4.1.5 LC/MS/MS - Drugs of Abuse (LCDOA) Confirmatory Analysis in Blood

4.1.5.1 Analytes

See toxicology scope of analysis in Toxicology Procedure Manual 1.14

4.1.5.2 Specimen Requirements

500 µL blood

4.1.5.3 General Description of Method

A confirmatory analysis for drugs in blood using liquid chromatography/tandem mass spectrometry

4.1.5.4 Equipment and Reagents

- QTRAP 4500 or LC-MS/MS equipped with validated column
- 11 mm autosampler vial with conical insert and appropriate caps
- 16 x 100 mm culture tube
- Strata X-Drug B Solid Phase Extraction Columns
- The usual assortment of laboratory glassware, pipettes, vortexers, centrifuges, extraction manifolds, vacuum pumps and turbovaps
- LC-MS grade methanol
- Reagent grade, or higher, acetonitrile
- High Purity formic acid
- LCMS grade water
- Certified reference materials, reference materials
- Mobile phase A, 0.1% formic acid in water
- Mobile phase B, 0.1% formic acid in methanol

4.1.5.5 Sample Preparation

A homogeneous blood sample is assured by gently rocking the specimen on the Labquake Shaker for at least 5 minutes. If the specimen is clotted, homogenizing glassware can be used to obtain a liquid sample. All sample handling will be performed in the biological safety cabinet using the universal biohazard handling techniques.

4.1.5.5.1 Unknowns

- Pipette 500 µL of sample into appropriately labeled 16 x 100 mm culture tube

4.1.5.5.2 Standards and Controls

- Pipette 500 µL of blank blood and 20 µL of the following methanol and acetonitrile standards and controls into appropriately labeled 16 x 100 culture tube:
  - Standards 1-6
  - Low Control
  - Medium Control
  - High Control
  - Negative Control

4.1.5.5.3 Internal Standards

- Pipette 20 µL of internal standard solution into each 16 x 100 mm culture tube

4.1.5.5.4 Additional Sample Preparation

- To negative and unknown samples, add 40 µL of 50:50 methanol:acetonitrile to each 16 x 100 mm culture tube
- Add 1.5 mL chilled 90:10 acetonitrile:methanol while vortexing each tube

Note: samples should be placed in -10°C to -25°C for approximately 20 minutes prior to centrifuging.

- Centrifuge samples at 3500 rpm for 10 minutes
- Transfer supernatant to clean, labeled culture tube
- Add 4 mL of aqueous 0.1% formic acid to each culture tube
- Vortex

4.1.5.6 Sample Extraction

4.1.5.6.1 Condition Strata X-Drug B Columns

- Add 2 mL methanol
- Add 2 mL DI water

4.1.5.6.2 Apply Samples

- Carefully, decant the sample mixture into its respective extraction column
- Allow gravity to pull the sample through the column

4.1.5.6.3 Wash Column

- Add 2 mL 0.1% formic acid in water, allow gravity to pull sample through the columns
- Add 2 mL methanol, allow gravity to pull sample through the columns
- Apply vacuum pump and dry for 3-4 minutes at >10 psi

4.1.5.6.4 Elute Analytes

Place clean, labeled 12 x 75 mm culture tubes into rack, place the rack inside the manifold tank ensuring that each column will elute into its respective tube.

NOTE: Elution solvent is prepared by adding ammonium hydroxide to isopropyl alcohol and then adding ethyl acetate.

- Add 1 mL ethyl acetate:isopropyl alcohol:ammonium hydroxide (70:20:10), allow gravity to pull elution solvent through column
- Add 1 mL ethyl acetate:isopropyl alcohol:ammonium hydroxide (70:20:10), allow gravity to pull elution solvent through column
- Apply vacuum pump to remove any remaining elution solvent from columns

NOTE: The final elution is performed two separate times, with 1 mL each time, collected in the same culture tube.

4.1.5.6.5 Evaporate Eluates

- Evaporate just to dryness in TurboVap (<40ºC)
- Reconstitute with 100 µL of 85:15 mobile phase A:mobile phase B
- Vortex
- Transfer to autosampler vials describes in 4.1.5.4
- Centrifuge vials for 10 minutes at 3500 rpm remove any existing air bubbles and prevent particulate matter from clogging LC lines

4.1.5.7 Instrument Preparation

4.1.5.7.1 Maintenance

- Check to ensure that the following maintenance has been completed:
  - Curtain plate has been cleaned within the last 7 days
  - Guard column has been changed within the last 2 months
  - Mobile phases have been prepared within the last 30 days and are of sufficient volume to complete the analytical run
- HPLC is connected to MS
- Correct mobile phases are being used

- Correct column is in place
- Waste container is not full
- Pump valves are closed
- Needle rinse has sufficient volume

4.1.5.7.2 Equilibrate

- Equilibrate until operating temperature (40ºC) is obtained
- Ensure that operating pressures are stable and record in log book
- Run at least two blanks to warm up the system

4.1.5.7.3 Retention Time Update

- Run appropriate standard and update retention times in acquisition and quantitation methods

4.1.5.8 Run Samples

- Subsequent to all steps in 4.1.5.7, samples may now be analyzed on the QTRAP 4500 or LC-MS/MS 4500

4.1.5.9 Data Analysis – Quantitative Analytes

4.1.5.9.1 Calibration Levels and Models

- Calibration Levels have been validated at the following concentrations (ng/mL):

Analyte	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6
amphetamine	10	60	120	180	240	300
benzoylecgonine	100	280	460	640	840	1000
buprenorphine	0.5	2.5	5	10	15	20
cocaine	5	20	40	60	80	100
codeine	10	60	120	180	240	300
fentanyl	0.5	2.5	5	10	15	20
hydrocodone	10	60	120	180	240	300
hydromorphone	5	14	23	32	41	50
morphine	10	60	120	180	240	300
oxycodone	10	60	120	180	240	300

oxymorphone	5	14	23	32	41	50

- Calibration models have been validated for each quantified analyte as follows:

Analyte	Curve Type	Weighting	Origin	Transition 1	Transition 2	I.S.
amphetamine	quadratic	Inverse (1/x)	Ignore	136.1/91.0	136.1/65.1	D11-amphetamine 147.0/98.1
benzoylecgonine	quadratic	None	Ignore	290.1/168.0	290.1/77.1	D8-benzoylecgonine 298.0/171.1
buprenorphine	linear	Inverse (1/x)	Ignore	468.1/55.2	468.1/414.2	D4-buprenorphine 472.2/59.2
cocaine	linear	Inverse (1/x)	Ignore	304.0/182.1	304.0/77.1	D3-cocaine 307.0/185.1
codeine	linear	Inverse (1/x)	Ignore	300.1/215.0	300.1/152.2	D6-codeine 306.0/218.0
fentanyl	linear	Inverse (1/x)	Ignore	337.2/105.0	337.2/188.0	D5-fentanyl 342.1/188.1
hydrocodone	linear	Inverse (1/x)	Ignore	300.0/171.0	300.0/199.0	D6-hydrocodone 306.0/202.1
hydromorphone	linear	Inverse (1/x)	Ignore	286.1/185.0	286.1/157.0	D6-hydromorphone 292.0/185.0
morphine	linear	Inverse (1/x)	Ignore	286.0/152.1	286.0/165.0	D6-morphine 292.0/152.1
oxycodone	quadratic	Inverse (1/x)	Ignore	316.1/256.1	316.1/241.0	D6-oxycodone 322.0/262.1
oxymorphone	linear	Inverse (1/x)	Ignore	302.0/227.1	302.0/198.0	D3-oxymorphone 305.0/230.0

- Calibration model information in the instrument method or MultiQuant software shall not be changed unless validated.
- A standard curve shall be generated with $r^2 \geq 0.95$ for each analyte.
- Evaluate the curve by examining the value of the calibrators. Values of ± 20% from the target concentration are acceptable, except for standard 1, which allows ± 30%.
- Verification of calibrator lot number and expiration date shall occur at the time of batch review.
- At least 4 calibrators must be included in the calibration curve.
- If the lowest or highest calibrators are removed from the curve, the change in working range may require a repeat analysis of casework samples that lie outside the new range. Alternately, the cases may be reported out as "< (new lowest calibrator)" or "> (new highest calibrator)" at the discretion of the unit supervisor.

4.1.5.9.2 Controls

- In addition to casework samples, each analytical batch shall contain a standard, low control, medium control, high control and negative control.

- Standard 1 shall be reinjected with 50% of the on column volume. This is performed in order to evaluate the processing method at the LRL for each analyte.
- Control values are acceptable when they are within +/- 20% of the target value. If one control is out of range, no action is necessary.
- If two controls are out of range the analyst shall consult with the unit supervisor who will determine whether:
    - Any affected casework samples will be re-extracted
    - The affected analyte will be reported qualitatively in positive casework samples
- The following target concentrations (ng/mL) for each control are given:

Analyte	LOW Control	MED Control	HIGH Control
amphetamine	20	100	250
benzoylecgonine	200	520	800
buprenorphine	1	8	16
cocaine	10	50	85
codeine	20	100	250
fentanyl	1	8	16
hydrocodone	20	100	250
hydromorphone	10	25	40
morphine	20	100	250
oxycodone	20	100	250
oxymorphone	10	25	40

4.1.5.9.3 Limits of Detection (LOD) and Lower Reporting Limit (LRL)

- The LOD and LRL (ng/mL) for each of the following analytes is as follows:

Analyte	LOD	LRL
amphetamine	3.375	5
benzoylecgonine	0.6	50
buprenorphine	0.188	0.25
cocaine	0.075	2.5
codeine	0.3	5
fentanyl	0.094	0.25
hydrocodone	1.875	5

hydromorphone	0.375	2.5
morphine	0.488	5
oxycodone	0.75	5
oxymorphone	0.375	2.5

NOTE: The LRL is determined as 50% of the area ratio between analyte and internal standard of calibrator 1. All concentrations listed in the previous table are estimates of the LRL.

NOTE: In the event that calibrator 1 is excluded from the curve, the area ratio of the LRL is determined by performing the following example calculation:

$$\frac{\text{amphetamine Std 1 = 10 ng/mL}}{\text{amphetamine Std 2 = 60 ng/mL}} = \frac{\text{amphetamine area ratio of Std 1 = X}}{\text{amphetamine area ratio of Std 2 = 1.2550}}$$

cross multiply and divide, X = .2092

multiply .2092 x 50% = 0.1046

NOTE: The calculations above can be simplified with the following equation:

$$\left(\frac{[Standard\ 1]}{[lowest\ standard\ used]} * area\ ratio\ of\ lowest\ standard\ used\right) * 50\% = area\ ratio\ reporting\ limit$$

4.1.5.9.4 Chromatographic and Mass Spectral Quality Control for Quantitative Analytes

- Chromatographic Quality
  - Chromatographic quality for LC/MS/MS transition data is defined as a reasonably symmetrical shaped peak consistent with those observed in calibrators and positive controls and is able to be differentiated from a negative control.
- Retention Time
  - The quantitative portion of this assay uses a Relative Retention Time (RRT) model for all analytes with a matched, deuterated internal standard. The RRT is calculated by diving the analyte retention time by the internal standard retention time. This is performed for all calibrators used in the calibration curve, averaged, and then given an acceptable value of ± 2.5%. Positive analytes must be within this range to be considered acceptable.

- Ion Ratios
  - The quantitative portion of this assay utilizes one transition ion as a quantitative ion and a second transition ion as a confirmatory ion. The ion ratio is calculated by dividing the area count of transition 2 by the area count of transition 1. The ratio between the two ions must be within ± 20% of the average of all of the calibrators used in the calibration curve to be acceptable.

NOTE: Chromatographic and Mass Spectral Quality Control shall be evaluated for all calibrators, controls and unknowns in an analytical batch.

4.1.5.10 Reporting the Results – Quantitative Analytes

- Quantitative results will be reported in ng/mL
- Quantitative results will be truncated to whole number (not rounded), except for fentanyl and buprenorphine which will be truncated to the tenth
- Analytes present at a concentration <LOQ with an area ratio of anayte to I.S. ≥50% of STD 1, or its equivalent in the event that STD 1 is excluded, will be reported as "< (LOQ)"
- Analytes present at a concentration <LOQ with an area ratio of analyte to I.S. <50% of STD 1, or its equivalent in the event that STD 1 is excluded, will be considered negative for reporting purposes
- If an analyte is present above the ULOQ, it will be reported as ">(ULOQ)"

4.1.5.11 Data Analysis – Qualitative Analytes

4.1.5.11.1 The following qualitative analytes are included in the scope of this confirmation:

Analyte	Transition 1	Transition 2	I.S.
6-acetylmorphine	328.0/165.0	328.0/211.2	D6-6-acetylmorphine 334.1/165.1
acetylfentanyl	323.0/188.1	323.0/105.1	D5-fentanyl 342.1/188.1
carfentanil	395.1/335.2	395.1/246.0	D5-fentanyl
methamphetamine	150.1/119.0	150.1/91.0	D11-methamphetamine 161.0/97.1
naloxone	328.0/212.1	328.0/268.2	D3-oxymorphone 305.0/230.0
norbuprenorphine	414.1/152.2	414.1/165.2	D4-buprenorphine 472.2/59.2

4.1.5.11.2 Levels have been validated at the following concentrations (ng/mL):

Analyte	STD 1	STD 2	STD 3	LOW Control	MED Control	HIGH Control
6-acetylmorphine	2	4	6	2	4	6
acetylfentanyl	0.5	1	2	0.5	1	2
carfentanil	0.5	1	2	0.5	1	2
methamphetamine	10	60	120	20	100	250
naloxone	12.5	25	50	12.5	25	50
norbuprenorphine	1.25	2.5	5	1.25	2.5	5

- Qualitative controls are analyzed to verify that the ion ratios and relative retention times of the analytes are correct. No quantitative values are calculated, however all three controls should be positive.

4.1.5.11.3 Limits of Detection (LOD) and Lower Reporting Limit (LRL)

- The LOD and LRL (ng/mL) for each of the following analytes is as follows:

Analyte	LOD	LRL
6-acetylmorphine	1	1
acetylfentanyl	0.3	STD 1
carfentanil	0.011	0.25
methamphetamine	3.375	5
naloxone	0.3	6.25
norbuprenorphine	0.938	STD 1

NOTE: The LRL is determined as 50% of the area ratio between analyte and internal standard of STD 1. All concentrations listed in the previous table are estimates of the LRL.

NOTE: In the event that calibrator 1 is excluded from the curve, see the example calculation provided in 4.1.5.9.3. to determine the LRL area ratio.

4.1.5.11.4 Chromatographic and Mass Spectral Quality Control for Qualitative Analytes

- Chromatographic Quality
  - Chromatographic quality for LC/MS/MS transition data is defined as a reasonably symmetrical shaped peak consistent with those observed in calibrators and positive controls and is able to be differentiated from a negative control.
- Retention Time
  - The qualitative portion of this assay uses a Relative Retention Time (RRT) model for all analytes which utilizes D5-fentanyl all analytes except methamphetamine. The RRT is calculated by diving the analyte retention time by the internal standard retention time. This is performed for STD 1, STD 2 and STD 3, averaged, and then given an acceptable value of ± 2.5%. Positive analytes must be within this range to be considered acceptable.
- Ion Ratios
  - The qualitative portion of this assay utilizes the area count of transition 2 and compares it to the area count of transition 1. The ratio is calculated by dividing the transition 2 area count by the transition 1 area count. The ratio between the two ions must be within ± 20% of the average of STD 1, STD 2 and STD 3 to be considered acceptable.

NOTE: Chromatographic and Mass Spectral Quality Control shall be evaluated for all calibrators, controls and unknowns in an analytical batch.

4.1.5.11.5 Reporting the Results – Qualitative Analytes

- If an analyte is present, and the area ratio of analyte to I.S. is ≥ 50% of the area ratio of STD 1, or its equivalent in the event that STD 1 is excluded, it will be reported as “Detected (Not Quantified)”.

4.1.5.12 Batch Review

- A Batch Review shall be conducted for all LCDOA analyses.
- For specific details regarding the batch review of both the quantitative and qualitative portions of this assay, see the LCDOA Batch Review Checklist with accompanying checklist instructions.