CONFIRMATION OF FENTANYL AND NORFENTANYL BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

38.1 METHOD

This test method may be used to confirm the presence of fentanyl (FEN) and metabolite norfentanyl (NFT) in biological specimens. The target compounds and corresponding internal standards are isolated from whole blood, serum, plasma, urine or other submitted biological specimens by solid-phase extraction (SPE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source.

38.2 SPECIMENS

The specimen volume is 0.5 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

The presence of ketamine in a specimen may cause interference with norfentanyl-d_5 internal standard, affecting chromatography and transition ratios (see 38.5 and 38.7).

NOTE: Matrix-matching of the full calibration curve and all positive control levels is not required for quantitation in serum/plasma or liver (tissue) homogenate samples, as determined through evaluation of alternate matrix (serum, liver homogenate) during method validation (see 38.4.3).

38.3 REAGENTS, MATERIALS AND EQUIPMENT

38.3.1 REAGENTS

NOTE: Laboratory general-use deionized water (DI H_2O) and reagent-grade organic solvents are used in reagent preparation, unless otherwise specified.

- Acetic acid, glacial
- 0.1M acetic acid

  Add 5.72 mL glacial acetic acid to 800 mL DI H_2O and mix. Dilute to 1 L with DI H_2O and mix. Store the acid in a glass bottle at room temperature for up to six months.
- Acetonitrile (ACN), reagent grade and LC-MS grade
- Ammonium hydroxide (NH_4OH), concentrated
- Certified blank blood and/or other biological matrices
- DI H_2O, laboratory general-use and LC-MS grade H_2O (or equivalent from a high-purity filtration system)
- Elution solvent

  To 20 mL isopropanol, add 2 mL concentrated NH_4OH and mix. Add 78 mL CH_2Cl_2 and mix. Store the elution solvent in a glass flask/bottle at room temperature and use on date of preparation only.

- Formic acid (concentrated)
- 0.1% Formic acid

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade H_2O in a 1 L flask and mix. Dilute to 1 L with LC-MS grade H_2O and mix. Store the solution in an amber glass bottle at room temperature for up to one year.

  NOTE: Filtration prior to use is not required for 0.1% formic acid unless DI H_2O must be used in place of LC-MS grade H_2O.
- Isopropanol (IPA)
- Methanol (MeOH), reagent grade and HPLC grade
- Methylene chloride (CH_2Cl_2, dichloromethane)
- 0.1M phosphate buffer (pH6)

  Dissolve 1.7 g Na_2HPO_4 and 12.14 g $NaH_2PO_4 \cdot H_2O$ in 800 mL DI H_2O and mix. Dilute to 1 L with DI H_2O and mix. Check the pH and, if necessary, adjust to 6 ± 0.5 with concentrated NaOH. Store the solution in a glass bottle at room temperature for up to one year.
- Sodium hydroxide (NaOH), concentrated
- Sodium phosphate, dibasic anhydrous (Na_2HPO_4)
- Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$)

NOTE: Adjustments to final volumes of prepared reagents are permitted as long as the proportions are maintained.

38.3.2 MATERIALS

- Polypropylene autosampler vials with integrated inserts and caps
- Disposable extraction tubes (16 x 100 mm recommended) and screw-cap or centrifuge tubes with closures
- Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200mg/6mL), or equivalent
- HPLC Column, Agilent Poroshell 120 EC-C18, 2.1 x 75 mm, 2.7 µM particle size, or equivalent
- Laboratory glassware (graduated cylinders, flasks)

38.3.3 EQUIPMENT

- Agilent HPLC (1100/1200 series or equivalent)
- Agilent MS-MS with API-ES source, (6420 or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipettes with disposable pipette tips
- General-use equipment (centrifuge, evaporator, pH meter/indicating paper, vacuum manifold, vortex mixer)

38.4 STANDARDS, CALIBRATORS AND CONTROLS

38.4.1 STANDARDS

- Working standard (WS): 1 ng/µL
- Working control standard (QC): 1 ng/µL
- Working internal standard (IS): 0.1 ng/µL

38.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 38.5 SAMPLE PREPARATION.

38.4.3 CONTROLS

38.4.3.1 At least one negative blood control and three positive blood controls are tested with every batch, prepared as described in 38.5.

38.4.3.2 One positive and one negative control must be included for each alternate matrix type tested in the batch, for qualitative or quantitative analysis.

38.4.3.3 Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls. When the batch contains more than 20 specimens, one of the positive controls is analyzed mid-run.

38.4.3.4 Positive controls in both blood and/or alternate matrices may be used to bracket case specimens. When analyzing compounds in multiple matrices, both blood and alternate matrix controls apply towards 10% of the batch.

38.5 SAMPLE PREPARATION

NOTE: NFT-d_5 must be carefully evaluated against criteria for acceptance for specimens that contain ketamine (see 38.2 and 38.7).

38.5.1 Label a clean extraction tube for each member of the test batch. (i.e., calibrator, control, case sample).

38.5.2 Add 3 mL 0.1M phosphate buffer (pH6) to each tube.

38.5.3 Using a calibrated pipette, add 0.5 mL of certified blank blood into each of the calibrator tubes, the positive control tubes and the negative control tube(s).

38.5.4 Prepare a 1:10 dilution of the working standard. (0.1 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the working standard with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

38.5.5 Prepare a 1:100 dilution of the working standard. (0.01 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the 1:10 dilution with 900 µL of ACN or MeOH in a labeled tube.

b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

38.5.6 Using a calibrated pipette, spike the calibrators according to the following table, using the prepared dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS
Calibrator 1 – 0.5 ng/mL	25	0.01 ng/µL	1:100
Calibrator 2 – 1.0 ng/mL	50	0.01 ng/µL	1:100
Calibrator 3 – 5.0 ng/mL	25	0.1 ng/µL	1:10
Calibrator 4 - 10 ng/mL	50	0.1 ng/µL	1:10
Calibrator 5 - 25 ng/mL	125	0.1 ng/µL	1:10

38.5.7 Prepare a 1:10 dilution of the working control standard. (0.1 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the working control standard with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

38.5.8 Prepare a 1:100 dilution of the working control standard. (0.01 ng/µL)

a. Using a calibrated pipette, combine 100 µL of the 1:10 dilution with 900 µL of ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation

38.5.9 Using a calibrated pipette, spike the positive controls according to the following table, using the prepared dilutions.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC
Low Control – 1.5 ng/mL	75	0.01 ng/µL	1:100
Mid Control – 8 ng/mL	40	0.1 ng/µL	1:10
High Control – 20 ng/mL	100	0.1 ng/µL	1:10

38.5.10 Using a calibrated pipette, sample 0.5 mL of each case specimen into its respective tube.

38.5.11 Using a calibrated pipette or verified repeater-pipette, add 50 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 10 ng/mL.

38.5.12 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500rpm (recommended for 16 x 100 mm tubes).

38.5.13 Place new, labeled SPE columns into the vacuum manifold.

38.5.14 Condition the SPE columns by passing each of the following solvents completely through under force of gravity.

a. 3 mL MeOH
b. 3 mL DI H_2O
c. 2 mL 0.1M phosphate buffer (pH6)

Do not let columns dry out between each conditioning step.

38.5.15 Transfer the contents of each tube to its respective SPE column and allow them to flow through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

38.5.16 Wash the SPE columns by passing each of the following solvents completely through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

a. 3 mL DI H_2O
b. 2 mL 0.1M acetic acid
c. 3 mL MeOH

38.5.17 Dry the columns for 10 minutes under vacuum.

38.5.18 Place clean, labeled centrifuge tubes in the collection rack underneath their corresponding SPE columns.

38.5.19 Pass 3 mL of elution solvent through each SPE column and collect the extracts.

38.5.20 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C.

38.5.21 Reconstitute the extracts with the addition of 50 µL mobile phase (95:5 0.1% formic acid in LC-MS grade H_2O:LC-MS grade ACN). Briefly vortex-mix the tubes. Centrifuge the tubes for 2 minutes at 2000 rpm (recommended) to collect the extracts at the bottom of the tubes.

38.5.22 Transfer the extracts to labeled polypropylene autosampler vials with integrated inserts and cap.

38.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – FENTANYL (instrumental parameters in Appendix B)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±3%, ion ratios ±20%) – Cal 4
- Result comparisons – all units in ng/mL
- Cal 1: truncated to two decimal places (acceptable range ±25%; 0.37 – 0.62 ng/mL)

  Cal 2-3, Pos Ctl 1, 2: truncated to one decimal place (acceptable range ±20%)

  Cal 4-5, Pos Ctl 3: truncated, whole integer values (acceptable range ±20%)

38.7 REPORTING

Results for fentanyl are reported in units of nanograms per milliliter (ng/mL), truncated to two significant figures. Qualitative results are reported for norfentanyl for concentrations ≥ the cal 1 (0.5 ng/mL) concentration, as "positive."

Where interference with NFT-d_5 is observed in case specimens containing ketamine (see 38.2 and 38.5), norfentanyl may be reported, provided all criteria for acceptance are met for norfentanyl.

38.8 METHOD PERFORMANCE

- Limit of detection: 0.1 ng/mL
- Lower limit of quantification: 0.5 ng/mL
- Dynamic range: 0.5 – 25 ng/mL
- Upper limit of quantitation: 25 ng/mL
- Upper limit of linearity: 150 ng/mL

38.9 REFERENCES

- A Black, B.E. O'Reilly and D. Sklerov, in-house method development
- T. Berg, B. Jorgenrud, D.H. Strand, Determination of buprenorphine, fentanyl and LSD in whole blood by UPLC – MS-MS*, Journal of Analytical Toxicology,* 37 (2013) 159-165.

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

Fentanyl
Fentanyl-d_5 (FEN-d_5)
Norfentanyl
Norfentanyl-d_5 (NFT-d_5)

APPENDIX B
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid in LC-MS grade H_2O
Solvent B	ACN (LC-MS grade)
Initial composition	95% A, 5% B
0 – 0.1 min	5% B
0.1 – 2.0 min	5% B
2.0 – 6.0 min	35% B
6.0 – 7.0 min	5% B
7.1 – 9.0 min	5% B
Column temp	40°C
Autosampler	
Injection volume	5 µL
Injection flush-port	active
Flush-port time	15 sec
Flush-port solvent	75:25 HPLC grade MeOH:LC-MS grade H_2O

MASS SPECTROMETER

Ion Mode	(+) MRM	Nebulizer gas	Nitrogen
Peak width	0.07 min	Nebulizer pressure	50 psi
Dwell time	50	Drying Gas	Nitrogen
Time segment 1	To waste	Drying gas flow	12 L/min
Time segment 2	EMV 200(+)	Drying gas temp	350°C
		Capillary voltage	4.0 kV

Compound	MRM Transitions
Fentanyl	337.2→ 188.0, 105.0
Fentanyl-D_5	342.1→ 188.1, 105.1
Norfentanyl	233.2→ 84.1, 55.2
Norfentanyl-D_5	238.0→ 84.1, 55.1

LIST OF CHANGES

Revision Date	Description	Page Number
8/11/16	Method approved by Washington State Toxicologist. See DRA dated 7/28/16. Method released for use in evidentiary testing as of 8/11/16.	All
7/10/17	A note was added to section 38.4.2 indicating that matrix-matching of calibrators and controls is not necessary based on method validation. Wording added to section 38.4.3 regarding dilution and standard volume testing. Specified use of calibrated pipettes for measurement of blank blood, specimens and standards throughout section 38.7 SAMPLE PREPARATION. Edited 38.10.2.2.d to indicate all positive controls must meet acceptability criteria to report quantitative results and added two article citations to references in 38.15. Other minor edits throughout.	1-10
8/5/19	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching and information on testing specimens containing ketamine in 38.2 SPECIMENS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Specified use of LC-MS grade deionized water and acetonitrile in 38.3.1. Criteria for batch acceptance (calibrators, controls) and specimen acceptability criteria, and specific data analysis and reporting information are now included in the General Requirements for Chromatographic Test Method Batch Analysis and Acceptance. Target compound/internal standard list added in APPENDIX A, with test method parameters moved to APPENDIX B. Formatting and minor edits throughout.	All
5/16/20	Edited NOTE in section 38.3.1; moved filtration information to NOTE in prep of 0.1% formic acid (no filtration required for prep with LC-MS grade H_2O). Changed references for “LC-MS grade DI H_2O” to “LC-MS grade H_2O.” NOTE regarding specific grade of H_2O and solvents used was removed from 38.5 (covered in 38.3.1). Use of mid-run control added in 38.4.3.3. Changed pipetted volumes in 38.5.4 – 38.5.5 and 38.5.7 – 38.5.8 from mL to µL. Other minor edits throughout.	1-7
4/24/21	Updated dynamic range to 0.5 – 25 ng/mL (38.5.6) and changed high positive control from 40 ng/mL to 20 ng/mL (38.5.9). Added mid control at 8 ng/mL. Updated 38.6 and 38.8 to reflect changes. Section 38.7 updated for qualitative reporting of norfentanyl. Updated transitions for target compounds ant internal standards in Appendix B. Re-validation performed to verify new dynamic range. See DRA dated 4/23/21.	3-7