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

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Maine HETL- Forensic Toxicology

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

Principle and Scope:

This method describes the procedures for the quantitative determination of Narcotics 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: Sufentanil shall not be diluted if >ULOQ.*

- 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.

  - **Norfentanyl**
  - **Sufentanil**
  - **Noroxycodone**
  - **6-Acetylcodeine**
  - **6-acetylmorphine**
  - **Heroin**
  - **Dihydrocodeine**
  - **Norhydrocodone**
  - **O-Desmethyltramadol**
  - **Trazodone**
  - **Meprobamate**
  - **Fentanyl**
  - **Codeine**
  - **Hydrocodone**
  - **Hydromorphone**
  - **Morphine**
  - **Oxycodone**
  - **Oxymorphone**
  - **Methadone**
  - **Tramadol**
  - **EDDP**

  - **Norfentanyl-D5**
  - **Sufentanil-d5**
  - **Fentanyl-D5**
  - **Codeine-d3**
  - **Hydrocodone-d3**
  - **Hydromorphone-D3**
  - **Morphine-d3**
  - **Oxycodone-d3**
  - **Oxymorphone-d3**
  - **Methadone-d3**
  - **Tramadol-13c, D3**
  - **Noroxycodone-D3**
  - **6-acetylmorphine-D3**
  - **Heroin-d9**
  - **Dihydrocodeine-d6**
  - **Norhydrocodone-d3**
  - **EDDP-D3**
  - **O-Desmethyl-cis-tramadol-D6**
  - **Trazodone-d6**
  - **Meprobamate-d3**

- **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.

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

- **Narcotics Parent 1/10µg/mL**: Add a portion of methanol to a 10 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 1/10µg/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)
Norfentanyl (1:10)	1000 (1:10 100ug/mL)	100 uL
Sufentanil	100	100 uL
Noroxycodone	1000	100 uL
6-Acetylcodeine	1000	100 uL
6-acetylmorphine	1000	100 uL
Heroin	1000	100 uL

Dihydrocodeine	1000	100 uL
Norhydrocodone	1000	100 uL
O-Desmethyltramadol	1000	100 uL
Trazodone	1000	100 uL
Meprobamate	1000	100 uL

- **Narcotics Stock A 100/1000 ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add the following standards or equivalents and parent stock to the volumetric flask to obtain a final concentration of 100/1000ng/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 (ug/mL)	Volume used (uL)
NAR Parent Stock	1-10	1000 uL
Pain Management Mix: *Fentanyl* *Codeine* *Hydrocodone* *Hydromorphone* *Morphine* *Oxycodone* *Oxymorphone* *Methadone* *Tramadol*	10-100	100 uL
EDDP	100	100 uL

- **Narcotics Stock B 10/100 ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Narcotics Working Stock 100/1000ng/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.

- **Narcotics Stock C 1/10 ng/mL:** Add a portion of methanol to a 10 mL volumetric flask then add 1mL of Narcotics Working Stock 10/100ng/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.

- **Narcotics Internal Standard 200/2000 ng/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 200/2000ng/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
Norfentanyl-D5	100 µg/mL	20 µL
Sufentanil-d5	100 µg/mL	20 µL
Fentanyl-D5	100 µg/mL	20 µL
Codeine-d3	100 µg/mL	200 µL
Hydrocodone-d3	100 µg/mL	200 µL
Hydromorphone-D3	100 µg/mL	200 µL
Morphine-d3	100 µg/mL	200 µL
Oxycodone-d3	100 µg/mL	200 µL
Oxymorphone-d3	100 µg/mL	200 µL
Methadone-d3	100 µg/mL	200 µL
Tramadol-13c, D3	100 µg/mL	200 µL
Noroxycodone-D3	100 µg/mL	200 µL
6-acetylmorphine-D3	100 µg/mL	200 µL
Heroin-d9	100 µg/mL	200 µL
Dihydrocodeine-d6	100 µg/mL	200 µL
Norhydrocodone-d3	100 µg/mL	200 µL
EDDP-D3	100 µg/mL	200 µL
O-Desmethyl-cis-tramadol-D6	100 µg/mL	200 µL
Trazodone-d6	100 µg/mL	200 µL
Meprobamate-d3	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 or from a different lot number. 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.

- **Narcotics QC Parent 1000/10000ng/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 1000/10000ng/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
Fentanyl	1 mg/mL	10 uL
Norfentanyl	1 mg/mL	10 uL
Sufentanil	1 mg/mL	10 uL
6-Acetylcodeine	1 mg/mL	100 uL
6-acetylmorphine	1 mg/mL	100 uL
Codeine	1 mg/mL	100 uL
Dihydrocodeine	1 mg/mL	100 uL
Heroin	1 mg/mL	100 uL
Hydrocodone	1 mg/mL	100 uL
Hydromorphone	1 mg/mL	100 uL
Methadone	1 mg/mL	100 uL
Morphine	1 mg/mL	100 uL
Norhydrocodone	1 mg/mL	100 uL
Noroxycodone	1 mg/mL	100 uL
Oxycodone	1 mg/mL	100 uL
Oxymorphone	1 mg/mL	100 uL
Tramadol	1 mg/mL	100 uL
EDDP	1 mg/mL	100 uL
Meprobamate	1 mg/mL	100 uL
O-Desmethyltramadol	1 mg/mL	100 uL
Trazodone	1 mg/mL	100 uL

- **Narcotics QC Stock A 100/1000 ng/mL**: Add a portion of methanol to a 10 mL volumetric flask. Add 1ml of Narcotics QC Parent Stock 1000/10000ng/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.

- **Narcotics QC Stock B 10/100ng/mL**: Add a portion of methanol to a 10 mL volumetric flask then add 1mL of the Quality Control Working Stock 100/1000ng/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 (100/1000 ng/mL)	Vol Stock B (10/100 ng/mL)	Vol Stock C (1/10 ng/mL)
	Group 1	Group 2	(uL)	(uL)	(uL)	(uL)
1	0.1	1	200			20
2	0.2	2	200			40
3	0.5	5	200			100
4	2.5	25	200		50	
5	5	50	200		100	
6	25	250	200	50		
7	40	400	200	80		
8	50	500	200	100		

Table 2:	Quality Control Levels				
Quality Control Level	Target Concentration Group 1	Target Concentration Group 2	Vol 100-1000 ng/mL Stock	Vol 10-100 ng/mL Stock	Blank Whole Blood
	ng/mL	ng/mL	(µL)	(µL)	(µL)
Low (QCL)	1	10		20	200
Medium (QCM)	5	50		100	200
High (QCH)	40	400	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 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 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	95	5
1.00	95	5
2.00	To MS	To MS
9.00	5	95
9.50	5	95
9.51	95	5
10.00	To Waste	To Waste
11.00	Stop	Stop

Table 4: Instrument Parameters for Target Analytes

Target Analyte	Precursor Ion (*m/z*)	Product Ion	
6-Acetylcodeine	342	Quant	225
		Qualifier	165

6-acetylmorphine	328	Quant	165
		Qualifier	211
Codeine	300	Quant	215
		Qualifier	165
Dihydrocodeine	302	Quant	199
		Qualifier	128
EDDP	278	Quant	234
		Qualifier	249
Fentanyl	337	Quant	105
		Qualifier	188
Heroin	370	Quant	165
		Qualifier	268
Hydrocodone	300	Quant	199
		Qualifier	171
Hydromorphone	286	Quant	185
		Qualifier	157
Methadone	310	Quant	265
		Qualifier	105
Morphine	286	Quant	152
		Qualifier	165
Norfentanyl	233	Quant	84
		Qualifier	55
Norhydrocodone	286	Quant	199
		Qualifier	128
Noroxycodone	302	Quant	187
		Qualifier	227
O-Desmethyltramadol	250	Quant	58
		Qualifier	250
Oxycodone	316	Quant	298
		Qualifier	241
Oxymorphone	302	Quant	284
		Qualifier	227
Sufentanil	387	Quant	238
		Qualifier	111
Tramadol	264	Quant	58
		Qualifier	264
Trazodone	372	Quant	176
		Qualifier	148
Meprobamate	219	Quant	158
		Qualifier	97

Table 5: Instrument Parameters for Internal Standards

Internal Standard	Precursor Ion (*m/z*)	Product Ion	
6-acetylmorphine-d3	331	Quant	165
		Qualifier	331
Codeine-d3	303	Quant	165
		Qualifier	215
Dihydrocodeine-d6	308	Quant	202
		Qualifier	308
EDDP-d3	281	Quant	234
		Qualifier	281
Fentanyl-d5	342	Quant	105
		Qualifier	188
Heroin-d9	379	Quant	272
		Qualifier	335
Hydrocodone-d3	303	Quant	199
		Qualifier	171
Hydromorphone-d3	289	Quant	185
		Qualifier	157
Methadone-d3	313	Quant	268
		Qualifier	105
Morphine-d3	289	Quant	152
		Qualifier	289
Norfentanyl-d5	238	Quant	84
		Qualifier	55
Norhydrocodone-d3	289	Quant	202
		Qualifier	152
Noroxycodone-d3	305	Quant	190
		Qualifier	305
O-Desmethyltramadol-d6	256	Quant	64
		Qualifier	256
Oxycodone-d3	319	Quant	301
		Qualifier	244
Oxymorphone-d3	305	Quant	230
		Qualifier	287
Sufentanil-d5	392	Quant	238
		Qualifier	392
Tramadol-d3	268	Quant	58
		Qualifier	268

Trazodone-d6	378	Quant	150
		Qualifier	182
Meprobamate-d3	222	Quant	161
		Qualifier	222

Identification, Quantitation & LLOD/LLOQ:

- 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
Fentanyl	0.5ng/mL	50ng/mL
Norfentanyl	0.5ng/mL	50ng/mL
Sufentanil	0.5ng/mL	50ng/mL
6-Acetylcodeine	5ng/mL	500ng/mL
6-acetylmorphine (6-MAM)	5ng/mL	500ng/mL
Codeine	5ng/mL	500ng/mL
Dihydrocodeine	5ng/mL	500ng/mL
Heroin	5ng/mL	500ng/mL
Hydrocodone	5ng/mL	500ng/mL
Hydromorphone	5ng/mL	500ng/mL
Methadone	5ng/mL	500ng/mL
Morphine	5ng/mL	500ng/mL
Norhydrocodone	5ng/mL	500ng/mL
Noroxycodone	5ng/mL	500ng/mL
Oxycodone	5ng/mL	500ng/mL
Oxymorphone	5ng/mL	500ng/mL
Tramadol	5ng/mL	500ng/mL
EDDP	5ng/mL	500ng/mL
Meprobamate	5ng/mL	500ng/mL
O-Desmethyltramadol	5ng/mL	500ng/mL
Trazodone	5ng/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: NAR082819EAF) 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
- Narcotics 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:

An automated and fully validated LC-MS/MS procedure for the simultaneous determination of 11 opioids used in palliative care, with 5 of their metabolites. Musshoff, Trafkowski, Kuepper, and Madea. Journal of Mass spectrometry 41:5 March 2006.

Simultaneous Determination of Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, and 6-Acetylmorphine in Urine, Serum, Plasma, Whole Blood, and Meconium by LC-MS-MS. Coles, Kushnir, Nelson, McMillin, and Urry. JAT 31 January/February 2007.

Accurate Pain Management Analysis in Under 5 Min on Raptor Biphenyl Superficially Porous Particle LC Columns. Lupo, Kahler, and Connolly. The Application Notebook: Pharma/Drug Discovery 20 September 2015

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

Revision:

REVISED BY	REV#	DATE	Revisions
LN	1	10/29/19	Table 6: LLOQ increased from 0.2 ng/mL to 0.5ng/mL for 3-methylfentanyl through 6-Acetylcodeine and 2 ng/mL to 5 ng/mL for 6-MAM through Trazodone.
LN	2	11/20/2019	Following a validation addendum evaluation all references to 3-methylfentanyl and butyrylfentanyl have been removed from this quantitative method.
LN	3	12/05/2019	All references to Buprenorphine and Norbuprenorphine have been removed as they are not a part of this quantitative method.
EAF	4	12/17/19	Updated dilution section & added qualifier ion ration report to batch documentation section.
EAF	5	3/27/20	Updated dilution section to include 1:10 dilution and 1:20 dilution.
EAF	6	4/13/20	Corrected spelling of compound of interest to “Trazodone”.

EAF	7	10/09/2020	Annual Review: Quality control stock prep added “or from a different lot number”. Table 6: removed LLOD column and combined LLOD/LLOQ/RL. 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: Batch files contents qualifier ion report added “when applicable” and removed STARLIMs batch sequence.
EAF	8	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.