SOP 404 - Fentanyl Extraction using Protein Precipitation for Quantification by Liquid Chromatography/Electrospray Mass Spectrometry/ Mass Spectrometry (LC/MS/MS)

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	9.2.1 – Updated # of calibrators 10.1.1.4 – Updated RT acceptance range 10.1.2.1 – Updated IRC acceptance range 10.1.3.2 – Updated Calibrator acceptance range 10.1.3.3 – Updated QC acceptance range	MSF – 05/07/2015
	9.1.1 – Updated reference 10.1.4 – Updated indent 10.1.3.3 – Added reference to QA Manual (Cal point exclusion)	MSF – 04/04/2016
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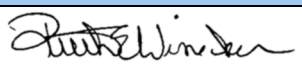
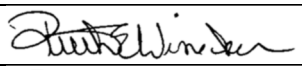
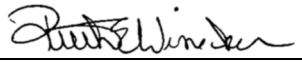
Ruth E. Winecker, Ph.D.		04/14/2015
Ruth E. Winecker, Ph.D.		06/28/2016
Ruth E. Winecker, Ph.D.		12/07/2017

SOP 404 - Fentanyl 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 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 acetone and identified by the retention times of precursor ions and ion ratios of the product ions.

1.2. Fentanyl was developed in the 1960's. A fat soluble and very short acting analgesic, fentanyl is a Mu receptor agonist prescribed for use in anesthesia and for pain management. Formulations include intravenous or intramuscular injection, transdermal patch (for chronic pain patients), a buccal film and dissolvable tablet as well as a lollipop (to dose pediatric patients before surgery and pediatric cancer patients). The range between a clinical dose and a toxic dose is very small and consequently this drug is extremely dangerous to non-tolerant individuals.

1.3. Because of the possibility of postmortem redistribution it is recommended that both peripheral blood and liver be analyzed for proper interpretation. As with any narcotic analgesic, interpretation of postmortem fentanyl concentrations must rely not only on the drug concentration but incorporate patient history, autopsy and scene findings.

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. Acetone, HPLC grade

3.4. Acetonitrile, HPLC grade

3.5. Deuterated Fentanyl Internal Standard Mix

3.6. Fentanyl Standard

3.7. Fentanyl QC Standard

3.8. Drug Free Blood, Urine, Liver Homogenate

3.9. Water with 0.1% formic acid

3.10. Acetonitrile with 0.1% formic acid

4. Standards, Controls, and Solutions

4.1. Fentanyl-d5 Stock Solution A (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 Stock Solution B (1000ng/mL)

4.2.1. Into a 10mL volumetric flask, add 1 ml of Fentanyl-d5 Stock Solution A (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. Fentanyl-d5 Internal Standard (10ng/mL)

4.3.1. Into a 10mL volumetric flask, add 0.1 ml of Fentanyl-d5 Stock Solution B (1000ng/mL) with a micropipette.

4.3.2. 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.

4.4. **Fentanyl 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.

4.5. **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 (TSQ01 & TSQ02): "Fentanyl"

6.1.2. Click here for instrument parameters.

6.2. Thermo Surveyor LC autosampler, or equivalent

6.3. Thermo Surveyor LC pump, or equivalent

6.4. Thermo TSQ triple quadrupole mass spectrometer

7. **Target Ions** (± 1 nominal mass)

    7.1. Fentanyl-d5 (**342** 188 105)
    7.2. Fentanyl (**337** 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 Fentanyl Standard Mix and the Fentanyl 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 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 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 TSQ 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 "Fentanyl" (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 linear regression resulting from the 5 standards 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	Fentanyl
LOD LOQ	0.25 ng/mL 1.0 ng/mL
Curve Linearity	1 - 500 ng/mL linear 1/x
Upper limit of quantification	500 ng/mL
Carryover	>500 ng/mL
Precision (n=15)	**Blood - L:** 3.59% **H:** 2.77% **Liver - L:** 3.45% **H:** 3.14%
Bias (n=15)	**Blood - L:** 4.04% **H:** 3.60% **Liver - L:** 2.26% **H:** 2.41%
Processed Sample Stability	7 days
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 >500ng/mL fentanyl 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

Scenario \ Dil	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. Erin Chambers, Diane M. Wagrowski-Diehl, Ziling Lu, Jeffrey R. Mazzeo, Journal of Chromatography B, 852(2007) 22-34.

15.2. Ronald C. Baselt, Disposition of Toxic Drugs and Chemicals in Man 6th edition, 430-433.

15.3. Levine, Barry. "Postmortem Forensic Toxicology." *Principles of Forensic Toxicology*. 2nd ed. Washington, DC: AACC, 2006. 67-79. Print.