

TENNESSEE BUREAU OF INVESTIGATION

Forensic Services Division

Toxicology Quality Assurance and Procedures Manual

8.10 Opioid Procedure (via LC/MS/MS)



8.10 OPIOIDS PROCEDURE (VIA LC/MS/MS)

8.10.1 Purpose

To quantitatively and/or qualitatively identify opioid related compounds in submitted evidence using a solid phase extraction column followed by instrumental analysis with liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS).

8.10.2 Specimen Requirements

Samples for this analysis shall be presumptively screened positive by either ELISA or GC/MS. There are instances of some opioid compounds that do not fall within either of these current screen capabilities (i.e. Naloxone). For these compounds it is acceptable to proceed with the analysis and two separate tests would be required for confirmation and reporting purposes. Acceptable samples for analysis include blood and/or urine with blood calibrators and controls. All other matrices will either need to have matrix match calibrators and controls or see alternative Matrices policy (section 6.6).

8.10.3 Apparatus and Equipment

- Disposable 10 mL culture tubes
- UCT Clean Screen XCEL I 130 mg/10 mL SPE Columns
- Disposable transfer pipettes
- Volumetric pipettes with disposable tips
- Assorted volumetric glassware
- Sample mixer
- Centrifuge
- Vacuum or positive pressure manifold
- Evaporation station
- 11 mm auto sampler vials, inserts, and caps
- 11 mm crimper
- LC/MS/MS Analyst compatible components

8.10.4 Reagents and Standards

- UCT Phosphate Buffer Pouches (disodium hydrogen phosphate/sodium dihydrogen phosphate)
- Disodium Hydrogen Phosphate (optional)
- Sodium Dihydrogen Phosphate (optional)
- 100 mM Phosphate Buffer
- Negative blood/urine or other matrix as needed
- Reference standards
- DI Water (H₂O)
- Methanol (CH₃OH)
- Acetonitrile (ACN) (CH₃CN)
- Glacial Acetic acid (GAA)(CH₃COOH)
- Isopropanol (IPA)((CH₃)₂CHOH)
- Ammonium Hydroxide (NH₄OH)



TENNESSEE BUREAU OF INVESTIGATION

Forensic Services Division

Toxicology Quality Assurance and Procedures Manual

8.10 Opioid Procedure (via LC/MS/MS)

Methylene Chloride (CH₂Cl₂)
1 M Ammonium Formate (NH₄HCO₂)
Formic Acid (CHO₂H)
Opioid Elution Solution ((CH₃)₂CHOH)/NH₄OH/CH₂Cl₂)
Mobile Phase A (99.6% HPLC suitable DI water, 0.2% 1 M ammonium formate, 0.2% formic acid)
Mobile Phase B (97.6% HPLC grade or equivalent methanol, 2% HPLC suitable DI water, 0.2% 1 M ammonium formate, 0.2% formic acid)
Reconstitution Solution (Mobile Phase A:Mobile Phase B 80:20)

8.10.5 Standard and Reagent Preparation

The following are examples of how to prepare the standards and reagents used in this procedure.

8.10.5.1 Standards

Stock Reference Standard Solution (1)

hydrocodone, morphine, oxycodone, codeine, and dihydrocodeine [2500 ng/mL] & fentanyl, hydromorphone, oxymorphone, 6-monoacetylmorphine, buprenorphine, norbuprenorphine, and naloxone [250 ng/mL]

Pipette 250 µL of each hydrocodone, morphine, oxycodone, codeine, and dihydrocodeine [1 mg/mL] certified reference standard and 25 µL of each fentanyl, hydromorphone, oxymorphone, 6-monoacetylmorphine, buprenorphine, norbuprenorphine, and naloxone [1 mg/mL] certified reference standard and dilute to 100 mL with acetonitrile.

Stock Reference Standard Solution (2)

hydrocodone, morphine, oxycodone, codeine, and dihydrocodeine [250 ng/mL] & fentanyl, hydromorphone, oxymorphone, 6-monoacetylmorphine, buprenorphine, norbuprenorphine, and naloxone [25 ng/mL]

Pipette 10 mL of Stock Reference Standard Solution 1 and dilute to 100 mL with acetonitrile.

Stock Reference Standard Control Solution

hydrocodone, morphine, oxycodone, codeine, and dihydrocodeine [2500 ng/mL] & fentanyl, hydromorphone, oxymorphone, 6-monoacetylmorphine, buprenorphine, norbuprenorphine, and naloxone [250 ng/mL]

Pipette 250 µL of each hydrocodone, morphine, oxycodone, codeine, and dihydrocodeine [1 mg/mL] certified reference standard and 25 µL of each fentanyl, hydromorphone, oxymorphone, 6-monoacetylmorphine, buprenorphine,



TENNESSEE BUREAU OF INVESTIGATION

Forensic Services Division

Toxicology Quality Assurance and Procedures Manual

8.10 Opioid Procedure (via LC/MS/MS)

norbuprenorphine, and naloxone [1 mg/mL] certified reference standard and dilute to 100 mL with acetonitrile.

Internal Standard Reference Solution

buprenorphine D4, morphine D3, and oxycodone D6) [100 ng/mL]

Pipette 100 μ L of each buprenorphine D4, morphine D3, and oxycodone D6 [100ug/mL] certified reference standard and dilute to 100 mL with acetonitrile.

Note: All reference standard solutions expire 6 months from the preparation date and shall be stored in the freezer when not in use.

Working Reference Calibrator/Control Standards Solution

To make the working reference calibrator/control standard solutions, add the following amounts to a final volume of 500 μ L with blood or urine.

CONCENTRATIONS	AMOUNT USED	STANDARD SOLUTION
1 ng/mL & 10 ng/mL	20 μ L	Stock Reference Solution 2
2 ng/mL & 20 ng/mL	40 μ L	Stock Reference Solution 2
5 ng/mL & 50 ng/mL	100 μ L	Stock Reference Solution 2
10 ng/mL & 100 ng/mL	20 μ L	Stock Reference Solution 1 AND Stock Reference Control Solution
20 ng/mL & 200 ng/mL	40 μ L	Stock Reference Solution 1
35 ng/mL & 350 ng/mL	70 μ L	Stock Reference Solution 1

8.10.5.2 Prepared Chemicals

Methanol/Water 1:1 (needle rinse)

Add 500 mL of methanol to a volumetric flask and dilute to 1000 mL with H₂O.

1 M Ammonium Formate

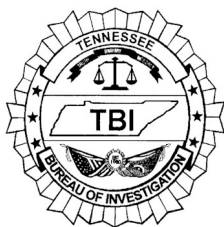
Dissolve 63 g of ammonium formate and dilute to 1000 mL with H₂O.

Mobile Phase A

Add 2 mL 1 M ammonium formate and 2 mL formic acid to H₂O and dilute to 1000 mL with H₂O.

Mobile Phase B

Add 2 mL 1 M ammonium formate and 2 mL formic acid to 20 mL H₂O and dilute to 1000 mL with methanol.



TENNESSEE BUREAU OF INVESTIGATION

Forensic Services Division

Toxicology Quality Assurance and Procedures Manual

8.10 Opioid Procedure (via LC/MS/MS)

100 mM Buffer Phosphate

Option 1: Fill a 1000 mL volumetric flask half full of DI H₂O. Then mix 1 pouch of phosphate buffer into the flask and bring to a final volume of 1000 mL with H₂O.

Option 2: Fill a 1000 mL volumetric flask half full of DI H₂O. Then add 1.7 g of disodium hydrogen phosphate and 12.14 g sodium dihydrogen phosphate into the flask and bring to a final volume of 1000 mL with DI H₂O. The pH of the buffer solution should be approximately 6.0.

2% Acetic Acid

Add 40 mL of glacial acetic acid to a volumetric flask and dilute to 2000 mL with methanol.

Opioid Elution Solution

First, thoroughly mix 20 mL isopropanol and 2 mL ammonium hydroxide, and then add 78 mL of methylene chloride into the solution. Prepare on day of extraction.

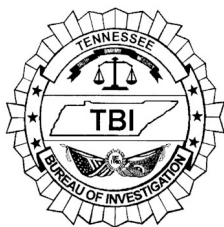
Note: If bubbles develop or separate layers form in the mixture after adding the methylene chloride, dispose of the solution and remake it.

Reconstitution Solution (80% Mobile Phase A and 20% Mobile Phase B)

Mix 80 mL Mobile Phase A and 20 mL Mobile Phase B.

8.10.6 Procedure

1. Allow all stock reference standards and case samples to equilibrate to room temperature before beginning procedure.
2. Label, check, and load/unload all samples in accordance with the "Sample Pipetting Check List" (see Appendix section).
3. Prepare working reference calibrator and/or control standards from reference standard solutions. See the example above.
4. Pipette 500 μ L of corresponding case sample, calibrator, positive control, or negative control into the appropriately labeled 10 mL culture tubes. Note: Smaller sample volumes may be analyzed on a case-by-case basis.
5. Pipette 100 μ L of internal standard into each sample to make a final concentration of 20 ng/mL.
6. Add 1 mL of 100 mM phosphate buffer, mix/vortex (caution when vortexing) for a minimum of 30 seconds, and let stand (incubate) for a minimum of 10 minutes.
7. After at least ten minutes add an additional 2 mL of 100 mM phosphate buffer and mix/vortex (caution when vortexing) for a minimum of 30 seconds.
8. Centrifuge at the maximum rpm level possible for 10 minutes or more. Note: if emulsion layer is present vortex and centrifuge again for a minimum of 5 minutes.
9. Decant the supernatant into labeled UCT Clean Screen XCEL I SPE Columns.
10. Using the positive pressure manifold pull the samples through the sorbent bed of the columns at a rate of 1- 2 mL per minute (2-4 psi).
11. Dry the SPE columns for a minimum of 1 minute at full positive pressure.
12. Wash the SPE column with 3 mL of DI H₂O at full positive pressure. Note: do not allow the column sorbent beds to become dry.
13. Wash the SPE column with 3 mL of 2% acetic acid at full positive pressure.



TENNESSEE BUREAU OF INVESTIGATION

Forensic Services Division

Toxicology Quality Assurance and Procedures Manual

8.10 Opioid Procedure (via LC/MS/MS)

14. Dry the SPE column thoroughly under full positive pressure for a minimum of 10 minutes.
15. Switch the solid phase extraction apparatus to clean labeled 10 mL culture tubes.
16. Add 3 mL of the elution solution to each SPE column and allow the samples to elute slowly. Gravity should be sufficient for this but should be followed by pressure at a 1-2 mL/minute rate (2-4 psi) after the majority of the eluent has passed through the sorbent bed of the SPE column.
17. Gently evaporate the samples to dryness with heat at approximately 50° C or less and a drying gas (e.g. Nitrogen) in an evaporation station (approximately 30 minutes).
18. Reconstitute the residue with 75 μ L of 80:20 Mobile Phase A to Mobile Phase B, mix/vortex, and centrifuge until separated at the maximum rpm level possible for approximately 10 minutes.
19. Transfer to 11 mm auto sample vial with insert; attempt to avoid transfer any of the particulate matter in the bottom of the tube, and seal with cap.
20. Analyze the samples by LC/MS/MS.

Note: Extracted and reconstituted samples are stable for analysis up to four days in 11 mm auto sampler vials refrigerated or at room temperature.

8.10.7 Reporting

8.10.7.1 Qualitative

8.10.7.1.1 Retention times of drugs identified and internal standards must fall within $\pm 2\%$ of calibrator or control standards.

Note: Some drug retention times are concentration dependent and a comparison of $\pm 2\%$ to a calibrator used in the calibration curve or control standard of similar concentration shall be acceptable.

8.10.7.1.2 Multiple reactions monitoring (MRM) ion ratios must fall within $\pm 20\%$ of the calibrators or control standard. If a calibration point is removed then the ion ratio range shall be recalculated from the calibrators used to establish the curve.

Note: Some drug MRM ion ratios are concentration dependent and a comparison of $\pm 20\%$ to a calibrator used in the curve or control standard of similar concentration shall be acceptable.

8.10.7.1.3 If the control standard concentration is outside the expected value, the drug may be reported as “positive” if the retention time criteria and ion ratio criteria are met.

If the control standard concentration is within the expected value range, the drug shall not be reported if the retention time criteria and/or ion ratio criteria are not met.

TENNESSEE BUREAU OF INVESTIGATION

Forensic Services Division



Toxicology Quality Assurance and Procedures Manual

8.10 Opioid Procedure (via LC/MS/MS)

8.10.7.1.4 Drug concentrations in casework may be reported as “positive” if the drug response ratio (i.e., area of drug/area of internal standard) is equal to or greater than the drug response ratio of the lowest calibrator used to establish the calibration curve.

8.10.7.1.5 Naloxone may be stated as presumptively positive and contact laboratory if further testing is needed on the case report if the sample is only analyzed once. Otherwise, Naloxone shall require two separate sample extractions for confirmation and /or quantitation.

Note: When o-desmethyltramadol is found in the Basic Extraction of a case sample and dihydrocodeine is found in the opioid extraction of a case sample, dihydrocodeine shall be reported as positive due to ion suppression.

8.10.7.2 Quantitative

8.10.7.2.1 All of the qualitative result criteria above must be met.

8.10.7.2.2 Sample drug concentrations greater than the highest calibrator level where results are not necessary for interpretation in the case may be reported as “greater than” The highest calibrator level.

Note 1: Drug concentrations of 300 ng/mL for 6-monoacetylmorphine, Buprenorphine, Fentanyl, Hydromorphone, Naloxone, Norbuprenorphine, and Oxymorphone produced no carryover using this procedure.

Note 2: Drug concentrations of 3000 ng/mL for Codeine, Dihydrocodeine, Hydrocodone, Morphine, and Oxycodone produced no carryover using this procedure.

8.10.7.2.3 Sample drug concentrations greater than the highest calibration level where the results are necessary for interpretation in the case shall be reanalyzed with a smaller sample amount (including dilution factor). When diluting a sample for reanalysis, the same matrix shall be used as the sample matrix for the diluent.

If the lowest calibrator levels are removed from the calibration curve, this change in calibration levels may require a repeat analysis of case specimens with calibration levels at the method LOQ when necessary for interpretation in a case.

8.10.7.2.4 Some opioid drug calibrations in this procedure are non-linear and the use of quadratic calibration models is appropriate. A weighted linear (1/x) model shall be used for buprenorphine, norbuprenorphine, naloxone, fentanyl, morphine, and oxymorphone. A weighted quadratic (1/x) model shall be used for hydromorphone, 6-monoacetylmorphine, oxycodone, hydrocodone, dihydrocodeine, and codeine. For the use of quadratic models, see section 6.4 Calibrators and Controls.



TENNESSEE BUREAU OF INVESTIGATION

Forensic Services Division

Toxicology Quality Assurance and Procedures Manual

8.10 Opioid Procedure (via LC/MS/MS)

8.10.7.3 Results

8.10.7.3.1 Any qualitative or quantitative report data not used in a case shall either be lined through and initialed or all the data used shall be highlighted.

8.10.7.3.2 Opiates, Fentanyl, and Buprenorphine drugs that have been previously reported as ELISA “presumptively not detected” do not need to be restated as negative on the report from the confirmatory test (ie Fentanyl not detected).

Opiates, Fentanyl and Buprenorphine drugs that have been previously tested as ELISA positive and have a drug concentration below the lowest calibrator level shall be reported as either no opiates detected, no fentanyl detected, and/or no buprenorphine detected.

8.10.7.3.3 Qualitative results shall be expressed as “positive” and include any clarifying remarks, if applicable.

8.10.7.3.4 Quantitative results shall be reported in ng/mL and truncated to the whole number.

8.10.7.3.5 When a definitive conclusion cannot be made, the reason shall be documented on the report (e.g., “insufficient sample for analysis”, “sample unsuitable for analysis”, “results are inconclusive due to sample condition”, etc.).

8.10.8 References:

1. L. K. Nahar, R. Andrews, and S. Paterson, “Validated Method for the Quantification of Buprenorphine in Postmortem Blood Using Solid-Phase Extraction and Two-Dimensional Gas Chromatography-Mass Spectrometry,” *Journal of Analytical Toxicology* (2015): 1-7
2. R. B. Dixon, F. Mbeunkui, and J. V. Weigel, “Stability Study of Opioids and Benzodiazepines in Urine Samples by Liquid Chromatography Tandem Mass Spectrometry,” *Journal of Analytical Science and Technology* (2015) 6:17
3. A. E. Steuer, A. Forss, A. M. Dally, and T. Kraemer, “Method Development and Validation for Simultaneous Quantification of 15 Drugs of Abuse and Prescription Drugs and & of their Metabolites in Whole Blood Relevant in the context of Driving Under the influence of Drugs—Usefulness of Multi-Analyte Calibration,” *Forensic Science International* 244 (2014) 92-101
4. S. Valtier, R. L. Mueck, and V. S. Bebart, “Quantitative Method for Analysis of Hydrocodone, Hydromorphone, and Norhydrocodone in Human Plasma by Liquid Chromatography-Tandem Mass Spectrometry,” *Journal of Chromatography B*. 925 (2013) 40-45
5. W. B. Fang, M. R. Lofwall, S. L. Walsh, and D. E. Moody, “Determination of Oxycodone, Noroxycodone, and Oxymorphone by High-Performance Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry in Human Matrices: In vivo and In vitro Applications,” *Journal of Analytical Toxicology* 2013;37:337-344

TENNESSEE BUREAU OF INVESTIGATION

Forensic Services Division

Toxicology Quality Assurance and Procedures Manual

8.10 Opioid Procedure (via LC/MS/MS)



6. S. Valtier, and V. S. Bebarta, "Excretion Profile of Hydrocodone, Hydromorphone, and Norhydrocodone in Urine Following Single Dose Administration of Hydrocodone to Healthy Volunteers," *Journal of Analytical Toxicology* 2012;36:507-514
7. N. B. Tiscione, X. Shan, I. Alford and D. T. Yeatman, "Quantitation of Opioids in Whole Blood by Election Impact-Gas Chromatography-Mass Spectrometry," *Journal of Analytical Toxicology* Vol.35, March 2011
8. R. Coles, M. M. Kushnir, G. J. Nelson, G. A. McMillin, and F. M. Urry, "Simultaneous Determination of Codeine, Morphine, Hydrocodone, Hydromorphone, Oxycodone, and 6-Acetylmorphine in Urine, Serum, Plasma, Whole, and Meconium by LC-MS-MS," *Journal of Analytical Toxicology*, Vol. 31, January/February 2007
9. J. D. Roper-Miller, M. K. Lambing, and R. E. Winecker, "Simultaneous Quantitation of Opioids in Blood by GC-EI-MS Analysis Following Deproteinization, Detautomerization of Keto Analytes, Solid-Phase Extraction, and Trimethylsilyl Derivatization," *Journal of Analytical Toxicology*, Vol. 26, October 2002
10. Agilent. "Comprehensive LC/MS Analysis of Opiates, Opioids, Benzodiazepines, Amphetamines, Illicits, and Metabolites in Urine." <https://www.agilent.com>, Agilent, 5 Aug. 2014, www.agilent.com/cs/library/applications/5991-1667EN.pdf. Publication Part Number: 5991-1667EN
11. Agilent. "Determination Of Opioids, Cocaine, and Cocaine Metabolites by Liquid Chromatography Mass Spectrometry Using ZORBAX Eclipse Plus C18 Columns." Search | Agilent, Agilent, 11 Dec. 2006, www.agilent.com/en-us/library/applications?N=129&Ntt=opiods&redirect=0. Publication Part Number: 5989-5906EN
12. Schlueter, Scott A., et al. "Determination of Opiates and Metabolites in Blood Using Electrospray LC/MS." Search | Agilent, Agilent, 13 Jan. 2017, www.agilent.com/en-us/library/applications?N=129&Ntt=opiates&redirect=0. Publication Part Number: 5988-4805EN
13. UCT. "EXTRACTION OF OPIATES FROM BLOOD/PLASMA/SERUM/URINE OR TISSUE USING CLEAN SCREEN XCEL ® I COLUMN." sampleprep.unitedchem.com, UCT United Chemical, sampleprep.unitedchem.com/media/at_assets/tech_doc_info/OPIATES_IN_BLOOD-XCEL_I.pdf.