SOP 417 – Fentalog Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

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SOP Name: **Furanylfentanyl Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)**		SOP #: **416**
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	**Revision:**	**Revision Date/Initials:**
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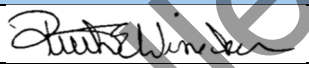
Approving Authority Name	**Approving Authority Signature**	**Approval Date**
Ruth E. Winecker, Ph.D.		08/25/2017

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1. **Principle of Assay**

1.1. This method is designed to confirm and quantitate fentanyl analogs in biological specimens by Liquid Chromatography Tandem Electrospray Mass Spectrometry (LC-MS/MS). Fentanyl analogs are extracted from biological matrices by protein precipitation with acetone and identified by retention time and ion ratio of product ions.

1.2. Fentanyl analogues are opioids with the main structural features of fentanyl that have been modified to bypass current United States drug laws and/or change the pharmacological profile of the drug. As such, they act at opioid receptors and decedents may have experienced the classic signs and symptoms of opioid toxicity (e.g., respiratory depression) prior to death.

1.3. Target Analytes

Target Analytes
Cyclopropyl Fentanyl
Methoxyacetyl Fentanyl

1.3.1.

2. **Specimens**

2.1. This procedure is applicable to urine, blood, serum, *bile, *gastric contents, and properly prepared **tissue specimens (typically 1:4 homogenates). A 0.1 mL (g) specimen amount (in duplicate) 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 0.1mL(g) aliquot shall be taken (volume permitting), spiked with appropriate QC, and analyzed to help to 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. Deuterated Internal Standard Mix (4.2)
3.5. Calibration Standard
3.6. QC Standard
3.7. Drug Free Blood, Urine, Liver Homogenate
3.8. Water with 0.1% formic acid
3.9. Acetonitrile with 0.1% formic acid

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

4.1. Cyclopropyl Fentanyl-d5 & Methoxyacetyl Fentanyl-d5 Stock Solution (1µg/mL)

4.1.1. Into a 10mL volumetric flask, add 0.1mL each Cyclopropyl Fenatnyl-d5 (100µg/mL) and Methoxyacetyl Fentanyl-d5 (100µg/mL) with a micropipette.

4.1.2. 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.

4.2. Cyclopropyl Fentanyl-d5 & Methoxyacetyl Fentanyl-d5 Working Solution (10ng/mL)

4.2.1. Into a 10mL volumetric flask, add 0.1 ml of Cyclopropyl Fentanyl-d5 & Methoxyacetyl Fentanyl-d5 Stock Solution (1µg/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. Fentalog 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” and “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” and “with 0.1% formic acid”.

5. **Equipment and Special Supplies**

5.1. Test Tubes, 16 x 100 mm
5.2. LC autosampler vials, 12 x 32 mm
5.3. Polyspring inserts, 5 mm O.D.
5.4. Centrifuge 2000 x g
5.5. Vortex mixer
5.6. Nitrogen evaporator

6. **Instrumentation and Parameters**
   - 6.1. Windows PC with Thermo LCQuan and Xcaliber software
     - 6.1.1. Instrument method (TSQ04): "Fentalog Quant_"
     - 6.1.2. Click here for instrument parameters.
   - 6.2. Dionex UltiMate 3000 LC autosampler, or equivalent
   - 6.3. Dionex UltiMate 3000 LC pump, or equivalent
   - 6.4. Thermo TSQ triple quadrupole mass spectrometer

7. **Target Ions** (± 1 nominal mass)

7.1.	Cyclopropyl Fentanyl	(**349** 105 188)
7.2.	Cyclopropyl Fenatnyl-d5	(**354** 105 188)
7.3.	Methoxyacetyl Fentanyl	(**353** 105 188)
7.4.	Methoxyacetyl Fentany-d5	(**358** 105 188)

   - 7.4.1. 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 qualification/confirmation.

8. **Procedure**
   - 8.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 16x100 mm test tubes.
   - 8.2. Add the appropriate quantity (according to the Standard & Control Worksheet) of Deuterated Internal Standard Mix (4.2) to all the tubes.
   - 8.3. Add the appropriate quantity (according to the Standard & Control Worksheet) of calibration standard and QC to the tubes labeled as standards and control, respectively, labeling test tubes as you go. Only internal standard should be present in the test tube labeled "Blank".
   - 8.4. Add 0.1mL of blank blood to all standards, controls, and blank test tubes (0.1 mL blank urine/0.1g blank liver homogenate to urine/liver blank and QC test tubes respectively).
   - 8.5. Add the appropriate amount of predetermined unknown specimen 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 16x100 test tubes, place in nitrogen evaporator, and evaporate at 55° C to dryness.

8.10. Remove dried specimens from nitrogen evaporator and reconstitute with 100µL of methanol. Vortex for 10 seconds and centrifuge at 2000 x g for 5 minutes.

8.11. Transfer approximately 100µL 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 (TSQ04).

8.12. 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 "Fentalog Quant" (SOP 055). It is used to calculate the internal standard response ratios, raw amounts, concentration, and ion ratios.

9.1.2. These calculations are computed as follows:

9.1.2.1. Response Ratio:

9.1.2.1.1. Response Ratio = response of the analytes quantifying product ion compared to that of the internal standard's quantifying product ion.

9.1.2.1.2. Response Ratio = QN_a / QN_{istd}

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

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

9.2. Calibration

9.2.1. A linear regression resulting from the 6 standards is used to quantitate the analytes in the load. 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

9.8.1.1.1. Ratio 1 = QL_1/QN

9.8.2. QL_1 = response of the quantifying product ion

9.8.3. QN = response of the qualifying product ion

10. **Quality Control**

10.1. Acceptance criteria

10.1.1. Chromatogram

10.1.1.1. Peaks must be Gaussian shaped (symmetrical).

10.1.1.2. Peaks must not exhibit extreme fronting or tailing.

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

10.1.1.4. The internal standard (ISTD) in each case should be inspected for evidence of signal enhancement and suppression. The area of the

quantifying ion should not be less than 50% or more than 200% of the average ISTD of the calibrators.

10.1.1.5. Retention time must not deviate outside ± 3% of target, based upon the retention time of the calibrators and controls.

10.1.2. Mass spectroscopy

10.1.2.1. 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).

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

10.1.3. Calibrators

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

10.1.3.2. Each calibrator, when calculated against the calibration curve, must not deviate outside ± 20% of the target value.

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

10.1.4. Controls

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

10.1.5. Blanks

10.1.5.1. 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.

10.1.6. Any deviation from the above criteria must be approved by a senior chemist.

11. **Validation of Method**

Parameter	Result (TSQ04)
Bias	**Blood:** Cyclopropyl Fentanyl - Low: -10.27% High: -5.06%
	Liver: Cyclopropyl Fentanyl - Low: -11.00% High: -5.60%

	Blood: Methoxyacetyl Fentanyl - Low: 0.01% High: 5.13%
	Liver: Methoxyacetyl Fentanyl - Low: 0.53% High: 4.21%
Precision	**Blood:** Cyclopropyl Fentanyl - Low: 2.93% High: 2.18%
	Liver: Cyclopropyl Fentanyl - Low: 3.90% High: 2.54%
	Blood: Methoxyacetyl Fentanyl - Low: 4.34% High: 4.39%
	Liver: Methoxyacetyl Fentanyl - Low: 6.47% High: 4.01%
Calibration model	Cyclopropyl Fentanyl - 1/x Weighting
	Methoxyacetyl Fentanyl - 1/x Weighting
Carryover	Tested to 2X high calibrator (1000ng/mL) with no visible amount of carryover.
Interference Studies	No interfering signal observed from Internal Standard, Non-target analytes, or matrix.
Ionization/Suppression: (Not needed if IS coelutes within 0.05 min.)	N/A - internal standards coelute within 0.05 minutes.
LOD (Calculate: 3.3xSD Y-intercept/Mean of Slope)	Cyclopropyl Fentanyl: **0.05 ng/mL**
	Methoxyacetyl Fentanyl: **0.05 ng/mL**
LOQ (Set to lowest calibrator with acceptable Bias/Precision).	**Blood: 0.05ng/mL** (Both Cyclopropyl and Methoxyacetyl Fentanyl)
	Liver: **1 ng/mL** (Both Cyclopropyl and Methoxyacetyl Fentanyl)

12. Reporting

12.1. All analytes concentrations shall be reported in ng/mL or ng/g as appropriate.

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

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

12.3.1. **Note:** Due to baseline noise associated with the matrix, the LOQ for liver specimens is 1ng/g (4ng/g with typical homogenate dilution factor).

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

Reporting Duplicates

- **Dilution factors of 1 and 2 (or other)**

Dil / Scenario	1	2	REPORT
A	**In curve**	**In curve**	**Average**
B	**In curve**	**BQL**	**"In" value**
C	**AQL**	**In curve**	**"In" value**
D	**In curve**	**ND (should be in)**	**Repeat**
E	**AQL/BQL**	**AQL/BQL**	**Less than/Greater than**
F	**BQL**	**ND**	**ND**
G	**In curve**	**ND (should be BQL)**	**"In" value**

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

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

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

12.3.1.4. ND = None Detected

12.4. Averaging reportable values

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

12.4.2. Enter calculated concentration for each specimen into toxlog.

12.5. Significant figures

12.5.1. Concentrations are truncated and reported with two significant figures in milligrams per liter (ng/mL).

13. **Preparation of Load**

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

13.1.1. Assignment 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**

14.1. Helander, Anders, Matilda Bäckberg, and Olof Beck. "Intoxications Involving the Fentanyl Analogs Acetylfentanyl, 4-methoxybutyrfentanyl and Furanylfentanyl: Results from the Swedish STRIDA Project." *Clinical Toxicology* 54.4 (2016): 324-32.

14.2. Palamalai, Vikram, Kalen N. Olson, Julie Kloss, Owen Middleton, Kelly Mills, A. Quinn Strobl, Lindsey C. Thomas, and Fred S. Apple. "Superiority of Postmortem Liver Fentanyl Concentrations over Peripheral Blood Influenced by Postmortem Interval for Determination of Fentanyl Toxicity." *Clinical Biochemistry* 46.7-8 (2013): 598-602.

14.3. Olson, Kalen N., Kristin Luckenbill, Jonathan Thompson, Owen Middleton, Roberta Geiselhart, Kelly M. Mills, Julie Kloss, and Fred S. Apple. "Postmortem Redistribution of Fentanyl in Blood." *American Journal of Clinical Pathology Am J Clin Pathol* 133.3 (2010): 447-53.

Other suggested reading:

14.4. Chambers, Erin, Diane M. Wagrowski-Diehl, Ziling Lu, and Jeffrey R. Mazzeo. "Systematic and Comprehensive Strategy for Reducing Matrix Effects in LC/MS/MS Analyses." Journal of Chromatography B 852.1-2 (2007): 22-34.