CONFIRMATION OF BUPRENORPHINE, NORBUPRENORPHINE, AND NALOXONE BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

36.1 POLICY

This test method may be used to confirm the presence of buprenorphine (BUP), norbuprenorphine (NBP), and naloxone (NLX) 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.

36.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and/or quantitation of BUP, NBP and NLX 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.

36.3 PRINCIPLE

The targeted compounds BUP, NBP and NLX, and corresponding internal standards BUP-d_4, NBP-d_3 and NLX-d_5, 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.

36.4 SPECIMENS

36.4.1 The specimen volume is 1.0 mL.

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

NOTE: Results from testing of tissue homogenate samples will be qualitative, wherever possible. If quantitation is necessary, testing batches will be evaluated on a batch-by-batch basis to determine the LLOQ.

36.4.3 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.

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

36.5 REAGENTS, MATERIALS AND EQUIPMENT

36.5.1 REAGENTS

36.5.1.1 Hydrochloric acid (HCl), concentrated

36.5.1.2 0.1M HCl

To 400 mL DI H_2O, add 4.2 mL concentrated HCl. Dilute to 500 mL with DI H_2O. Store in a glass bottle at room temperature for up to 6 months. Adjustments to final volume are permitted as long as the proportions are maintained.

36.5.1.3 Acetonitrile (ACN)

36.5.1.4 Ammonium hydroxide (NH_4OH), concentrated

36.5.1.5 Certified blank blood

36.5.1.6 Deionized water (DI H_2O)

36.5.1.7 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.

36.5.1.8 Formic acid (concentrated)

36.5.1.9 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 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.

36.5.1.10 Isopropanol (IPA)

36.5.1.11 Methanol (MeOH)

36.5.1.12 Methylene chloride (dichloromethane, CH_2Cl_2)

36.5.1.13 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.

36.5.1.14 Sodium hydroxide (NaOH), concentrated

36.5.1.15 Sodium phosphate, dibasic anhydrous (Na_2HPO_4)

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

36.5.2 MATERIALS

36.5.2.1 Autosampler vials, inserts and caps

36.5.2.2 Disposable 16 x 100mm tubes with closures

36.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

36.5.2.4 Disposable pipette tips

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

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

36.5.2.7 Laboratory glassware (graduated cylinders, flasks)

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

36.5.2.9 Volumetric glassware (flasks)

36.5.3 EQUIPMENT

36.5.3.1 Agilent HPLC (1100/1200 series), Shimadzu HPLC, or equivalent

36.5.3.2 Agilent MS-MS with API-ES source (6410/6420), Sciex API 3200 MS-MS, or equivalent

36.5.3.3 Calibrated, adjustable piston pipettes

36.5.3.4 Centrifuge

36.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

36.5.3.6 pH Meter and/or indicating pH paper

36.5.3.7 Solvent filtration apparatus

36.5.3.8 Vortex mixer

36.5.3.9 Vacuum manifold

36.6 STANDARDS, CALIBRATORS AND CONTROLS

36.6.1 STANDARDS

36.6.1.1 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 and positive controls, and the working internal standard.

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

a. Buprenorphine: 1.0 mg/mL
b. Buprenorphine-D_4: 0.1 mg/mL
c. Naloxone: 1.0 mg/mL
d. Naloxone-D_5: 0.1 mg/mL
e. Norbuprenorphine: 0.1 mg/mL
f. Norbuprenorphine-D_3: 0.1 mg/mL

36.6.1.3 Working standard (1.0 ng/µL)

a. Using a calibrated pipette, measure 25 µL each of BUP and NLX stock standards and 250 µL NBP 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 1.0 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.

36.6.1.4 Working internal standard (0.1 ng/µL)

a. Using a calibrated pipette, measure 25 µL each of stock internal standards into a 25mL 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.

36.6.2 CALIBRATORS

36.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 36.7 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 (see NOTE in 36.4.2).

36.6.3 CONTROLS

36.6.3.1 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 – see NOTE in 36.4.2.)

36.6.3.2 Positive Controls

a. Three 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 36.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 36.7 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 (see NOTE in 36.4.2).

36.7 SAMPLE PREPARATION

NOTE: Flunitrazepam and 7-aminoflunitrazepam may cause interference, affecting chromatography and transition ratios of buprenorphine and norbuprenorphine, respectively.

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

36.7.2 Add 2 mL of 0.1M phosphate buffer pH6 to each tube.

36.7.3 Add 1 mL of certified blank whole blood into each of the six calibrator tubes, the positive control tubes and the negative control tube(s).

36.7.4 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.

36.7.5 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.

36.7.6 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 - 0.20 ng/mL	20	0.01 ng/µL
Calibrator 2 - 0.50 ng/mL	50	0.01 ng/µL
Calibrator 3 - 1.0 ng/mL	100	0.01 ng/µL
Calibrator 4 - 5.0 ng/mL	50	0.1 ng/µL
Calibrator 5 - 20 ng/mL	20	1.0 ng/µL
Calibrator 6 - 50 ng/mL	50	1.0 ng/µL

36.7.7 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.

36.7.8 Prepare a 1:100 dilution of the working control 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.

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

Control Description	Volume (µL) Added	Control Working Standard
Control 1 - 0.6 ng/mL	60	0.01 ng/µL
Control 2 - 15 ng/mL	15	1.0 ng/µL
Control 3 - 40 ng/mL	40	1.0 ng/µL

36.7.10 If in-house positive controls are being used, transfer 1 mL of each into their labeled tubes.

36.7.11 Sample 1 mL of each case sample into its respective tube.

36.7.12 Add 100 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 10 ng/mL.

36.7.13 Add 2 mL of 0.1M phosphate buffer pH6 to each tube again.

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

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

36.7.16 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.

36.7.17 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.)

36.7.18 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. 3 mL 0.1M HCl
c. 3 mL MeOH

36.7.19 Dry the columns for 10 minutes under vacuum.

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

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

36.7.22 Transfer the tubes to the evaporator and evaporate the extracts to dryness

at 40°C.

36.7.23 Reconstitute the extracts by the addition of 50 µL mobile phase (85:15 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.

36.7.24 Transfer the extracts to labeled autosampler vials and cap.

URINE ANALYSIS

For analysis of urine specimens, include a negative urine control and a positive urine control spiked to 0.5 ng/mL, as described for Calibrator 2 in 36.7.6. This will be used as the cutoff level for qualitative reporting of target compounds in urine specimens.

36.8 INSTRUMENTAL PARAMETERS

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

36.9 DATA ANALYSIS

36.9.1 Analysis of the batch data is conducted using the MassHunter (Agilent) or MultiQuant (Sciex) quantitative instrumental data analysis software.

36.9.2 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.

36.9.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves.

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

36.10 CRITERIA FOR BATCH ACCEPTANCE

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

36.10.1 CALIBRATORS AND CALIBRATION CURVES

36.10.1.1 Chromatographic peaks for BUP, NBP and NLX and internal standards shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

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

36.10.1.3 Quantitative results for BUP, NBP and NLX 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 calibrators 1 and 2, result comparison will use results to two decimal place (as seen on report), for calibrators 3 and 4, result comparison will use results truncated to one decimal place, and for calibrators 5 and 6, result comparison will use the whole integer, truncated results in units of ng/mL.

36.10.1.4 The calibration curves for BUP, NBP and NLX shall have a correlation coefficient ≥0.99.

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

36.10.2 CONTROLS

36.10.2.1 The negative control(s) shall not identify BUP, NBP or NLX above its 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.

36.10.2.2 Positive controls

a. Chromatographic peaks for BUP, NBP and NLX 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 BUP, NBP and NLX in each control shall be within ±20% of their target values. These are inclusive ranges. For the low positive control, result comparison will use results to two decimal places (as seen on report), and for the mid and high controls, result comparison will use the whole integer, truncated results, in units of ng/mL.
d. At least two positive controls must meet these criteria for all compounds for the batch to be accepted.

36.10.2.3 The urine positive control shall meet those criteria in 36.10.2.2.a and 36.10.2.2.b, above. The quantitative results, when calculated against the blood curve shall be within ±20% of the target value (0.5 ng/mL).

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

36.11 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.

36.11.1 Any chromatographic peak for BUP, NBP and NLX shall appear symmetrical.

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

36.11.3 The quantitative results for BUP, NBP and NLX must be within the dynamic range of the test method.

36.11.4 Target compounds in urine specimens must meet those criteria in 36.11.1 and 36.11.2 for qualitative reporting.

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

36.12 REPORTING

36.12.1 Results for BUP, NBP and NLX are reported in units of nanograms per milliliter (ng/mL).

36.12.1.1 Results are truncated to two significant figures for reporting.

a. Example 1: BPN is measured as 4.82 ng/mL.

b. The result is truncated to 4.8 ng/mL (two significant figures) and reported.

c. Example 2: NLX is measured as 16.18 ng/mL.

d. The result is truncated to 16.1 ng/mL, but reported as 16 ng/mL (two significant figures).

36.12.2 Qualitative results for all target compounds are reported for urine values ≥0.5 ng/mL (cutoff level).

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

36.13 METHOD PERFORMANCE

36.13.1 Limit of detection: BUP, NLX – 0.05 ng/mL
NBP – 0.2 ng/mL

36.13.2 Lower limit of quantification: BUP, NBP, NLX – 0.2 ng/mL

36.13.3 Dynamic range: 0.2 – 50 ng/mL

36.13.4 Upper limit of quantitation: 50 ng/mL

36.13.5 Upper limit of linearity: 120 ng/mL

36.14 TRACEABILITY

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

36.15 REFERENCES

36.15.1 A. Black, B.E. O'Reilly, D. Sklerov, in-house development.

36.15.2 United Chemical Technologies (UCT) Solid Phase Extraction Applications Manual (2013) 149-150.

36.15.3 I. Dioumaeva, LC/MS/MS of Buprenorphine in Whole Blood Using Agilent Bond Elut Plexa PCX and an Agilent Poroshell 120 column, Agilent Application Note 5990-9930EN, Feb 2013.

APPENDIX A
INSTRUMENTAL PARAMETERS

Agilent LC-MSMS System

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid
Solvent B	ACN
Initial composition	85% A, 15% B
0 – 2.0 min	70% B
2.0 – 2.1 min	95% B
2.1 – 5.5 min	95% B
5.5 – 5.51 min	15% B
Post time	4.0 min
Column temp	40°C
Autosampler	
Injection volume	10 µL
Injection flush-port	Active
Flush-port time/volume	10 sec/1 mL
Flush-port solvent	75:25 MeOH:DI H_2O

MASS SPECTROMETER

Scan type	(+) MRM	Nebulizer gas	Nitrogen
Ion mode	ESI	Nebulizer pressure	35 psi
Peak width	0.07	Drying gas	Nitrogen
Resolution (MS1)	Wide	Drying gas flow	12 L/min
Resolution (MS2)	Unit	Drying gas temp	350 °C
Dwell time	50 msec	Capillary voltage	2.8 kV
Time segment 1 (Time 0 min)	To waste		
Time segment 2 (Time 0.3 min)	To MS (EMV +400)		
Time segment 3 (Time 5.6 min)	To waste		

Compound	MRM Transitions
Naloxone-d5	333.2→315.1, 212.0
Naloxone	328.1→310.0, 212.0
Norbuprenorphine-d3	417.3→83.1, 57.1
Norbuprenorphine	414.3→83.1, 57.1
Buprenorphine-d4	472.4→59.1, 400.2
Buprenorphine	468.3→55.1, 396.1

Shimadzu/Sciex LC-MSMS System

SHIMADZU LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid
Solvent B	ACN
Initial composition	85% A, 15% B
0 – 2.0 min	70% B
2.0 – 2.1 min	95% B
2.1 – 5.5 min	95% B
5.5 – 5.51 min	15% B
Post time	4.0 min
Column temp	40°C
Autosampler	
Injection volume	10 µL
Rinsing volume	1000 µL
Rinsing solvent	75:25 MeOH:DI H_2O
Cooler temperature	25ºC

SCIEX MASS SPECTROMETER

Scan type	(+) MRM	Curtain/collision gas	Nitrogen
Ion mode	ESI	Curtain gas flow	30 L/min
Resolution (Q1)	Unit	Collision gas flow	6 L/min
Resolution (Q3)	Unit	Gas 1 temp	70ºC
Dwell time	50 msec	Gas 2 temp	70ºC
Valve position A (Time 0 min)	To waste	Ion voltage	2.0 kV
Valve position B (Time 0.1 min)	To MS	Interface temp	550ºC
Valve position A (Time 4.5 min)	To waste		

Compound	MRM Transitions
Naloxone-d5	333.1→315.1, 212.2
Naloxone	328.1→310.1, 212.0
Norbuprenorphine-d3	417.0→83.1, 55.1
Norbuprenorphine	414.0→83.3, 57.2
Buprenorphine-d4	472.1→59.1, 400.2
Buprenorphine	468.1→55.2, 396

LIST OF CHANGES

Revision Date	Description	Page Number
09/14/15	Method approved by Washington State Toxicologist. See DRA dated 8/25/15. Method released for use in evidentiary testing as of 09/14/15.	All
4/18/16	Added note regarding CRM expiration dates in 36.6.1.3 and 36.6.1.4 and clarification to 36.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Edited 36.12.1 and removed example in 36.12.1.1 e-f to reflect that only two significant figures are used for reporting. Other minor edits throughout.	1, 4, 9-11