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

38.1 POLICY

This test method may be used to confirm the presence of fentanyl (FEN) and metabolite norfentanyl (NFT) in biological samples. Reporting of results following the application of this method will be contingent upon a thorough review and acceptance of quality control data and the qualification of individual results under the criteria for acceptance.

Any adjustments or deviations from the procedures below must be approved by a member of TLD Management, and appropriately documented in the batch file.

38.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of fentanyl and norfentanyl present in biological specimens. This procedure will serve as the laboratory document describing sample preparation, instrumental analysis, data analysis, criteria for acceptance and reporting of the specified compounds.

38.3 PRINCIPLE

The targeted compounds and corresponding internal standards are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of solid-phase extraction (SPE). Following SPE, the specimens, now termed extracts, are injected into a high performance liquid chromatograph (HPLC) where they are separated between a liquid mobile and liquid stationary phase. Each compound exits the HPLC at a reproducible time which is termed its retention time.

The HPLC is coupled to a tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source. As each ionized compound is drawn into the high vacuum region of the instrument, selected-ion and multiple-reaction monitoring is used to measure the mass-to-charge ratios of each compound and its related fragments. Multiple-point, internal standard calibration is used to generate a calibration curve. The concentration of any target compound identified in a sample is determined from its calibration curve.

38.4 SPECIMENS

38.4.1 The specimen volume is 0.5 mL.

38.4.2 Specimens include whole blood, serum, plasma, urine, and tissue homogenate.

38.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; however, this should be done in addition to testing the standard specimen volume, unless sample quantity dictates otherwise.

38.4.4 Analysis of larger specimen volumes must be approved and documented.

38.5 REAGENTS, MATERIALS AND EQUIPMENT

38.5.1 REAGENTS

38.5.1.1 Acetic acid, glacial

38.5.1.2 0.1M acetic acid

Add 5.72 mL glacial acetic acid to 800 mL DI H_2O. Dilute to 1 L with DI H_2O and mix. Store in a glass bottle at room temperature for up to six months. Adjustments to final volume are permitted as long as proportions are maintained.

38.5.1.3 Acetonitrile (ACN)

38.5.1.4 Ammonium hydroxide (NH_4OH), concentrated

38.5.1.5 Certified blank blood

38.5.1.6 Deionized water (DI H_2O)

38.5.1.7 Elution solvent

To 20 mL isopropanol, add 2 mL concentrated NH_4OH and mix. Add 78 mL CH_2Cl_2 and mix. Store in glass flask/bottle at room temperature and use on date of preparation only. Adjustments to final volume are permitted as long as the proportions of the elution solvent are maintained.

38.5.1.8 Formic acid (concentrated)

38.5.1.9 0.1% Formic acid

Add 1 mL of concentrated formic acid to 800 mL DI H_2O in a 1 L flask. Dilute to 1 L with DI H_2O 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. Adjustments to final volume are permitted as long as the proportions are maintained.

38.5.1.10 Isopropanol (IPA)

38.5.1.11 Methanol (MeOH)

38.5.1.12 Methylene chloride (dichloromethane, CH_2Cl_2)

38.5.1.13 0.1M phosphate buffer (pH6)

Dissolve 1.7 g Na_2HPO_4 and 12.14 g NaH_2PO_4 in 800 mL DI H_2O. 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. Adjustments to final volume are permitted as long as the proportions are maintained.

38.5.1.14 Sodium hydroxide (NaOH), concentrated

38.5.1.15 Sodium phosphate, dibasic anhydrous (Na_2HPO_4)

38.5.1.16 Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \bullet H_2O$)

38.5.2 MATERIALS

38.5.2.1 Autosampler vials, inserts and caps

38.5.2.2 Disposable 16 x 100mm tubes with closures

38.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

38.5.2.4 Disposable pipette tips

38.5.2.5 Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200mg/6mL), or equivalent

38.5.2.6 HPLC Column, Agilent Poroshell 120 EC-C18, 2.1x75 mm, 2.7µM particle size, or equivalent

38.5.2.7 Laboratory glassware (graduated cylinders, flasks)

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

38.5.2.9 Volumetric glassware (flasks)

38.5.3 EQUIPMENT

38.5.3.1 Shimadzu HPLC, or equivalent

38.5.3.2 Sciex API 3200 MS-MS, or equivalent

38.5.3.3 Calibrated, adjustable piston pipettes

38.5.3.4 Centrifuge

38.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

38.5.3.6 pH Meter and/or indicating pH paper

38.5.3.7 Solvent filtration apparatus

38.5.3.8 Vortex mixer

38.5.3.9 Vacuum manifold

38.6 STANDARDS, CALIBRATORS AND CONTROLS

38.6.1 STANDARDS

38.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of working standards which in turn are used to produce calibrators, positive controls and the working internal standard.

38.6.1.2 Stock standards and stock internal standards are purchased from an approved reference material supplier and include the following:

a. Fentanyl: 1.0 mg/mL
b. Fentanyl-D_5: 0.1 mg/mL
c. Norfentanyl oxalate: 1.0 mg/mL
d. Norfentanyl-D_5: 0.1mg/mL

38.6.1.3 Working standard (1.0 ng/µL)

a. Using a calibrated pipette, measure 25 µL of each stock standard into a 25 mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working standard is 10 ng/µL. The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

38.6.1.4 Working internal standard (0.1 ng/µL)

a. Using a calibrated pipette, measure 50 µL of each stock internal standard into a 50-mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working internal standard is 0.1 ng/µL. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation (if a CRM expires prior to one year from date of preparation, the prepared solution expiration date becomes the first day of the month in which the CRM with the earliest expiration date expires). Volumes may be adjusted, provided that proportions remain constant.

38.6.2 CALIBRATORS

38.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 38.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure.

38.6.3 CONTROLS

38.6.3.1 Negative Control

a. At least one negative whole blood control is tested with every batch. The negative control is prepared using certified blank blood.
b. When testing different sample types, wherever possible, include a negative control prepared from that matrix. (For example, when analyzing whole blood and urine samples the batch shall include at least one negative whole blood control and at least one negative urine control.)

38.6.3.2 Positive Controls

a. Two positive whole blood controls are tested with every batch. The positive controls are prepared using certified blank blood to which the designated volume of control working standard has been added.
b. Control stock standards are obtained from an approved reference material supplier.
c. The control stock standards must be either a different lot number or from a different supplier to those used in producing the working standard. If the same lot must be used, the working control standard must be prepared by someone other than the person that prepared the working standard.
d. The control working standard (1.0 ng/µL) is prepared as described in 38.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 38.7 SAMPLE PREPARATION. Alternatively, quality assurance personnel may provide in-house positive controls.
f. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix.

38.7 SAMPLE PREPARATION

NOTE: The presence of ketamine in a specimen may cause interference with norfentanyl-d_5 internal standard, affecting chromatography and transition ratios.

38.7.1 Label a clean 16 x 100mm tube for each member of the test batch. (i.e. Calibrator, control, case sample).

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

38.7.3 Add 0.5 mL of certified blank whole blood into each of the six calibrator tubes, the positive control tubes and the negative control tube(s).

38.7.4 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.

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

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

38.7.6 Using the working standard and the prepared dilutions, spike the calibrators according to the following table.

Calibrator Description	Volume (µL) Added	Working Standard
Calibrator 1 – 0.5 ng/mL	25	0.01 ng/µL
Calibrator 2 – 1.0 ng/mL	50	0.01 ng/µL
Calibrator 3 – 5.0 ng/mL	25	0.1 ng/µL
Calibrator 4 - 10 ng/mL	50	0.1 ng/µL
Calibrator 5 - 25 ng/mL	125	0.1 ng/µL
Calibrator 6 - 50 ng/mL	25	1.0 ng/µL

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

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

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

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

38.7.9 Using the control working standard and prepared dilution, spike the positive controls according to the following table.

Control Description	Volume (µL) Added	Control Working Standard
Low Control – 1.5 ng/mL	75	0.01 ng/µL
High Control – 40 ng/mL	20	1.0 ng/µL

38.7.10 If in-house positive controls are being used, transfer 0.5 mL of each into their labeled tubes.

38.7.11 Sample 0.5 mL of each case sample into its respective tube.

38.7.12 Add 50 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 10 ng/mL.

38.7.13 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500rpm.

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

38.7.15 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.

38.7.16 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.)

38.7.17 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

38.7.18 Dry the columns for 10 minutes under vacuum.

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

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

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

38.7.22 Reconstitute the extracts with the addition of 50 µL mobile phase (95:5 0.1% formic acid: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.

38.7.23 Transfer the extracts to labeled polypropylene autosampler vials and cap.

38.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a batch worklist and set the data file/path in Analyst to the date of the test. After entering all vial locations, sample descriptions, comments and/or lot numbers in the sequence table ensure that the method listing in the table is Fentanyl.dam (Shimadzu/Sciex) for each line. As needed, the sequence may conclude with an injection that rinses the column (e.g., using method RINSE.dam), or this may be done manually.

38.9 DATA ANALYSIS

38.9.1 Analysis of the batch data is conducted using MultiQuant quantitative instrumental data analysis software.

38.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression with a 1/a (inverse of concentration) weighting factor. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

38.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves (reports do not need to be generated for batch entries added for column rinse or shutdown at the conclusion of acquisition).

38.9.4 Technical review of the batch is conducted according to the criteria listed below.

38.10 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

38.10.1 CALIBRATORS AND CALIBRATION CURVES

38.10.1.1 Chromatographic peaks for target compounds and internal standards shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

38.10.1.2 Retention times shall be within ±3% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.

38.10.1.3 Quantitative results for target compounds in each calibrator shall be within ±20% of their target values with the exception of calibrator 1 which shall be within ±25% of their targets. These are inclusive ranges. For calibrator 1, result comparisons will use two decimal places (as seen on report), for calibrators 2-3, comparisons will use results truncated to one decimal place, and for calibrators 4-6, comparisons will use the whole integer, truncated results in units of ng/mL.

38.10.1.4 The calibration curves for target compounds shall have a correlation coefficient ≥0.99.

38.10.1.5 The failure to meet any of these criteria for one compound does not invalidate the acceptability of the other compound.

38.10.2 CONTROLS

38.10.2.1 The negative control(s) shall not identify target compounds above their limit of detection. Identification is based on a) acceptable retention time matching, b) distinct peaks present for all selected ions, and c) acceptable ion ratios.

38.10.2.2 Positive controls

a. Chromatographic peaks for target compounds and internal standards shall appear symmetrical.
b. Retention times shall be within ±3% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.
c. Quantitative results for target compounds in each control shall be within ±20% of their target values. These are inclusive ranges. For the low control, comparisons will use the results truncated to one decimal place, and for the high control, comparisons will use the whole integer, truncated results in units of ng/mL.
d. At least one positive control must meet these criteria for both compounds for the batch to be accepted.

38.10.2.3 The failure to meet any of these criteria for one compound does not invalidate the acceptability of the other compound.

38.11 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

38.11.1 Any chromatographic peak for target compounds shall appear symmetrical.

38.11.2 The retention times for target compounds are ±3% and the ion ratios are within ±20% of those in calibrator 4. These are inclusive ranges.

38.11.3 The quantitative results for target compounds must be within the dynamic range of the test method.

38.11.4 When dilutions of case samples are tested, the quantitative result(s) before multiplication shall be within the dynamic range of the test method.

38.12 REPORTING

38.12.1 Results for target compounds are reported in units of nanograms per milliliter (ng/mL).

38.12.1.1 Results are truncated to two significant figures for reporting.

a. Example 1: fentanyl is measured as 3.75 ng/mL.

b. The result is truncated to 3.7 ng/mL (two significant figures) and reported.

38.12.2 When multiple dilutions are analyzed, the smallest dilution within the dynamic range is reported.

38.13 METHOD PERFORMANCE

38.13.1 Limit of detection: 0.1 ng/mL

38.13.2 Lower limit of quantification: 0.5 ng/mL

38.13.3 Dynamic range: 0.5 – 50 ng/mL

38.13.4 Upper limit of quantitation: 50 ng/mL

38.13.5 Upper limit of linearity: 150 ng/mL

38.14 TRACEABILITY

Traceability of the reference materials to SI units is provided through the certificate of analysis provided by the approved reference material supplier.

38.15 REFERENCES

38.15.1 A. Black and B.E. O'Reilly, in-house method development.

APPENDIX A
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
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	1000uL
Flush-port solvent	75:25 MeOH:DI H_2O
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 (Time 0)	To waste	Ion voltage	2.0 kV
Time segment 2 (Time 3.0 min)	To MS	Interface Temp	550°C
Time segment 3 (Time 8.0 min)	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