FORENSIC TOXICOLOGY LABORATORY
OFFICE OF CHIEF MEDICAL EXAMINER
CITY OF NEW YORK

BENZOYLECGONINE, COCAINE, EBE, MORPHINE, CODEINE, 6-MAM, OXYCODONE, OXYMORPHONE, HYDROCODONE AND HYDROMORPHONE by LCMS ANALYSIS

PRINCIPLE

Morphine, as the major metabolite of heroin (diacetylmorphine), is a possible indication of heroin use. Morphine is also a drug with wide medical use. 6-monoacetylmorphine (6-MAM) is an intermediate metabolite of heroin to morphine; its presence in biological specimens indicates heroin use. Codeine is a narcotic analgesic found in many pharmaceutical preparations alone or in combination with non-narcotic analgesics, antihistamines and other drugs. Oxycodone, a semisynthetic narcotic analgesic, is found in many pharmaceutical preparations alone or in combination with non-narcotic analgesics. Oxymorphone, a metabolite of oxycodone, is also found in pharmaceutical preparations alone. Hydrocodone is a narcotic analgesic found in many pharmaceutical preparations alone or in combination with non-narcotic analgesics, antihistamines and other drugs. Hydromorphone, a metabolite of hydrocodone, is structurally similar to morphine and is also a drug with medical use.

Benzoylecgonine (BE) is the major metabolite of cocaine, and is a marker of cocaine use. Ethylbenzoylecgonine (EBE, cocaethylene) is produced in the body when cocaine and ethanol are used concurrently. EBE can have significant pharmacological activity.

The silyl derivatives of morphine and hydromorphone are very similar when analyzed by GCMS; the structures of these two drugs differing only by the position of a double bond. These compounds are readily separated and identified without derivatization by high performance liquid chromatography, followed by atmospheric pressure electro-spray ionization mass spectrometry (LCMS).

These drugs are extracted from biological specimens using solid phase extraction. Drugs are temporarily bound to a sorbent in the solid phase cartridge as the prepared sample is poured through the column. The column is washed to remove interfering compounds, followed by the elution of drugs from the column. The eluate is evaporated and reconstituted in LCMS Mobile Phase “A”. The resulting solution is analyzed by LCMS.

SAFETY

The handling of all biological specimens and reagents is performed within the guidelines which are detailed in the Safety and Health manual.

SPECIMEN PREPARATION

The procedure is routinely applied to the following biological specimens and their aliquots unless otherwise specified:

Blood	1.0 mL of the undiluted specimen
Urine	1.0 mL for qualitative identification
Bile	1.0 mL of the undiluted specimen
Brain	1.0 mL of a 1:3 homogenate
Gastric Contents	1.0 mL of a 1:10 dilution
Liver	1.0 mL of a 1:5 homogenate
Vitreous Humor	1.0 mL of the undiluted specimen

DILUTION OF SPECIMENS

Specimens are diluted as follows:

Brain 1:3	5.0 g of brain homogenized with 10 mL of distilled water.
Liver 1:5	5.0 g of liver homogenized with 20 mL of distilled water.
Gastric 1:10	2.0 mL of liquid *q.s.* to 20 mL of distilled water, or 2.0 g of a solid specimen homogenized with 18 mL of distilled water.

Note: *Use a homogenate which was prepared within two weeks. Do not use homogenates older than two weeks unless low sample size requires it. Discuss with supervisor and note in case record. The entire submitted amount of gastric contents needs to be homogenized prior to sampling.*

REAGENTS AND MATERIALS

All chemicals should be analytical reagent grade or better.

1. **Deionized water** (distilled can be substituted)
2. **Methanol** (Fisher Scientific - ACS Certified)
3. **Methylene Chloride CH_2Cl_2**
4. **2-Propanol C_3H_8O** (isopropanol, IPA)
5. **Ammonium Hydroxide, NH_4OH** (Fisher Scientific)

   Note: *Ammonium hydroxide will break down to ammonia and water and the ammonia will evaporate if the container is not kept closed. This will cause a pH decrease, making the reagent unsuitable for solid phase extraction. Use small lots of working solution (500 mL bottles), open the bottle only briefly to remove aliquots and recap immediately. If the solution appears old, discard and use a fresh bottle.*

6. **100 mM phosphate buffer (pH 6.0)**

Dissolve 3.4 g Na_2HPO_4 and 24.2 g NaH_2P0_4 H_2O in 1600 mL DI H_2O.

Dilute to 2000 mL using DI H_2O. Mix. Adjust pH to 6.0 ± 0.1 with 100 mM monobasic sodium phosphate (lowers pH) or 100 mM dibasic sodium phosphate (raises pH).

Store at 5 ºC in glass.

Stability: 1 month. Inspect each day before use for contamination.

7. **Hydrochloric Acid, 100 mM**

   Add 8.4 mL concentrated HCl to 800 mL Deionized water. Q.S. to 1000 mL with deionized H_2O.

   Store at room temperature in glass or plastic.

   Stability: 6 months. Inspect each day before use for contamination.

8. **Eluting solvent, CH_2Cl_2 /IPA/NH_4OH (78/20/2).** Prepare fresh each day of use.
9. **Morphine d_3** (internal standard), Cerilliant or equivalent, 1.0 mg/mL in 1 mL acetonitrile, 99% pure (or equivalent).
10. **BE d_3** (internal standard), Cerilliant or equivalent, 1.0 mg/mL in 1mL acetonitrile, 99% pure (or equivalent).
11. **Cocaine d_3** (internal standard), Cerilliant or equivalent, 1.0 mg/mL in 1 mL acetonitrile, 99% pure (or equivalent).
12. **Oxycodone d_6** (internal standard), Cerilliant or equivalent, 1.0 mg/mL in 1 mL methanol, 99% pure (or equivalent).
13. **Codeine d_6** (internal standard), Cerilliant or equivalent, 1.0 mg/mL in 1 mL methanol, 99% pure (or equivalent).
14. **Morphine** Cerilliant or equivalent, 1.0 mg/mL in 1 mL acetonitrile, 99% pure or equivalent.
15. **Codeine** Cerilliant or equivalent, 1.0 mg/mL in 1 mL acetonitrile, 99% pure or equivalent.
16. **6-MAM** Cerilliant or equivalent, 1.0 mg/mL in 1 mL acetonitrile, 99% pure or equivalent.
17. **BE** Cerilliant or equivalent, 1.0 mg/mL in 1 mL acetonitrile, 99% pure or equivalent.
18. **Cocaine** Cerilliant or equivalent, 1.0 mg/mL in 1 mL acetonitrile, 99% pure or equivalent.
19. **EBE** Cerilliant or equivalent, 1.0 mg/mL in 1 mL acetonitrile, 99% pure or equivalent.
20. **Hydrocodone** Cerilliant or equivalent, 1.0 mg/mL in 1 mL methanol, 99% pure or equivalent.
21. **Oxycodone** Cerilliant or equivalent, 1.0 mg/mL in 1 mL methanol, 99% pure or equivalent.
22. **Hydromorphone** Cerilliant or equivalent, 1.0 mg/mL in 1 mL methanol, 99% pure or equivalent.

23. **Oxymorphone** Cerilliant or equivalent, 1.0 mg/mL in 1 mL methanol, 99% pure or equivalent.

24. **Morphine d_3/Oxycodone d_6/Codeine d_6/BE d_3/Cocaine d_3 working internal standard solution (10 mg/L)**

    a. Pipet 1.0 mL of 1.0 mg/mL Morphine d_3, 1.0 mL of 1.0 mg/mL Oxycodone d_6, 1.0 mL of 1.0 mg/mL Codeine d_6, 1.0 mL of 1.0 mg/mL BE d_3, and 1.0 mL of 1.0 mg/mL Cocaine d_3 into a 100 mL volumetric flask. Q.S. to 100 mL with acetonitrile.

    b. Transfer into properly labeled container – Morphine d_3/BE d_3/Cocaine d_3/Oxycodone d_6/Codeine d_6 (10 mg/L).

    Note: *Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.*

    c. Store at 4 °C.

25. **BE/Morphine/Codeine/6-MAM/Cocaine/EBE working calibrator solution (100 mg/L)**

    a. Transfer 5 mL of 1 mg/mL primary standard solution to a 50 mL volumetric flask.

    b. Q.S. to 50 mL with acetonitrile.

    c. Transfer into a properly labeled container.

    Note: *Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.*

    d. Store at 4 °C.

26. **BE/Morphine/Codeine/6-MAM/Cocaine/EBE working calibrator solution (10 mg/L)**

    a. Transfer 5 mL of 100 mg/L calibrator solution to a 50 mL volumetric flask.

    b. Q.S. to 50 mL with acetonitrile.

    c. Transfer into properly labeled container.

    Note: *Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.*

    d. Store at 4° C.

27. **Oxycodone, Oxymorphone, Hydrocodone, Hydromorphone working calibrator solution (100 mg/L)**

    a. Transfer 1.0 mL of 1 mg/mL primary standard solution of the four analytes to a 10 mL volumetric flask.

b. Q.S. to 10 mL with methanol.

c. Transfer into a properly labeled container.

Note: Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.

d. Store at 4 ^{o}C.

28. **Oxycodone, Oxymorphone, Hydrocodone, Hydromorphone working calibrator solution (10 mg/L)**

a. Transfer 1.0 mL of 100 mg/L working calibrator solution to a 10 mL volumetric flask.

b. Q.S. to 10 mL with methanol.

c. Transfer into a properly labeled container.

Note: Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.

d. Store at 4 ^{o}C.

29. **BE/Morphine/Codeine/6-MAM/Cocaine/EBE working control solution (100 mg/L)**

a. Transfer 5 mL of 1 mg/mL primary standard to a 50 mL volumetric flask.

b. Q.S. to 50 mL with acetonitrile.

c. Transfer to properly labeled container.

Note: *Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.*

d. Store at 4^{o} C.

30. **BE/Morphine/Codeine/6-MAM/Cocaine/EBE working control solution (10 mg/L)**

a. Transfer 5 mL of 100 mg/L control solution to a 50 mL volumetric flask.

b. Q.S. to 50 mL with acetonitrile.

c. Transfer into properly labeled container.

Note: *Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.*

d. Store at 4^{o} C.

31. **Oxycodone, Oxymorphone, Hydrocodone, Hydromorphone working control solution (100 mg/L)**

    a. Transfer 1.0 mL of 1 mg/mL primary standard solution of the four analytes to a 10 mL volumetric flask.

    b. Q.S. to 10 mL with methanol.

    c. Transfer into a properly labeled container.

    > ***Note:*** *Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.*

    d. Store at 4 ^{o}C.

32. **Oxycodone, Oxymorphone, Hydrocodone, Hydromorphone working control solution (10 mg/L)**

    a. Transfer 1.0 mL of 100 mg/L working control solution to a 10 mL volumetric flask.

    b. Q.S. to 10 mL with methanol.

    c. Transfer into a properly labeled container.

    > ***Note:*** *Include identity, concentration, solvent, lot number, date prepared, and initials of analyst.*

    d. Store at 4^{o}C.

33. **QAS External Blood Control**

    This is an unassayed blood control purchased through an external vendor with morphine, codeine, benzoylecgonine, EBE, and cocaine at a nominal concentration of 300 ng/mL. For each new lot of QAS control, the control is run at least 15 times, and the average of the values for each analyte becomes the new target value. The acceptable control range is ± 20% of the established target.

34. **QAS External Oxycodone/Oxymorphone/Hydrocodone/Hydromorphone Blood Control**

    This is an unassayed external blood control purchased through Quality Assurance Corp. at a nominal concentration of 300 ng/mL. For each new lot of QAS control, the control is run at least 15 times, and the average of the values for each analyte becomes the new target value. The acceptable control range is ± 20 %.

35. **Negative blood, serum, brain, liver**

    Calf or sheep blood obtained from outside source or equivalent. Sodium fluoride is added as a preservative, and stored frozen (-10°C or lower). Human serum obtained from outside source or equivalent, and stored frozen (-10°C or lower). Calf brain and liver obtained from

outside source or equivalent. Homogenized and stored frozen (-10°C or lower). All matrices are validated as negative by in-house analysis.

36. **Polycrom Clin II Solid Phase Extraction Columns**, CEREX Polycrom II, SPEware
37. **System 48 Processor** connected to nitrogen source.
38. **Waste Rack, SPE Rack, Collection Tube Rack.**
39. **Concentrator (Turbovap or SPEware)** connected to a nitrogen source.
40. **Vacuum Filtration Apparatus.**
41. **HPLC Columns,** Agilent Zorbax Eclipse XDB-C18 part # 927975-902, Rapid Resolution HT column, 4.6 x 50 mm, 1.8 micron; Restek Ultra II Biphenyl part # 9609335, 4.6 x 30 mm, 3 micron.

LCMS MOBILE PHASES

"A" 1 mM Ammonium Acetate

1. Add 0.154 g ammonium acetate to a 2 L volumetric flask.
2. Add approximately 800 mL of deionized water to the 2 L flask and mix.
3. Add 400 µL of trifluoroacetic acid to the flask and mix.
4. Add 100 ml (5%) methanol to the flask and mix.
5. Q.S. to 2L mark with deionized water and mix.
6. Filter the solution using vacuum filtration apparatus.
7. Transfer mobile phase back to the 2L volumetric flask.

"B" 2 mM Ammonium Acetate

1. Add 0.154 g ammonium acetate to a 1 L volumetric flask.
2. Add 500 mL of methanol to the 1 L flask and mix.
3. Add 200 µL of trifluoroacetic acid to the flask and mix.
4. Q.S. to 1L mark with acetonitrile and mix.
5. Filter the solution using vacuum filtration apparatus.
6. Transfer mobile phase back to the 1L volumetric flask.

EXTRACTION PROCEDURE

For quantitative analysis, prepare all calibrators and controls as listed below in #2 and #3.

For qualitative analysis, prepare a single point calibrator at 1.0 mg/L, a blank, and two positive controls (0.01 mg/L, and 0.2 mg/L). See below.

1. Aliquot 1 mL of validated negative matrix into each 16 x 125 mm tube labeled calibrator(s) or control(s). Aliquot 1 mL of sample into each appropriately labeled 16 x 125 mm tube.

***Note**: Deionized water is used as the negative matrix for urine and gastric specimens.*

2. Five calibrators are prepared as follows using negative matrix:

   0.05mg/L - add 5 µL of 10 mg/L BE/Morphine/Codeine/6-MAM/Cocaine/EBE working working calibrator solution and 5 µL of 10 mg/L Oxycodone/Oxymorphone/Hydrocodone/Hydromorphone working calibrator solution.

   0.10mg/L - add 10 µL of 10 mg/L BE/Morphine/Codeine/6-MAM/Cocaine/EBE working calibrator solution and 10 µL of 10 mg/L Oxycodone/Oxymorphone/Hydrocodone/Hydromorphone working calibrator solution.

   0.5mg/L - add 5 µL of 100 mg/L BE/Morphine/Codeine/6-MAM/Cocaine/EBE working calibrator solution and 5 µL of 100 mg/L Oxycodone/Oxymorphone/Hydrocodone/Hydromorphone working calibrator solution.

   1.0mg/L - add 10 µL of 100 mg/L BE/Morphine/Codeine/6-MAM/Cocaine/EBE working calibrator solution and 10 µL of 100 mg/L Oxycodone/Oxymorphone/Hydrocodone/Hydromorphone working calibrator solution.

   1.5mg/L - add 15 µL of 100 mg/L BE/Morphine/Codeine/6-MAM/Cocaine/EBE working calibrator solution and 15 µL of 100 mg/L Oxycodone/Oxymorphone/Hydrocodone/Hydromorphone working calibrator solution.

3. Positive controls are prepared using negative matrix as follows:

   0.01 mg/L – add 1 µL of 10 mg/L BE/Morphine/Codeine/6-MAM/Cocaine/EBE working calibrator solution and 1 µL of 10 mg/L Oxycodone/Oxymorphone/Hydrocodone/Hydromorphone working calibrator solution.

   0.025 mg/L – add 2.5 µL of 10 mg/L BE/Morphine/Codeine/6-MAM/Cocaine/EBE working calibrator solution and 2.5 µL of 10 mg/L Oxycodone/Oxymorphone/Hydrocodone/Hydromorphone working calibrator solution.

   0.20mg/L – add 20 µL of 10 mg/L BE/Morphine/Codeine/6-MAM/Cocaine/EBE working calibrator solution and 20 µL of 10 mg/L Oxycodone/Oxymorphone/Hydrocodone/Hydromorphone working calibrator solution.

4. For blood batches, an external control (QAS) is included with approximate values of 0.3 mg/L, containing BE, Morphine, Codeine, Cocaine and EBE.

   An additional external control, also supplied by QAS, for Oxycodone, Oxymorphone, Hydrocodone, and Hydromorphone is included with approximate values of 0.3 mg/L.

5. A matrix matched blank must be included for each matrix type in the batch.
6. Add 50 µL of 10 mg/L working internal standard solution to all tubes. The concentration of the internal standard in each sample is 0.5 mg/L.
7. Add 2 mL 100 mM phosphate buffer pH 6.0. Mix by Vortex for 30 seconds, then sonicate for 15 minutes. Additional buffer (up to 4 mL total) can be used for complex matrices.
8. Centrifuge sample for 10 minutes at 3000 rpm.
9. Decant the supernatant into the Polychrom Clin II column and apply nitrogen at a pressure of 2-4 psi.
10. Wash Column (All wash steps are pressurized at 2-4psi).

    Pour 1 mL DI H_2O onto column
    Pour 1 mL 100 mM HCl onto column
    Pour 1 mL CH_3OH onto column
    Pour 1 mL Ethyl Acetate onto column
    Dry column for 2 minutes at Max Flow.
11. Prepare Elution Solvent

    CH_2Cl_2 /IPA/NH_4OH (78/20/2) by mixing IPA/NH_4OH, followed by CH_2Cl_2.

    Note: *Prepare elution solvent each day of use.*
12. Elute Drugs

    Place labeled 10 mL conical centrifuge tubes under each column to collect eluate by gravity. Elute with 2 mL.
13. Dry under nitrogen at 40 ^{0}C to absolute dryness.
14. Reconstitute with 300 µL of mobile phase A. Mix by Vortex. Centrifuge.
15. Label autosampler vials indicating aliquot and toxicology number (eg. 1-YY-xxxx), specimen type, dilution, analyst and date.
16. Transfer reconstituted extract to an insert placed in a labeled autosampler vial. Cap immediately to avoid possible contamination from other samples. Do not wait until all transfers have been made to seal the vials. Samples are ready for MS injection.
17. Create batch sequence as specified in Instrument Setup.
18. Enter the date extracted in the Dataease database, so the samples are not duplicated by another analyst.

INSTRUMENTATION – LCMS 1

Agilent LCMSD series 1100, with 1100 HPLC, G1313A Autosampler, and Agilent Chemstation with appropriate software. The method name for this assay is LCMS1OPBE_50.M, utilizing the Zorbax Eclipse XDB-C18 Rapid Resolution HT column.

The following ions are monitored for each drug:

Morphine d_3 IS	289.1, 290.1
Morphine	286.1, 287.1
Oxymorphone	302.1, 303.1
Hydromorphone	286.1, 287.1
Codeine d_6 IS	306.2, 307.2
Codeine	300.1, 301.1
Oxycodone	316.1, 317.1
Oxycodone d_6 IS	322.1, 323.1
Hydrocodone	300.1, 301.1
6-MAM	328.1, 329.1
BE d_3 IS	293.1, 294.1
BE	290.1, 291.1
Cocaine	304.1, 305.1
Cocaine d_3 IS	307.1, 308.1
EBE	318.1, 319.1

Method Information For: C:\ CHEM\1\METHODS\LCMS1OPBE_50.M

Run Time Checklist:

(X) Save Copy of Method With Data
( ) Pre-Run Cmd/Macro
(X) Data Acquisition
( ) Data Analysis
( ) Post-Run Cmd/Macro

Method Comments:

This method is for Opiates/BE/Coc/EBE analysis.

1100 High Pressure Gradient Pump 1

Control	
Column Flow	0.600 ml/min
Stoptime	18.00 min
Posttime	6.00 min
Solvents	
Solvent A 1	100%

Solvent B 1	0%
Pressure Limits	
Minimum Pressure	0 bar
Maximum Pressure	400 bar
Auxiliary	
Maximal Flow Ramp	100.00 ml/min^2
Compressibility A	50*10^{-6}/bar
Minimal Stroke A	Auto
Compressibility B	115*10^{-6}/bar
Minimal Stroke B	Auto
Store Parameters	
Store Ratio A	Yes
Store Ratio B	Yes
Store Flow	Yes
Store Pressure	Yes

Timetable		
Time	Solv.B	Flow
0.00	0.0	0.600
2.50	5.0	0.600
6.00	10.0	
13.00	25.0	0.600
16.00	100.0	
16.20		1.000
18.00	100.0	1.000

Agilent 1100 Diode Array Detector 1

Signals			
SIGNAL	STORE	SIGNAL,BW	REF,BW [NM]
A:	YES	220 8	360 10
B:	NO	254 16	360 100
C:	NO	210 8	360 100
D:	NO	230 16	360 100
E:	NO	280 16	360 100

Spectrum	
Store Spectra	All
Range from	190 nm
Range to	325 nm
Range step	2.00 nm
Threshold	1.00 mAU
Time	
Stoptime	16.50 min
Posttime	Off
Required lamps	
UV lamp required	Yes
Vis lamp required	No
Autobalance	
Prerun balancing	Yes
Postrun balancing	No
Margin for negative Absorbance	100 mAU
Peakwidth	> 0.05 min
Slit	4 nm
Analog Outputs	
Zero offset ana. out. 1	5%
Zero offset ana. out. 2	5%
Attenuation ana. out. 1	1000 mAU
Attenuation ana. out. 2	1000 mAU
Timetable is empty	

Mass Spectrometer Detector

General Information	
Use MSD	Enabled
Ionization Mode	API - ES
Tune File	Atunes.tun
StopTime	16.50
Time Filter	Enabled
Data Storage	Full
Peakwidth	0.20 min

Signals					
{Signal 1}					
Polarity	Positive				
Fragmentor Ramp	Not applicable				
Percent Cycle Time	90%				
SIM Parameters					
Time (min)	Group Name	Sim Ion	Fragmentor	Sim Resol.	Actual Dwell
0.00	Group 1	462.10	70	High	534
		463.10	70		534
0.8	Group 2	286.10	70	High	177
		287.10	70		177
		289.10	70		177
		290.10	70		177
		302.10	70		177
		303.10	70		177
6.0	Group 3	300.10	70	High	105
		301.10	70		105
		306.20	70		105
		307.20	70		105
		316.10	70		105
		317.10	70		105
		322.10	70		105
		323.10	70		105
		328.10	70		105
		329.10	70		105
12.0	Group 4	290.10	70	High	105
		291.10	70		105
		293.10	70		105
		294.10	70		105
		304.10	70		105
		305.10	70		105
		307.10	70		105
		308.10	70		105
		318.10	70		105
		319.10	70		105
[Signal 2}					
Polarity	Positive				
Fragmentor Ramp	Disabled				
Percent Cycle Time	10%				

Scan Parameters					
Time	Mass range low	Mass range hi	Fragmentor	Threshold	Step size
0.00	100.00	500.00	70	150	0.10
Spray chamber					
[MSZones]					
Gas temp	350 ^{0} C		Max temp	350 ^{0} C	
Drying Gas	11.0 L/min		Max DryGas	13.0 L/min	
Neb Pres	45 psig		Max Pres	60 psig	
VCap (Positive)	3500 V				
VCap (Negative)	3500 V				

Gain is a dynamic variable, and should be set accordingly as needed for analysis.

END OF MS ACQUISITION PARAMETERS

FIA Series

FIA Series in this Method : Disabled

Time Setting
Time between Injections : 0.73 min

Agilent 1100 Autosampler 1

Injection	
Injection Mode	Standard
Injection Volume	10.0 µL **
Auxiliary	
Drawspeed	200 µL/min
Ejectspeed	200 µL/min
Draw Position	0.0 mm
Time	
Stoptime	As pump
Posttime	Off

******** *Injection volume is set to 5.0 µL for LCMS1UROPBE_50.m, used for qualitative analysis of urine samples. All other instrument parameters are as established for LCMS1OPBE_50.m.*

Agilent 1100 Column Thermostat 1

Temperature settings	
Left temperature	50 ^{0} C
Right temperature	Same as left
Enable analysis	When Temp is within ± 0.8 ^{0} C
Store left temperature	No
Store right temperature	Yes
Time	
Stoptime	As pump
Posttime	Off
Column switching valve	Column 2
Timetable is empty	

Analysis by an alternate stationary phase may be necessary for reasons discussed below, in the Acceptance Criteria section. Instrument parameters not specified below remain as stated above in the LCMS1OpBE_50.m method.

LCMS1MOR.m / LCMS1HYM.m

1100 High Pressure Gradient Pump 1

Timetable		
Time	Solv.B	Flow
0.00	0.0	0.600
2.50	5.0	0.600
6.00	10.0	
10.00	50.0	0.600
13.00	100.0	1.000

Mass Spectrometer Detector

Time (min)	Group Name	SIM Ion
0.80	Group 2	286.1
		287.1
		289.1
		290.1

Column:
ULTRA biphenyl II 3 µm, 30 mm x 4.6 mm

LCMS1OXYM.m

1100 High Pressure Gradient Pump 1

Timetable		
Time	Solv.B	Flow
0.00	0.0	0.600
2.50	5.0	0.600
6.00	10.0	
10.00	50.0	0.600
13.00	100.0	1.000

Mass Spectrometer Detector

Time (min)	Group Name	SIM Ion
0.80	Group 2	302.1
		303.1
		289.1
		290.1

Column:
ULTRA biphenyl II 3 µm, 30 mm x 4.6 mm

LCMS1COCBE.m

1100 High Pressure Gradient Pump 1

Timetable		
Time	Solv.B	Flow
0.00	0.0	0.600
6.00	50.0	
9.00	100.0	
9.50		1.000
11.00	100.0	1.000

Mass Spectrometer Detector

Time (min)	Group Name	SIM Ion
0.80	Group 4	290.1
		291.1
		293.1
		294.1
		304.1
		305.1
		307.1
		308.1
		318.1
		319.1

Column:
ULTRA biphenyl II 3 µm, 30 mm x 4.6 mm

INSTRUMENTATION – LCMS 2

Agilent LCMSD series 6130, with 1200 HPLC, G1367C High Performance Autosampler SL 1, and Agilent Chemstation with appropriate software. The method name for this assay is LCMS2OPBE50.M

The following ions are monitored for each drug:

Morphine d_3 IS	289.1, 290.1
Morphine	286.1, 287.1
Oxymorphone	302.1, 303.1
Hydromorphone	286.1, 287.1
Codeine d_6 IS	306.2, 307.2
Codeine	300.1, 301.1
Oxycodone d_6 IS	322.1, 323.1
Oxycodone	316.1, 317.1
Hydrocodone	300.1, 301.1
6-MAM	328.1, 329.1
BE d_3 IS	293.1, 294.1
BE	290.1, 291.1
Cocaine	304.1, 305.1
Cocaine d_3 IS	307.1, 308.1
EBE	318.1, 319.1

Method Information For: C:\ CHEM\1\METHODS\LCMS2OPBE50.M

Run Time Checklist:

(X) Save Copy of Method With Data

( ) Pre-Run Cmd/Macro
(X) Data Acquisition
( ) Data Analysis
( ) Post-Run Cmd/Macro

Method Comments: This method is for Opiates/BE/Coc/EBE analysis.

1200 High Pressure Gradient Pump 1

Control	
Column Flow	0.600 ml/min
Stoptime	19.00 min
Posttime	5.00 min
Solvents	
Solvent A 1	100%
Solvent B 1	0%
Pressure Limits	
Minimum Pressure	0 bar
Maximum Pressure	400 bar
Auxiliary	
Maximal Flow Ramp	100.00 ml/min^2
Minimal Stroke A	Auto
Minimal Stroke B	Auto
Store Parameters	
Store Ratio A	Yes
Store Ratio B	Yes
Store Flow	Yes
Store Pressure	Yes

Timetable		
Time	Solv.B	Flow
0.00	0.0	0.600
2.50	0.0	0.600
6.00	10.0	0.600
13.00	25.0	0.600
16.00	100.0	0.600
19.00	100.0	1.000

AGILENT 1100/1200 DIODE ARRAY DETECTOR 1

Signals			
SIGNAL	STORE	SIGNAL,BW	REFERENCE,BW [NM]
A:	YES	220 8	360 10
B:	NO	254 16	360 100
C:	No	210 8	360 100
D:	No	230 16	360 100
E:	No	280 16	360 100

Spectrum	
Store Spectra	All
Range from	190 nm
Range to	325 nm
Range step	2.00 nm
Threshold	1.00 mAU
Time	
Stoptime	As Pump
Posttime	off
Required lamps	
UV lamp required	yes
Vis lamp required	no
Autobalance	
Prerun balancing	yes
Postrun balancing	no
Margin for negative Absorbance	100 mAU
Peakwidth	> 0.0025 min
Slit	4 nm
Analog Outputs	
Zero offset ana. out. 1	5%
Zero offset ana. out. 2	5%
Attenuation ana. out. 1	1000 mAU
Attenuation ana. out. 2	1000 mAU
Timetable is empty	

Mass Spectrometer Detector

Signals					
{Signal 1}					
Polarity Percent Cycle	Positive 90%				
Fragmentor Ramp	Not applicable				
SIM Parameters					
Time (min)	Group Name	Sim Ion	Fragmentor	Sim Resol.	Actual Dwell
0.00	Group 1	462.10	70	High	534
		463.10	70		534
0.8	Group 2	286.10	70	High	177
		287.10	70		177
		289.10	70		177
		290.10	70		177
		302.10	70		177
		303.10	70		177
6.50	Group 3	290.10	70	High	105
		291.10	70		105
		293.10	70		105
		294.10	70		105
		300.10	70		105
		301.10	70		105
		316.10	70		105
		317.10	70		105
		322.10	70		105
		323.10	70		105
		328.10	70		105
		329.10	70		105
12.00	Group 4	304.10	70	High	105
		305.10	70		105
		307.10	70		105
		308.10	70		105
		318.10	70		105
		319.10	70		105
[Signal 2]					
Polarity	Positive				
Percent Cycle	10%				
Fragmentor Ramp	Disabled				
Scan					

Parameters					
Time	Mass range low	Mass range hi	Fragmentor	Threshold	Step size
0.00	100.00	500.00	70	150	0.10
[Signal 3]					
Not active					
[Signal 4]					
Not active					
Spray chamber					
[MSZones]					
Gas temp	350 ^{0} C		Max Temp	350 ^{0} C	
Drying Gas	11.0 L/min		Max DryGas	13.0 L/min	
Neb Pres	45 psig		Max Pres:	60 psig	
VCap (Positive)	3500 V				
VCap (Negative)	3500 V				
[Time Table]					
Time Table is	Empty.				

Gain is a dynamic variable, and should be set accordingly as needed for analysis.

General Information	
Use MSD	Enabled
Ionization Mode	API – ES
Tune File	Atunes.tun
StopTime	16.50
Time Filter	Enabled
Data Storage	Full
Peakwidth	0.20 min

END OF MS ACQUISITION PARAMETERS

FIA Series

FIA Series in this Method : Disabled

Time Setting
Time between Injections : 0.2 min

Agilent 1200 High Performance Autosampler SL 1

Injection	
Injection Mode	Needle Wash
Injection Volume	10.0 µL**
Auxiliary	
Drawspeed	200 µL/min
Ejectspeed	200 µL/min
Draw Position	0.0 mm
Wash Mode	Wash in Flushport
Stoptime	No Limit
Posttime	Off

*** Injection volume is set to 5.0 µL for LCMS2UROPBE50.m, used for qualitative analysis of urine samples. All other instrument parameters are as established for LCMS2OPBE50.m.*

Agilent 1200 Column Thermostat 1

Temperature settings	
Left temperature	40 ^{0} C
Right temperature	Same as left
Enable analysis	When Temp is within ± 0.8 ^{0} C
Store left temperature	Yes
Store right temperature	No
Time	
Stoptime	As pump
Posttime	off
Column-switching valve	Column 2
Timetable is empty	

INSTRUMENT SETUP

An acceptable Checktune must be obtained each day that samples are run. Refer to the SOP entitled “LCMS Autotune” for instructions.

Prepare a sequence using the following steps.

1. Click on the Easy Sequence Setup tab.
2. Load an appropriate Easy Sequence Setup template. If one does not exist, see #16.
3. Click on the “Samples” tab.

4. Edit the number of samples to be run (include all blanks, cases, controls, and control reinjections).
5. Edit the data file name under Data Information. The data file should read LCMS#mmddX<c>. # represents instrument number. X indicates the batch being run. Reset the counter.
6. Edit the sequence name under Sequence Information. The sequence should read LCMS#mmddyyX<c>.
7. Save the updated easy sequence template.
8. Now, click on the Easy Sequence tab. Open the Easy Sequence Setup that was previously modified and saved (.est).
9. Click “Fill samples”.
10. Fill in sample names and information. Indicate lot numbers, dilutions and any comments as needed for calibrators, blanks, controls and cases.
11. Save Easy Sequence as LCMS#mmddyyX.es.
12. “Preview/Print Sequence” for loading of vials. Using the printed Sequence List, verify that the vials are loaded in their correct location. For LCMS1, vials are loaded in numerical sequence (i.e: 1-100). For LCMS2, vials are loaded onto one of two separate wellplates (i.e: P1-A-01 to P1-A-09 through P1-F-01 to P1-F-09; P2-A-01 through P2-F-09).
13. Verify Sequence Queue is in play mode. Save and add Easy Sequence to the queue.
14. The complete sequence cannot be printed correctly before the sequence is started. Once the sequence is running, click “Sequence” from the main toolbar, then “Print Sequence”. Select Sequence Parameters, Sample Info Part, and Method and Injection part. Click Print.
15. Date and initial the chain of custody label on the sequence printout, listing any comments, transfers or exceptions.
16. If an easy sequence template must be created or modified, follow these steps.
    a) Select extended parameters. Check shutdown and select standby from the drop down menu. Not ready timeout should be set to 0.0. Click OK.
    b) Click on the calibration tab.
    c) Select cyclic calibration mode.
    d) Click on calibrant icon, drag and drop into sequence start box.
    e) Fill out calibrant information (vial info, injections/vial, calibration level). Select No Update for both response factor and retention time.
    f) Repeat for each calibrator.
    g) Enter Calibration Interval as 50 (or > number of injections needed for entire batch). The interval unit is injections.
    h) Drag blank icon to sequence end box. Select a Shutdown method.
    i) Review Easy Sequence template overview.

j) Save Easy Sequence Setup template.

k) Return to step 3 and continue through #15.

DATA TRANSFER AND PROCESSING

1. After the batch acquisition has completed, the data files will reside on the local Chemstation and OpenLab ECM. Go to the acquiring instrument and log into a session of Enhanced Data Analysis. Select “Custom tool 3”. This custom tool parses the three types of data (SIM, Scan, and UV-VIS) into two separate batches (SIM/Scan and UV) and uploads them to ECM. Highlight all files from the appropriate subdirectory, changing the path if necessary. Data files are located in C:\Chem32\1\data. Click the → Arrow and Process.

2. Next, download only the SIM/Scan data files to a local processing terminal for processing and reviewing. Log onto the processing terminal and load Data Analysis. Under ECM toolbar, click Retrieve Entire Sequence from ECM. Double click LCMS, the appropriate instrument, month, and subdirectory (LCMS#MMDDYYAxms) and click OK.

3. Data Files will download to the local processing terminal. The progress of this transfer will appear at the bottom of the data analysis screen. If the operation is cancelled, log in to OpenLab ECM again and repeat step 2.

4. A SIM GCMS method is used to process data, rather than the LCMS acquisition method. An appropriate processing method is located in the Method folder under the monthly folder of the appropriate acquiring instrument. To load the method: Select Load Method from the ECM drop down menu. Select the method from the Method folder in the monthly folder of the appropriate acquiring instrument. Click OK. Save this method to the processing terminal by selecting Save method from the Method drop down menu, the path should be C:\msdchem\1\ecm\retrieve\ LCMS#MMDDYYAxms. Save to the correct location in OpenLab ECM.

5. Process the calibrators. Select Tools from the toolbar, DoLIST, and Quant, No Report (QT 1). Press Add, and OK. Select the files for this action to be performed on, in this case, calibrators only. Verify that the selected files are located in the correct subdirectory. Change the path if necessary. Click the → Arrow and Process.

6. Review the integrations of the targeted compounds for each calibrator checking that the ion peaks are present and integrated correctly (i.e., the baseline is the most scientifically accurate one that can be drawn). Select View from the toolbar, QEDIT. Answer appropriately when prompted to save changes made to quantitation results when moving from file to file. Return to Data Analysis by selecting View from the toolbar, return to Data Analysis.

7. Update the existing calibration table (all levels). Select Calibrate, Update, Quick Levels Update. When prompted to clear responses, select YES. When asked to requant files before update, select NO. Select single data file/level option. Select the appropriate data file to associate with calibration level 1 (50ng/mL). Click OK. Repeat for remaining calibration levels (100, 500, 1000, 1500 ng/mL). Select level 3 when prompted to update retention times.

Load the file associated with level 3 (500 ng/mL), by selecting File, Load Data File. Select Calibrate, Update One Level. Do NOT requant. Select Update One Level, select only Replace Qualifier Ion Relative Responses, and choose the corresponding existing level ID (#3). Click Do

Update.

8. Review the Compound database. Double click on the internal standard listed on the left to reveal the compounds quantitated with it. Select the calibration tab to reveal compound responses, calibration curves, and r^2. To disable a point on the calibration curve for a compound, delete its response from the table. Click OK or Cancel when review is complete.

9. Save Method locally before proceeding. Select Method from the toolbar, Save method, make sure that the path is correct. Do not save to OpenLab ECM at this time.

10. Requantitate the calibrators with the updated calibration curve. Select Tools from the toolbar, DoLIST, Requant, no report (QT 2), Add, and OK. Remove any existing commands. Select files to process. Click the → Arrow and Process. Review with QEDIT. Evaluate the responses, retention times and ion ratios.

11. Regression correlation coefficient (r^2) for each analyte must be equal to or greater than 0.99.

12. Process controls and cases. Select Tools from the toolbar, DoLIST, Quant, No Report (QT 1), Add, and OK. Select appropriate files. Click the → Arrow and Process. Review with QEDIT. Verify multipliers/dilution factors are applied.

13. When review is complete, return to Data Analysis. Select report format by choosing Quantitate from the toolbar, Report Options. Check SIM style report and uncheck Internal Standards. Press OK.

14. To print reports, select Tools from the toolbar, DoLIST, Profile Quant w/o Calculations (QT 0,1,'P'), Add, and OK. Select files to print, click the → Arrow and Process.

15. Save files to ECM. Select ECM from the toolbar, select "Save multiple data files to ECM". Select all files.

16. Save method to ECM. Select ECM from the toolbar, Save Method to ECM. Make sure data path is correct.

ACCEPTANCE CRITERIA

1. Acceptance range for calibrators is ± 20% of the target. Maximum two out of five calibrators may be dropped if outside of the acceptable range. However, the remaining acceptable calibrators must be re-processed and quantitative values for cases reported within the dynamic range of the acceptable calibration range.
2. Blood controls must pass integration, retention time, and peak shape criteria. Additionally, they must be within ± 20 % of the target value. The blank must not contain detectable amounts of target analytes or significant interfering peaks.
2. Ion ratios must be within ± 20% of the target ion ratio, as determined by the midpoint calibrator, for blood samples. For non-blood samples, the ion ratios may be accepted within ± 30%, if the analyte has met acceptance criteria in a blood matrix.

***Note**: Sometimes ion ratios will be out in exceptionally low or high concentrations, or in cases of coelutions. The analyst must evaluate this, and determine other appropriate actions as needed, such as reinjection (using methods described on pages 15-16).*

3. Make sufficient copies of the controls (in-house and external), calibration report, and the sequence list, enough to attach a set for each case in the batch.

REPORTING

After the batch has been reviewed and printed, it must be reported, using the following guidelines:

1. Each case printout must have a copy of the sequence and all controls appended.
2. Results are reported in mg/L. SA and EP cases are reported in ng/mL.
3. Concentrations greater than 0.05 mg/L but less than 1.0 mg/L are reported to two decimal points (i.e., 0.275 mg/L is reported as 0.27 mg/L).
4. Concentrations greater than 1.0 mg/L are reported to one decimal point (i.e. 2.75 mg/L is reported as 2.7 mg/L).
5. Concentrations less than 0.05 mg/L but greater than 0.005 mg/L are reported as “detected, less than 0.05 mg/L”.
6. Concentrations less than 0.005 mg/L are reported as “not detected.”
7. Sample concentrations greater than the highest acceptable calibrator must be re-extracted with suitable dilution to bring it within the limits of the calibration curve.
8. Review other findings in the case, especially immunoassay and GC results to see if they are consistent with the LCMS findings. If there are discrepancies, schedule additional testing to resolve it. If in doubt, consult with a supervisor.
9. If the positive blood matrix controls are ± 30% of target, the samples may be reported qualitatively, as detected or not detected. If quantitative results are needed, consult a supervisor.
10. Atropine, isobaric to BE, can be detected under the primary C-18 methods. This analyte is reported qualitatively as “detected” or “not detected”, provided that a standard has been included in the batch.

REFERENCES

Agilent 1100 Series LCMS Systems. Installation Guide.

Agilent 1100 Series LCMS Systems. Users Guide.

Agilent 1100 Series LCMS Systems. Standard Operating Procedures.

SPEware Corp. Cerex Applications Manual.

System 48 Processor. Users Guide.

Turbovap. Users Guide.

Agilent 1290 Infinity Series LCMS Systems – System Manual

Baselt, R.C, “Disposition of Toxic Drugs and Chemicals in Man.” Fifth Ed (2000).