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

Contents

Maine HETL- Forensic Toxicology

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

Principle and Scope:

This method describes the procedures for the quantitative determination of Buprenorphine and Norbuprenorphine 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 an HPLC 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

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 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	500µL	500µL
1:4	250µL	750µL

- If a case sample is found to have insufficient quantity to extract and analyze as a neat sample, a dilution may 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. Alternatively, if a case sample if found to have insufficient quantity to extract and analyze as a neat sample then extraction and confirmation analysis by the Qualitative B panel shall be performed and the sample shall be reported out as a qualitative result.
- 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:

- Hexane
- Ethyl acetate
- Methanol - High purity LC-MS quality
- Water - High purity LC-MS quality
- Formic Acid
- Water with 0.1% Formic Acid- High purity LC-MS quality, Fisher brand or equivalent
- Ammonium Formate

- **Stock Standard Solutions** – Individual 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.
    - **Buprenorphine 100 µg/mL**
    - **Buprenorphine -d4 100 µg/mL**
    - **Norbuprenorphine 100 µg/mL**
    - **Norbuprenorphine-d3 100 µg/mL**

- **Blank Blood matrix**- approved vendor supplied and checked for quality control 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.

- **80:20 Hexane: Ethyl Acetate**- To a graduated cylinder or 100 mL volumetric flask, add 80 mL Hexane and QS to 100 mL with ethyl acetate. Different volumes may be prepared as long as proportions are kept the same. This reagent is good for one month or until the earliest expiration date of any components used in the making.

- **80:20 Water with 0.1% Formic Acid and 5mM Ammonium Formate: Methanol -**To a graduated cylinder or a 100mL volumetric flask add 20mL 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 Buprenorphine working stock preparations shall be recorded in the LCMS Reagent Preparation Log.

- **Buprenorphine Stock A 1000ng/mL:** Add a portion of methanol to a 10 mL volumetric flask. Using only Cerilliant stock or equivalent add 100µL of Buprenorphine 100 µg/mL Stock, and 100µL of Norbuprenorphine 100 µg/mL Stock or equivalents to the volumetric flask to obtain a final concentration of 1000ng/mL. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months or until the earliest expiration date of any components used in the making.

- **Buprenorphine Stock B 100ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Buprenorphine Stock A 1000 ng/mL to the volumetric flask. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months or until the earliest expiration date of any components used in the making.

- **Buprenorphine Stock C 10ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Buprenorphine Stock B 100 ng/mL to the volumetric flask. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months or until the earliest expiration date of any components used in the making.

- **Buprenorphine Internal Standard 2000 ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 200 uL of each Norbuprenorphine-d3 (100 ug/mL) and Buprenorphine-d4 (100 ug/mL) 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 months or until the earliest expiration date of any components used in the making.

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 utilizing a different product/lot number/dilution factor. 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.

- **Buprenorphine QC Stock A 2000 ng/mL**: Add a portion of methanol to a 10 mL volumetric flask. Using Lipomed stock or equivalent add 20µL of Buprenorphine 1mg/mL Stock and 20µL of Norbuprenorphine 1mg/mL stock 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 months or until the earliest expiration date of any components used in the making.

- **Buprenorphine QC Stock B 200ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of the Buprenorphine QC Stock A 2000 ng/mL to the volumetric flask. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months or until the earliest expiration date of any components used in the making.

- **Buprenorphine QC Stock C 20ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of the Buprenorphine QC Stock B 200 ng/mL to the volumetric flask. QS to 10mL with LCMS grade Methanol. This working stock is good for 12 months 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 (1000 ng/mL)	Vol Stock B (100 ng/mL)	Vol Stock C (10 ng/mL)
		(uL)	(uL)	(uL)	(uL)
1	0.25	1000			25
2	0.5	1000			50
3	1	1000			100
4	5	1000		50	
5	20	1000	20		
6	50	1000	50		
7	80	1000	80		
8	100	1000	100		

Table 2: Quality Control Levels					
Quality Control Level	Target Concentration	Vol 2000 ng/mL QC Stock A	Vol 200 ng/mL QC Stock B	Vol 20 ng/mL QC Stock C	Blank Whole Blood
	ng/mL	(µL)	(µL)	(µL)	(µL)
Low (QCL)	2			100	1000
Medium (QCM)	20		100		1000
High (QCH)	80	40			1000
Negative (Neg)	--				1000

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 in glass tubes.
3. Quality Controls are prepared as per Table 2: Quality Control Levels in glass tubes.

4. Transfer 1mL of each case sample into a glass tube.
5. Pulse vortex
6. Add 25µL Internal Standard to each 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 3 mL 80:20 Hexane: Ethyl acetate to each tube and cap.
9. Pulse Vortex 1 minute
10. Centrifuge at high speed 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 temp w/ Nitrogen
13. Reconstitute in 150 µL 80:20 mobile phase Water w/0.1% Formic Acid and 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 – Raptor Biphenyl 2.7um 5x3.0mm
- LC Column- Raptor Biphenyl 2.7um 100x2.1mm
- Flow rate 0.5 mL/Min
- Mobile phase A: Water with 0.1% Formic Acid with 5mM Ammonium Formate
- Mobile Phase B: Methanol
- Injection volume 50 uL
- LC oven 40ºC

Table 3: LC Pump Gradient

Time	% A: Water with 0.1% Formic Acid and 5mM Ammonium Formate	% B: Methanol
0	60	40
1.0	To MS	To MS
1.9	60	40
2.0	45	55
5.0	45	55
9.0	5	95
9.5	5	95
9.75	60	40
10.0	To Waste	To Waste
11.0	Stop	Stop

Table 4: Instrument Parameters for Target Analytes

Target Analyte	Precursor Ion (*m/z*)	Product Ion	
Buprenorphine	468	Quant	101
		Qualifier	396
Norbuprenorphine	414	Quant	187
		Qualifier	101

Table 5: Instrument Parameters for Internal Standards

Internal Standard	Precursor Ion (*m/z*)	Product Ion	
Buprenorphine-d4	472	Quant	415
		Qualifier	240
Norbuprenorphine-d3	417	Quant	343
		Qualifier	83

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 Quantitation:** the limits of detection/limit of quantitation has been determined as illustrated in Table 6: Limits of Detection/Limit of Quantitation and are linear within

the range indicated. If the lowest or highest calibrator of this indicated range has been dropped then the quantification window shall be adjusted to reflect the change as needed.

Table 6:	Limits of Detection/Limit of Quantitation	
Compound	**Lower LOD/LOQ**	**Upper LOD/LOQ**
Buprenorphine	0.5ng/mL	100ng/mL
Norbuprenorphine	0.5ng/mL	100ng/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: BUP052920EAF) folder shall contain:

- Raw/Summary data from the instrument for all calibrators and quality controls
- Calibration Report from the instrument
- LC-MSMS Calibration & QC Review Form
- Internal Standard Area Report
- Qualifier Ion Ratio Report
- Instrument Sequence Table
- STARLIMs Batch Sequence
- Buprenorphine 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 narcotics in blood.

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