concentration of 20µg/mL. QS to 5 mL 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)
Amphetamine	1000 ug/mL	100 uL
Benzoylecgonine	1000 ug/mL	100 uL
Cocaethylene	1000 ug/mL	100 uL
Cocaine	1000 ug/mL	100 uL
Ketamine	1000 ug/mL	100 uL
MDA	1000 ug/mL	100 uL
MDMA	1000 ug/mL	100 uL
Methamphetamine	1000 ug/mL	100 uL
Methylphenidate	1000 ug/mL	100 uL
Norketamine	1000 ug/mL	100 uL
PCP	1000 ug/mL	100 uL

- **Stimulants Stock A 2000ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Stimulants Parent Stock (20µg/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.

- **Stimulants Stock B 200ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Stimulants Working Stock A (2000ng/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.

- **Stimulants Stock C 20ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Stimulants Working Stock B (20ng/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.

- **Stimulants Internal Standard 2000ng/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 2000ng/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
Amphetamine-d8	100 µg/mL	200 µL
Benzoylecgonine-d8	100 µg/mL	200 µL
Cocaine-d3	100 µg/mL	200 µL
Ketamine-d4	100 µg/mL	200 µL
MDA-d5	100 µg/mL	200 µL
MDMA-d5	100 µg/mL	200 µL
Methamphetamine-d5	100 µg/mL	200 µL
Methylphenidate-d9	100 µg/mL	200 µL
Norketamine-d4	100 µg/mL	200 µL
PCP-d5	100 µg/mL	200 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. 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.

- **Stimulant QC Parent 20µg/mL**: Add a portion of methanol to a 5.0 mL volumetric flask. Using only Lipomed stock or equivalent add the following or equivalents to the volumetric flask to obtain a final concentration of 20µg/mL. QS to 5 mL 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
Amphetamine	1000 µg/mL	100 uL
Benzoylecgonine	1000 µg/mL	100 uL
Cocaethylene	1000 µg/mL	100 uL
Cocaine	1000 µg/mL	100 uL
Ketamine	1000 µg/mL	100 uL
MDA	1000 µg/mL	100 uL
MDMA	1000 µg/mL	100 uL
Methamphetamine	1000 µg/mL	100 uL
Methylphenidate	1000 µg/mL	100 uL
Norketamine	1000 µg/mL	100 uL
PCP	1000 µg/mL	100 uL

- **Stimulant QC Stock A 2000ng/mL**: Add a portion of methanol to a 10 mL volumetric flask. Add 1mL of Quality Control Parent Stock (20µg/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.

- **Stimulant QC Stock B 200ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of the Quality Control Working Stock A (2000ng/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 (2000 ng/mL)	Vol Stock B (200 ng/mL)	Vol Stock C (20 ng/mL)
		(uL)	(uL)	(uL)	(uL)
1	5	200			50
2	10	200			100
3	20	200		20	
4	50	200		50	
5	100	200		100	
6	200	200	20		
7	400	200	40		
8	500	200	50		

Table 2: Quality Control Levels				
Quality Control Level	Target Concentration Group 1	Vol QC Stock A (2000ng/mL)	Vol QC Stock B (200ng/mL)	Blank Whole Blood
	ng/mL	(µL)	(µL)	(µL)
Low (QCL)	25		25	200
Medium (QCM)	100		100	200
High (QCH)	300	30		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 200 µL 80:20 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 10 uL
- LC oven 40ºC

Table 3: LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid 5mM Ammonium Formate	% B: Methanol
0.01	90	10
0.25	To MS	To MS
9.00	5	95
9.01	To Waste	To Waste
12.00	5	95
12.01	90	10
13.00	Stop	Stop

Table 4: Instrument Parameters for Target Analytes

Target Analyte	Precursor Ion (*m/z*)	Product Ion	
Amphetamine	136	Quant	91
		Qualifier	119
Methamphetamine	150	Quant	91
		Qualifier	119
MDA	180	Quant	105
		Qualifier	163
MDMA	194	Quant	163
		Qualifier	105

Norketamine	224	Quant	207
		Qualifier	125
Ketamine	238	Quant	125
		Qualifier	207
Cocaethylene	318	Quant	82
		Qualifier	196
Methylphenidate	234	Quant	84
		Qualifier	56
Cocaine	304	Quant	182
		Qualifier	82
Benzoylecgonine	290	Quant	168
		Qualifier	105
PCP	244	Quant	159
		Qualifier	91

Table 5: Instrument Parameters for Internal Standards

Internal Standard	Precursor Ion (*m/z*)	Product Ion	
Amphetamine-d8	144	Quant	97
		Qualifier	127
Methamphetamine-d5	155	Quant	92
		Qualifier	121
MDA-d5	185	Quant	168
		Qualifier	110
MDMA-d5	199	Quant	165
		Qualifier	135
Norketamine-d4	228	Quant	129
		Qualifier	211
Ketamine-d4	242	Quant	129
		Qualifier	224
Methylphenidate-d9	243	Quant	93
		Qualifier	243
Cocaine-d3	307	Quant	185
		Qualifier	85
Benzoylecgonine-d8	298	Quant	171
		Qualifier	298
PCP-d5	249	Quant	96
		Qualifier	164

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/LLOD/LLOQ	ULOQ
Amphetamine	10ng/mL	500ng/mL
Benzoylecgonine	10ng/mL	500ng/mL
Cocaethylene	10ng/mL	500ng/mL
Cocaine	10ng/mL	500ng/mL
Ketamine	10ng/mL	500ng/mL
MDA	10ng/mL	500ng/mL
MDMA	10ng/mL	500ng/mL
Methamphetamine	10ng/mL	500ng/mL
Methylphenidate	10ng/mL	500ng/mL
Norketamine	10ng/mL	500ng/mL
PCP	10ng/mL	500ng/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: STIM082819EAF) folder shall contain:

- Raw/Summary data from the instrument for all calibrators and quality controls
- Calibration Report from the instrument
- LC-MSMS Batch Form
- Internal Standard Area Report
- Qualifier Ion Ratio Report *(when applicable)*
- Instrument Sequence Table
- Stimulants 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.

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

Simultaneous screening for 238 drugs in blood by liquid chromatography-ion spray tandem mass spectrometry with multiple-reaction monitoring. Gergov, Ojanpera, & Vuori. Journal of Chromatography B, 795 41-53 2003.

Revision:

REVISED BY	REV#	DATE	Revisions
EAF	1	12/17/19	Update dilution section, corrected table 6 LLODs from 10ng/mL to 5ng/mL, added qualifier ion ration report to batch documentation section.
EAF	2	3/27/20	Updated dilution section to include 1:10 dilution and 1:20 dilution.
EAF	3	10/09/2020	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	03/03/2021	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.