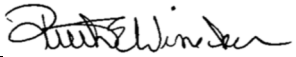
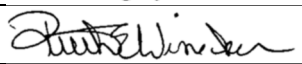
SOP 414 – Acetylfentanyl Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

Table of Contents

SOP 414 – Acetylfentanyl Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

SOP Name:		SOP #:
Acetylfentanyl Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)		**414**
North Carolina Office of the Chief Medical Examiner Toxicology Laboratory	**Revision:**	**Revision Date/Initials:**
	9.2.1 – Initial Document Preparation	MSF – 02/04/2016
	12.4.1 –Updated reporting units	MSF - 03/03/2016
	8.14, 9.1.1 – Updated references 10.1.4 – Corrected indent 10.1.3.3 – Added reference to QA Manual (Cal point exclusion)	MSF – 04/04/2016
	11.1 – Updated Validation Table	MSF – 06/09/2016
	1 – Updated Introduction 4 – Added standard prep instructions 6 – Updated instrument parameters	MSF – 09/21/16 MSF – 05/25/2017 MSF – 06/05/2017
	12.4.1 – updated reporting units	MSF – 11/15/2017

Approving Authority Name	**Approving Authority Signature**	**Approval Date**
Ruth E. Winecker, Ph.D.		08/25/2016
Ruth E. Winecker, Ph.D.		12/07/2017

SOP 414 – Acetylfentanyl Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

1. Principle of Assay

1.1. This method is designed to confirm and quantitate acetyl fentanyl in biological specimens by Liquid Chromatography Tandem Electrospray Mass Spectrometry (LC-MS/MS). The drug is extracted from its biological matrix by protein precipitation with methanol and identified by the retention times and ion ratios of the product ions.

1.2. Acetyl fentanyl is another drug in the ever-growing class of "novel psychoactive substances" (NPS, note: this abbreviation is often used, synonymously, with "new psychoactive substances"). The vast majority of NPS drugs are derivatives of known legal or illicit drugs, and are meant to circumvent existing drug laws. They are commonly sold in retail stores or on-line, often with the caveat of "not meant for human consumption." The design and synthesis of such drugs are often pulled from patents and scientific publications, with very little known about activity or toxicity in humans.

The first mass, public awareness of acetyl fentanyl occurred in June, 2013, when the CDC issued a health advisory and recommendations for laboratory testing (1). This alert was spurred by 14 acetyl fentanyl related deaths in Rhode Island, 10 of which occurred in March, 2013 (2). While commonly reported as "15 times stronger than heroin" or "5 times less potent than fentanyl", there are no known pharmacokinetic, pharmacodynamic, or toxicity studies in higher mammals or humans. While sharing the same structural backbone of fentanyl, acetyl fentanyl is known to act as an agonist on opioid receptors and expected to have central analgesic effects, as well as adverse effects of respiratory depression.

Because it has not been studied in humans, there are no known "therapeutic doses", and due to the way it is produced and sold, users may not know the concentration or purity of acetyl fentanyl, nor even know they are using it at all, if it is being sold as an adulterant to heroin or claimed to be another drug entirely. Outside of this laboratory, acetyl fentanyl related deaths have been reported throughout much of the United States and abroad (3, 4, 5)

Like fentanyl, acetyl fentanyl has been shown to exhibit postmortem redistribution (3). As with any drug, particularly one likely to be combined with other opioid analgesics, and CNS depressants such as benzodiazepines, interpretation of postmortem acetyl fentanyl concentrations must rely not only on the drug concentration, but incorporate patient history, autopsy and scene findings.

Acetyl fentanyl was listed as a Schedule I controlled substance under the Controlled Substances Act in May 2015 (6).

2. **Specimens**

2.1. This procedure is applicable to urine, blood, serum, vitreous humor, properly prepared tissue specimens (typically 1:4 homogenates), bile*, and gastric contents*.

2.2. A 0.1 mL (g) sample size (in duplicate) is generally employed for urine, blood, serum, bile, and gastric contents, and a 0.1g sample size (in duplicate) for tissue homogenate (unless a dilution is required) so that the calibration curve encompasses the expected range of unknown specimens.

2.2.1. *For non-typical matrices, an additional 0.1mL 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. Acetonitrile, HPLC grade

3.4. Deuterated Fentanyl Internal Standard Mix

3.5. Acetylfentanyl Standard

3.6. Acetylfentanyl 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. **Fentanyl-d5 Stock Solution (10µg/mL)**

4.1.1. Into a 10mL volumetric flask, add the contents of 1 ampule (~1mL) of Fentanyl-d5 (Cerilliant – 100µg/mL).

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. **Fentanyl-d5 Internal Standard (100ng/mL)**

4.2.1. Into a 10mL volumetric flask, add 0.1 ml of Fentanyl-d5 Stock Solution (10µg/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. **Acetylfentanyl 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.

4.5. **Water with 0.1% formic acid**

4.5.1. To a 4L bottle of HPLC grade water, add 4 mL of formic acid

4.5.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

4.6. **Acetonitrile with 0.1% formic acid**

4.6.1. To a 4L bottle of HPLC grade acetonitrile, add 4 mL of formic acid

4.6.2. Label bottle as "LC/MS" and "with 0.1% formic acid".

5. **Equipment and Special Supplies**

5.1. Test Tubes, 13 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 (TSQ02): "Acetyl Fentanyl"

6.1.2. Click here for instrument parameters.

6.1.3.

6.2. Thermo Surveyor LC autosampler, or equivalent

6.3. Thermo Surveyor LC pump, or equivalent

6.4. Thermo TSQ triple quadrupole mass spectrometer

6.4.1. =

7. **Target Ions** (± 1 nominal mass)

7.1. Fentanyl-d5 (**342** 188 105)
7.2. Acetylfentanyl (**323** 188 105)

7.2.1. 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.

8. **Procedure**

8.1. Prepare a colored tape label for each standard, blank, control, and specimen to be placed on 13x100 mm test tubes.

8.2. Add the appropriate quantity (according to the Standard and Control Worksheet) of the Deuterated Fentanyl Internal Standard Mix to all the tubes.

8.3. Add the appropriate quantity (according to the Standard and Control Worksheet) of the Acetylfentanyl Standard and the Acetylfentanyl QC Standard Mix 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, control, and blank test tubes (0.1 mL blank urine/0.1g blank liver homogenate to urine/liver blank and QC test tubes).

8.5. Add the appropriate amount of unknown specimen, labeling test tubes as you go (See Specimens section).

8.6. Vortex all test tubes for 10 seconds.

8.7. Add 3mL methanol to each tube and vortex for 20 seconds.

8.8. Centrifuge at 2000 x g for 10 minutes.

8.9. Decant the top methanol layer into clean 13x100 test tubes transferring the tape label as you go. Place in nitrogen evaporator.

8.10. Evaporate under a stream of nitrogen at 55° C to dryness.

8.11. Remove dried specimens from nitrogen evaporator and reconstitute with 300µL of methanol.

8.12. Vortex for 10 seconds and centrifuge at 2000 x g for 5 minutes.

8.13. Transfer each extract to appropriately labeled autosampler vials fitted with 100 µL polyspring insert and place in the autosampler tray of the Thermo TSQ02 triple-quadropole LC/MS/MS.

8.14. Build and initiate sequence as directed in SOP-053.

9. **Calculations**

9.1. Quantitative Ion ratios

9.1.1. The method for processing the data using the Thermo LCQuan software is "Acetylfentanyl" (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 standards.

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 quadratic regression resulting from 6 calibrators is used to quantitate the analytes in the case. 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 sharing parent/product ions must have baseline resolution.

10.1.1.3. 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.

10.1.1.4. 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 3).

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 (± 25% at LOQ).

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

10.1.4. Controls - must calculate within ± 20% of the target.

10.1.5. Blanks

10.1.5.1. Blanks should not contain any target analyte with a response ratio > 10% that of the low calibrator (LOQ).

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

11. **Validation of Method**

11.1. The method validation plan was written and is stored with the validation data. The plan was followed to determine this assay's linearity, precision, limit of detection, limit of quantitation, and carryover threshold. The validation results are as follows:

Parameter	Acetylfentanyl
LOD LOQ	4 ng/mL 10 ng/mL
Calibration Model	10 - 1000 ng/mL - Quadratic 1/x
Upper limit of quantification	1000 ng/mL
Carryover	>1000 ng/mL
Precision (n=15)	**Blood - L:** 8.38% **H:** 5.72% **Liver - L:** 9.96% **H:** 8.97%
Bias (n=15)	**Blood - L:** 9.28% **H:** 4.09% **Liver - L:** 3.24% **H:** -0.09%
Processed Sample Stability	Not Evaluated
Interference (Matrix/non-target analytes)	None Identified

11.2.

11.2.1. To rule out carryover, a case specimen injected immediately following a specimen with >1000ng/mL Acetylfentanyl shall be re-injected (along with appropriate QC) or repeated.

12. **Reporting**

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

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

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

12.2.1.

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

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

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

12.2.1.4. ND = None Detected

12.3. Averaging reportable values

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

12.3.2. Enter calculated concentration for each specimen into toxlog.

12.4. Significant figures

12.4.1. Concentrations are truncated and reported with two significant figures in **ng/mL**.

13. **Reinjection**

13.1. A sample may be reinjected due to autosampler failure, apparent low recovery, to check for carry-over or to meet ion ratio and/or retention time criteria. Reinjected sample(s) must be followed by reinjection of either the duplicate case sample(s) or matrix-matched calibrator or control. All reinjected samples must meet the QA/QC criteria.

13.2. See the QA/QC Manual for laboratory guidelines.

14. **Preparation of Load**

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

14.1.1. Assignment sheet

14.1.2. Comments or note to file if applicable

14.1.3. Load summary

14.1.4. Specimen worklist

14.1.5. Chain of custody (Specimen)

14.1.6. Aliquot chain of custody

14.1.7. Standard and control worksheet

14.1.8. Sequence summaries/calibration reports – paper clipped

14.1.9. Calibrator data - paper clipped

14.1.10. Blank matrix data - paper clipped

14.1.11. Control data - paper clipped

14.1.12. Specimen data – stapled

15. **References**

15.1. CDC Health Advisory: Recommendations for Laboratory Testing for Acetyl Fentanyl and Patient Evaluation and Treatment for Overdose with Synthetic Opioid. (2013, June 20). Retrieved from https://emergency.cdc.gov/han/han00350.asp

15.2. Lozier, Matthew J., Molly Boyd, Christina Stanley, Laurie Ogilvie, Ewa King, Colleen Martin, and Lauren Lewis. "Acetyl Fentanyl, a Novel Fentanyl Analog, Causes 14 Overdose Deaths in Rhode Island, March–May 2013." *Journal of Medical Toxicology J. Med. Toxicol.* 11.2 (2015): 208-17.

15.3. Poklis, Justin, Alphonse Poklis, Carl Wolf, Mary Mainland, Laura Hair, Kelly Devers, Leszek Chrostowski, Elise Arbefeville, Michele Merves, and Julia Pearson. "Postmortem Tissue Distribution of Acetyl Fentanyl, Fentanyl and Their Respective Nor-metabolites Analyzed by Ultrahigh Performance Liquid Chromatography with Tandem Mass Spectrometry." *Forensic Science International* 257 (2015): 435-41.

15.4. Fort, Chelsea, Byron Curtis, Clay Nichols, and Cheryl Niblo. "Acetyl Fentanyl Toxicity: Two Case Reports." *Journal of Analytical Toxicology J Anal Toxicol* (2016): 1-4.

15.5. 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.

15.6. Schedules of Controlled Substances: Temporary Placement of Acetyl Fentanyl into Schedule I. (2015, May 21). Retrieved from http://www.deadiversion.usdoj.gov/fed_regs/rules/2015/fr0521_7.htm