

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: Unless use of LC-MS grade (or equivalent from a high-purity filtration system) deionized water (DI H₂O) is specified, laboratory general-use DI H₂O is used in reagent preparation. Organic solvents are reagent grade unless otherwise specified.

- Acetic acid, glacial
- 0.1M acetic acid
Add 5.72 mL glacial acetic acid to 800 mL DI H₂O. Dilute to 1 L with DI H₂O 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 (NH₄OH), concentrated
- Certified blank blood and/or other biological matrices
- DI H₂O, laboratory general-use and LC-MS grade (or equivalent from a high-purity filtration system)
- Elution solvent
To 20 mL isopropanol, add 2 mL concentrated NH₄OH and mix. Add 78 mL CH₂Cl₂ 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 DI H₂O in a 1 L flask. Dilute to 1 L with LC-MS grade DI H₂O and mix. Filter this solution prior to use on the HPLC. Store the solution in a glass bottle at room temperature for up to one year.

- Isopropanol (IPA)
- Methanol (MeOH)
- Methylene chloride (CH₂Cl₂, dichloromethane)
- 0.1M phosphate buffer (pH6)

Dissolve 1.7 g Na₂HPO₄ and 12.14 g NaH₂PO₄ • H₂O in 800 mL DI H₂O. Dilute to 1 L with DI H₂O 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 (Na₂HPO₄)
- Sodium phosphate, monobasic monohydrate (NaH₂PO₄ • H₂O)

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)
- Solvent filters (0.45 µm pore size; reduced cellulose, other)

38.3.3 EQUIPMENT

- Shimadzu HPLC, or equivalent
- Sciex API 3200 MS-MS, or equivalent
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipettes with disposable pipette tips
- General-use equipment (centrifuge, evaporator, pH meter/indicating paper, solvent filtration apparatus, vacuum manifold, vortex mixer)

38.4 STANDARDS, CALIBRATORS AND CONTROLS

38.4.1 STANDARDS

- Working standard: 1 ng/μL
- Working control standard: 1 ng/μL
- Working internal standard: 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 whole blood control and two positive whole 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.
- 38.4.3.4 Positive controls in both whole blood and/or alternate matrices may be used to bracket case specimens. When analyzing compounds in multiple matrices, both whole blood and alternate matrix controls apply towards 10% of the batch.

38.5 SAMPLE PREPARATION

NOTE: NFT-d₅ must be carefully evaluated against criteria for acceptance for specimens that contain ketamine (see 38.2 and 38.7).

NOTE: Laboratory general-use DI H₂O and reagent grade ACN are used in sample preparation. Reconstitution of extracts (38.5.21) uses LC-MS grade ACN and 0.1% formic acid prepared using LC-MS grade DI H₂O (or equivalent).

- 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 whole 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 0.1 mL of the working standard with 0.9 mL 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)
- Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
 - 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 working standard and the prepared dilutions.

Calibrator Description	Volume (μL) Added	Standard Concentration	Dilution of WS (or 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
Calibrator 6 - 50 ng/mL	25	1.0 ng/μL	WS

- 38.5.7 Prepare a 1:10 dilution of the control working standard. (0.1 ng/μL)
- Using a calibrated pipette, combine 0.1 mL of the control working standard with 0.9 mL of ACN or MeOH in a labeled tube.
 - Cap and vortex mix. This dilution shall be disposed of after control preparation.
- 38.5.8 Prepare a 1:100 dilution of the control working standard. (0.01 ng/μL)
- Using a calibrated pipette, combine 0.1 mL of the 1:10 dilution with 0.9 mL of ACN or MeOH in a labeled tube.
 - 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 control working standard and prepared dilution.

Control Description	Volume (μL) Added	Standard Concentration	Dilution of QC (or QC)
Low Control – 1.5 ng/mL	75	0.01 ng/μL	1:100
High Control – 40 ng/mL	20	1.0 ng/μL	QC

- 38.5.10 Using a calibrated pipette, sample 0.5 mL of each case sample 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.
- 3 mL MeOH
 - 3 mL DI H₂O
 - 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.)
- 3 mL DI H₂O
 - 2 mL 0.1M acetic acid
 - 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:LC-MS grade ACN). Briefly vortex-mix the tubes. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm 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 $\pm 3\%$, ion ratios $\pm 20\%$) – Cal 4
- Result comparisons – all units in ng/mL
- Cal 1: truncated to two decimal places (acceptable range 0.37 – 0.62 ng/mL)
Cal 2-3, Pos Ctl 1: truncated to one decimal place
Cal 4-6, Pos Ctl 2: truncated, whole integer values

38.7 REPORTING

Results for target compounds are reported in units of nanograms per milliliter (ng/mL), truncated to two significant figures for reporting.

Where interference with NFT-d₅ is observed in case specimens containing ketamine (see 38.2 and 38.5), norfentanyl is not reported.

38.8 METHOD PERFORMANCE

- Limit of detection: 0.1 ng/mL
- Lower limit of quantification: 0.5 ng/mL
- Dynamic range: 0.5 – 50 ng/mL
- Upper limit of quantitation: 50 ng/mL
- Upper limit of linearity: 150 ng/mL

38.9 REFERENCES

- Black, A. and B.E. O'Reilly, in-house method development
- Patel, N. Sharma, M. Sanyal, P. Shrivastav, An accurate, rapid and sensitive determination of tramadol and its active metabolite *o*-desmethyltramadol in human plasma by LC-MS/MS, *Journal of Pharmaceutical and Biomedical Analysis*, 49 (2009) 354-366.
- 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-d₅ (FEN-d₅)
 Norfentanyl
 Norfentanyl-d₅ (NFT-d₅)

APPENDIX B
 INSTRUMENTAL PARAMETERS

Shimadzu/Sciex LC-MSMS System

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid
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
Rinsing Volume	1000 uL
Flush-port solvent	75:25 MeOH:DI H ₂ O
Cooler Temperature	25°C

MASS SPECTROMETER

Scan type	(+) sMRM	Curtain/collision gas	Nitrogen
Ion mode	ESI	Curtain gas flow	30 L/min
Resolution (MS1)	Unit	Collision gas flow	6 L/min
Resolution (MS2)	Unit	Gas 1 Temp	70°C
Target Scan Time	0.5 sec	Gas 2 Temp	70°C
Time segment 1	To waste	Ion voltage	2.0 kV
Time segment 2 (all transitions)	To MS	Interface Temp	550°C
Time segment 3	To Waste		
Compound	MRM Transitions		
Fentanyl	337.1→ 188.3, 105.2		
Fentanyl-D ₅	342.1→ 137.3, 105.2		
Norfentanyl	233.1→ 84.2, 55.1		
Norfentanyl-D ₅	238.2→ 84.3, 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