FENTANYL SCREEN BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

48.1 METHOD

This test method may be used to identify the presence of fentanyl (FEN) in biological specimens. The target compound and deuterated internal standard are isolated from whole blood, serum, plasma or other submitted biological specimens through precipitation of blood with acetonitrile. The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a tandem mass spectrometer (MS-MS) detector equipped with an Agilent jet stream (AJS) atmospheric pressure electrospray ionization source.

48.2 SPECIMENS

The specimen volume is 0.2 mL. For any matrix other than whole blood (serum, tissue homogenate) matrix matching of all calibrators and controls is required. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

NOTE: During validation it was determined that isobutyrl fentanyl and acetyl fentanyl have the same retention time as fentanyl. Acryl fentanyl elutes just prior to fentanyl, and exhibits an ion ration failure.

48.3 REAGENTS, MATERIALS AND EQUIPMENT

48.3.1 REAGENTS

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

- Acetonitrile (ACN), reagent grade and LC-MS grade
- 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)
- 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.
- Methanol (MeOH), reagent grade and HPLC grade

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

48.3.2 MATERIALS

- Polypropylene autosampler vials with integrated inserts and caps

- Disposable extraction tubes (12 x 75 mm recommended) and screw-cap or centrifuge tubes with closures
- HPLC Column, Agilent Zorbax RRHD Eclipse Plus C18, 2.1 x 50 mm, 1.8 µM particle size, or equivalent
- Laboratory glassware (graduated cylinders, flasks)

48.3.3 EQUIPMENT

- Agilent HPLC 1200 series or equivalent
- Agilent MS-MS with AJS source, (Ultivo or equivalent)
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipettes with disposable pipette tips
- General-use equipment (centrifuge, evaporator, vortex mixer)

48.4 STANDARDS, CALIBRATORS AND CONTROLS

48.4.1 STANDARDS

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

48.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 48.5 SAMPLE PREPARATION. For analysis of alternate matrices, all calibrators are prepared in that matrix.

48.4.3 CONTROLS

48.4.3.1 At least one negative blood control and three positive blood controls are tested with every batch, prepared as described in 48.5. For analysis of alternate matrices, all controls (positive and negative) are prepared in that matrix.

48.4.3.2 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 20 or more specimens, a positive control must be analyzed mid-run.

48.4.3.3 When analyzing compounds in matrices other than blood, 48.4.3.2 is duplicated for those matrices.

48.5 SAMPLE PREPARATION

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

48.5.2 Using a calibrated pipette, add 0.2 mL of certified blank blood into each of the calibrator tubes, the positive control tubes and the negative control tube(s).

48.5.3 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.

48.5.4 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.

48.5.5 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	10	0.01 ng/µL	1:100
Calibrator 2 – 1.0 ng/mL	20	0.01 ng/µL	1:100
Calibrator 3 – 10 ng/mL	20	0.1 ng/µL	1:10

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

a. Using a calibrated pipette, combine 100 µL of the control 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 control preparation.

48.5.7 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

48.5.8 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	30	0.01 ng/µL	1:100
Mid Control – 8 ng/mL	16	0.1 ng/µL	1:10
High Control – 25 ng/mL	50	0.1 ng/µL	1:10

48.5.9 Using a calibrated pipette, sample 0.2 mL of each case specimen into its respective tube.

48.5.10 Using a calibrated pipette or verified repeater-pipette, add 20 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 10 ng/mL.

48.5.11 Using a calibrated pipette or verified repeater-pipette, add 800 µL of the acetonitrile to each tube.

48.5.12 Cap the tubes and briefly vortex mix for at least 15 seconds. Centrifuge the tubes for 10 minutes at 3500rpm.

48.5.13 Decant the supernatant to a conical centrifuge tube, and evaporate the extracts to dryness at 40°C.

48.5.14 Reconstitute the extracts with the addition of 50 µL 0.1% formic acid in LC-MS grade H_2O. Briefly vortex-mix the tubes. Centrifuge the tubes for 10 minutes at 3500 rpm to collect the extracts at the bottom of the tubes.

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

48.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – FenScreen (instrumental parameters in Appendix B)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±3%, ion ratios ±20%) – Cal 3
- 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, Pos Ctl 1: truncated to one decimal place (acceptable range ±20%)

  Cal 3, Pos Ctl 2-3: truncated, whole integer values (acceptable range ±20%)

48.7 REPORTING

Results at or above the cutoff concentration (Cal 1) are reported as positive for all matrices, provided that the specimen results meet all criteria for acceptance (e.g., retention time, chromatography, transition ratios), including the results > Cal 3 concentrations (10 ng/mL).

48.8 METHOD PERFORMANCE

- Limit of detection: 0.05 ng/mL
- Lower limit of qualitative reporting: 0.5 ng/mL
- Dynamic range: 0.5 – 10 ng/mL

48.9 REFERENCES

- D. Sklerov, in-house method development
- S.Marin, J. Hughes, B. Lawlor, C. Clark, G. McMillin, Rapid Screening for 67 Drugs and Metabolites in Serum or Plasma by Accurate-Mass LC-TOF-MS, J Anal Tox. 36: 477-486 (2012).
- WSP Toxicology Fentanyl confirmation method TCf12738_Fentanyl LCMSMS_Rev 3_20200516.

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

Fentanyl (FEN)
Fentanyl-d_5 (FEN-d_5)

APPENDIX B
INSTRUMENTAL PARAMETERS

Agilent LC-(AJS) MSMS System

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 – 1 min	5% B
1 – 4.5 min	30% B
4.5 – 5.0 min	95% B
5.0 – 5.5 min	5% B
5.5 – 7.0 min	5% B
Hold to 8 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	EMV	Set in tune
Time filter width	0.07 min	Capillary voltage	3.5kV
Resolution	Unit	Nebulizer pressure	40 psi
Sheath gas flow	12 L/min	Drying/sheath gas	Nitrogen
Sheath gas temp	400° C	Drying gas flow	11 L/min
Nozzle voltage	2000V	Drying gas temp	350° C

Compounds	MRM Transitions
Fentanyl	337.2 → 105, 188
Fentanyl-d5	342.1 → 105.1, 188.1

LIST OF CHANGES

Revision Date	Description	Page Number
4/16/2021	Method approved by Washington State Toxicologist. See DRA dated 3/23/2021. Method released for use in evidentiary testing on 4/16/2021.	All
4/10/2023	Removed urine matrix from acceptable matrices to run on this assay in 48.1 and 48.2. Updated 48.4.3.2 to change requirement for mid-run control from more than 20 specimens to 20 or more specimens.	1, 2