INTEROFFICE COMMUNICATION

WASHINGTON STATE PATROL

TO: Dr. Fiona J. Couper, Toxicology Laboratory Division

FROM: Ms. Amanda Black, Toxicology Laboratory Division

SUBJECT: Diphenhydramine and dextromethorphan quantitation

DATE: June 13, 2019

The confirmation test method "*Confirmation of select basic drugs by liquid chromatography – tandem mass spectrometry*" (TCb12744) was validated and approved for use in casework as of 8/7/2018. The method is used for quantitation/confirmation of bupropion, citalopram, venlafaxine, o-desmethylvenlafaxine, diphenhydramine, dextromethorphan and cyclobenzaprine.

During method validation, whole blood pooled controls were prepared, with replicates analyzed, at multiple levels (30, 400, 800, 1500 and 5000 ng/mL). Spiked control replicates were also prepared at 30, 400 and 800 ng/mL. The within-run and between-day precision and accuracy results from analysis of both pooled and spiked replicates across the range of the calibration curve are attached (as reviewed with validation records 7/2018). Performance in pooled and spiked replicates was acceptable for all compounds, including for dilutions of high concentration pools (1500 and 5000 ng/mL).

Prior to the implementation of the LC-MSMS test method, the GC-NPD/GC-MS SIM test method *"Basic Drug Identification/Confirmation by Gas Chromatography – Mass Spectrometry/Nitrogen Phosphorus Detection"* (TCb12714) was used for quantitation of bupropion, citalopram, venlafaxine, tramadol, diphenhydramine and dextromethorphan. Use of the GC method was discontinued 8/7/2018 (upon implementation of the LC-MSMS test method), except for NPD quantitation of tramadol and SIM analysis of tramadol, methadone and lidocaine.

Since the implementation of the LC-MSMS method, results in case specimens have been checked for agreement with estimated concentrations from the basic drug screen, and have been routinely consistent with screening estimates. However, Forensic Scientists recently attempting LC-MSMS test method certification have experienced low recovery of diphenhydramine and dextromethorphan in spiked test samples, when compared to target concentrations, particularly in samples with target concentrations greater than 1000 ng/mL (analyzed at full volume and dilution). This occurred with multiple scientists, different preparations of standard/internal standard solutions and different LC-MSMS instruments.

LC-MSMS materials, equipment, instrumentation and test method acquisition/data analysis have been evaluated to determine the cause, but no specific issue has been identified. While evaluation of the LC-MSMS method continues, testing was performed to demonstrate that the GC-NPD/SIM test method previously used for diphenhydramine and dextromethorphan quantitation is appropriate for use and to verify continued performance of the LC-MSMS test method for bupropion, citalopram and venlafaxine (o-desmethylvenlafaxine and cyclobenzaprine are not included in the GC-NPD/SIM method).

Forensic Scientist Asa Louis analyzed a batch of case specimens with the LC-MSMS and GC-NPD test methods, and Andrew Gingras analyzed the lower-concentration cases with the GC-MS SIM test method. The resulting data supports that the GC-NPD and GC-MS SIM methods previously used for quantitation of diphenhydramine and dextromethorphan (until replaced with LC-MSMS) continue to be fit-for-purpose. The data also confirms performance of the LC-MSMS method for bupropion, citalopram and venlafaxine. A summary spreadsheet of comparison values is attached for review.

To gather additional information on LC-MSMS performance, Asa analyzed a dilution control in the LC-MSMS batch, which contained diphenhydramine and venlafaxine spiked to a target of 2000 ng/mL in 2 mL whole blood (analyzed at full volume and 1:10 dilution). Both dilution and full volume results were within ±20% of the spiked target.

Performance of diphenhydramine and dextromethorphan in the GC-NPD and GC-MS SIM testing batch demonstrate this method continues to be fit-for-purpose for quantitative analysis of these compounds.

Results of the case comparisons confirm performance of the new LC-MSMS method for case specimens. However, since the performance of spiked certification samples remains inconsistent for diphenhydramine and dextromethorphan, I recommend use of the GC-NPD or GC-MS SIM methods for quantitation of these compounds, pending further evaluation of LC-MSMS method. Results for diphenhydramine and dextromethorphan may be reported qualitatively from the LC-MSMS method.

AB:ab

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Concur. Recommendations approved.

6-14-19

CONFIRMATION OF SELECT BASIC DRUGS BY LIQUID CHROMATOGRAPHY - TANDEM MASS SPECTROMETRY

44.1 METHOD

Quantitation of diphenhydramine and dextromethorphan performed using TCb12714 - approved as of 6/14/19. Both compounds reported qualitatively from this method. AB 6/18/19

This test method may be used to confirm the presence of select basic drugs in biological samples. Bupropion (BPN), citalopram (CIT), cyclobenzaprine (CBZ), dextromethorphan (DXM), diphenhydramine (DPH), venlafaxine (VEN) and O-desmethylvenlafaxine (ODV) and internal standards (BPN-d_9, CBZ-d_3,VEN-d_6 and ODV-d_6) are isolated from biological matrices by solid phase extraction (SPE). The extracts are injected into a high performance liquid chromatograph (HPLC) coupled to a tandem mass spectrometer (MS-MS) detector equipped with an atmospheric pressure electrospray ionization source.

44.2 SPECIMENS

The specimen volume is 0.5 mL. Specimens include, but are not limited to, whole blood, serum, plasma, urine, and tissue homogenate. Dilutions of specimens may be analyzed at the Forensic Scientist's discretion.

The presence of ketamine in a specimen may cause interference with both transitions for bupropion. Where a specimen contains both ketamine and bupropion, an alternative test method must be used for qualitative/quantitative analysis of bupropion.

NOTE: Method validation established that matrix-matching of the full calibration curve and all positive control levels is required for quantitation in serum/plasma specimens or liver (tissue) homogenate specimens (see 44.4.2 and 44.4.3).

44.3 REAGENTS, MATERIALS AND EQUIPMENT

44.3.1 REAGENTS

NOTE: Unless use of LC-MS grade (or equivalent from a high-purity filtration system) deionized water (DI H_2O) is specified, laboratory general-use DI H_2O is used in reagent preparation. Organic solvents are reagent grade unless otherwise specified.

- Acetic acid (glacial)
- 0.1M Acetic acid

  Add 5.72 mL glacial acetic acid to 800 mL DI H_2O. Dilute to 1 L with DI H_2O and mix. Store the solution in a glass bottle for up to six months.
- Acetonitrile (ACN), reagent and LC-MS grade
- Ammonium hydroxide (concentrated)
- Certified blank blood and/or other biological matrices
- DI H_2O, laboratory general-use and LC-MS grade (or equivalent from a high purity filtration system)

- Elution solvent

  To 20 mL isopropanol, add 2 mL concentrated ammonium hydroxide and mix. Add 78 mL methylene chloride and mix. Store the solvent in a glass flask/bottle at room temperature and use on date of preparation only.

- Formic acid (concentrated)
- 0.1% Formic acid

  Add 1 mL of concentrated formic acid to 800 mL LC-MS grade DI H_2O in a 1 L flask. Dilute to 1 L with LC-MS grade DI H_2O and mix. Filter this solution prior to use on the HPLC. Store the acid in an amber glass bottle at room temperature for up to one year.

- Isopropanol (IPA)
- Methanol (MeOH), HPLC grade
- Methylene chloride (dichloromethane, CH_2Cl_2)
- 0.1M Phosphate buffer (pH6)

  Dissolve 1.7 g Na_2HPO_4 and 12.14 g $NaH_2PO_4 \bullet H_2O$ 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 buffer in a glass bottle at room temperature for up to one year.

- Sodium hydroxide (NaOH), concentrated
- Sodium phosphate, dibasic anhydrous (Na_2HPO_4)
- Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \bullet H_2O$)

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

44.3.2 MATERIALS

- Disposable extraction tubes (16 x 100mm recommended) and screw-cap or centrifuge tubes with closures
- Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206 200 mg/6 mL), or equivalent
- HPLC Column, Phenomenex Kinetex® 2.6µm Biphenyl 100Å, 50x30mm, or equivalent
- Laboratory glassware (graduated cylinders, flasks)
- Polypropylene autosampler vials with integrated inserts and caps
- Solvent filters (0.45 µm pore size; reduced cellulose, other)

44.3.3 EQUIPMENT

- Shimadzu HPLC, or equivalent
- Sciex API 3200 MS, or equivalent

- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, pH meter or pH paper, solvent filtration apparatus, vacuum manifold, vortex mixer)

44.4 STANDARDS, CALIBRATORS AND CONTROLS

44.4.1 STANDARDS

- Working standard: 10 ng/µL
- Working control standard: 10 ng/µL
- Working internal standard: 1 ng/µL

44.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 44.5 SAMPLE PREPARATION. Quantitation in serum/plasma or liver (tissue) homogenate specimens requires that a calibration curve be prepared in blank matrix. If testing only serum/plasma or liver (tissue) homogenate specimens, a whole blood calibration curve is not required.

44.4.3 CONTROLS

44.4.3.1 At least one negative whole blood control and three positive whole blood controls are tested with every batch, prepared as described in 44.5. For quantitative analysis of serum/plasma or liver (tissue) homogenate specimens only, whole blood controls are not required.

44.4.3.2 Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls.

44.4.3.3 For qualitative analysis of any alternate matrices, one negative control and one positive control must be included for each alternate matrix type tested in the batch.

44.4.3.4 For quantitative analysis of serum/plasma or liver (tissue) homogenate specimens, matrix-matching of the full calibration curve and positive controls (to meet 10% and bracket specimens in that matrix) is required.

44.5 SAMPLE PREPARATION

NOTE: Laboratory general-use DI H_2O is used in sample preparation. 0.1% Formic acid used in reconstitution (44.5.22) is prepared using LC-MS grade DI H_2O (or equivalent). Organic solvents used in sample preparation are reagent grade, with the exception of HPLC grade MeOH.

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

44.5.2 Add 3 mL 0.1M phosphate buffer pH 6 into each tube.

44.5.3 Using a calibrated pipette, add 0.5 mL of certified blank whole blood into each of the calibrator tubes, positive control tubes, and negative control tube(s).

44.5.4 Prepare a 1:10 dilution of the working standard. (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.

44.5.5 Prepare a 1:100 dilution of the working standard. (0.1 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.

44.5.1 Using a calibrated pipette, spike the calibrators according to the following table, using the prepared working standard dilutions.

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS
Calibrator 1 - 10 ng/mL	50	0.1 ng/µL	1:100
Calibrator 2 - 25 ng/mL	125	0.1 ng/µL	1:100
Calibrator 3 - 50 ng/mL	25	1 ng/µL	1:10
Calibrator 4 - 100 ng/mL	50	1 ng/µL	1:10
Calibrator 5 - 250 ng/mL	125	1 ng/µL	1:10
Calibrator 6 - 500 ng/mL	25	10 ng/µL	WS
Calibrator 7 - 1000 ng/mL	50	10 ng/µL	WS

44.5.2 Prepare a 1:10 dilution of the control working standard. (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.

44.5.3 Prepare a 1:100 dilution of the control working standard. (0.1 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.

44.5.4 Using a calibrated pipette, spike the positive controls according to the following table, using the prepared dilutions of the control working standard.

Control Description	Volume (µL) Added	Standard Concentration	Dilution of QC
Control 1 - 30 ng/mL	150	0.1 ng/µL	1:100
Control 2 - 400 ng/mL	20	10 ng/µL	QC
Control 3 - 800 ng/mL	40	10 ng/µL	QC

44.5.5 Using a calibrated pipette, sample 0.5 mL of each case specimen into its respective tube.

44.5.6 Using a calibrated pipette or verified repeater-pipette, add 125 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 250 ng/mL.

44.5.7 Cap the tubes and briefly vortex mix.

44.5.8 Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).

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

44.5.10 Condition the SPE columns by passing each of the following reagents/solvents completely through under force of gravity.

a. 3 mL MeOH
b. 3 mL DI H_2O
c. 2 mL 0.1M phosphate buffer pH 6

Do not let columns dry out between each conditioning step.

44.5.11 Transfer the contents of each extraction tube to its respective SPE column and allow to flow through under force of gravity. (Moderate, positive pressure or vacuum may be applied if the flow is insufficient.)

44.5.12 Wash the SPE columns by passing each of the following reagents/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 acetic acid
c. 3 mL MeOH

44.5.13 Dry the columns for 10 minutes under vacuum.

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

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

44.5.16 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 40°C.

44.5.17 Reconstitute the extracts with the addition of 500 µL mobile phase (90:10 0.1% formic acid: MeOH) to each tube and briefly vortex mix. If necessary, centrifuge the tubes for 2 minutes at 2000 rpm to collect the extracts at the bottom of the tubes.

44.5.18 Transfer approximately 200µL of the extracts to labeled polypropylene autosampler vials with integrated inserts and cap.

44.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – BASIC (instrumental parameters in Appendix A)
- Calibration curve – quadratic, 1/a weighting factor
- Updating calibrator (retention times ±3%, ion ratios ±20%) – Cal 4
- Result comparisons –

  Cal 1: truncated to one decimal place in units of ng/mL (acceptable range 7.5 – 12.5 ng/mL)

  Cals 2-7, Ctls 1-3: truncated, whole integer values in units of ng/mL

Quadratic calibration curves must include all calibration points. Removal of one or more calibration points from a target compound calibration curve will prohibit quantitative reporting for that compound.

NOTE: Target compound O-desmethylvenlafaxine is isomeric with tramadol. A peak for O-desmethylvenlafaxine qualifier transition m/z 246.3, at a retention time outside the range of acceptability, may appear in specimens that contain tramadol.

44.7 REPORTING

Quantitation of diphenhydramine and dextromethorphan performed using TCb12714 - approved as of 6/14/19. Qualitative reporting of those compounds from this assay. AB 6/18/19

Results are converted from units of nanograms per milliliter (ng/mL) to units of milligrams per liter (mg/L), and truncated to two significant figures for reporting.

44.8 METHOD PERFORMANCE

- Limit of detection: 5 ng/mL (0.05 mg/L)
- Lower limit of quantification: 10 ng/mL (0.01 mg/L)
- Dynamic range: 10 – 1000 ng/mL (0.01 – 1.0 mg/L)
- Upper limit of quantitation: 1000 ng/mL (1.0 mg/L)

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARDS

Bupropion
Bupropion-d_9
Citalopram
Cyclobenzaprine
Cyclobenzaprine-d_3
Dextromethorphan
Diphenhydramine
O-desmethylvenlafaxine
O-desmethylvenlafaxine-d_6
Venlafaxine
Venlafaxine-d_6

APPENDIX B
INSTRUMENTAL PARAMETERS

LIQUID CHROMATOGRAPH

Gradient Elution	
Flow rate	0.5 mL/min
Solvent A	0.1% Formic acid
Solvent B	MeOH
Initial composition	90% A, 10% B
0-1.00 min	10% B
1.00-6.00 min	98% B
6.00-8.00	98% B
8.00-8.10	10% B
8.10-10.00	10% B
Column temp	40°C
Autosampler	
Injection volume	5.0 µL
Rinsing Volume	1000 µL
Flush-port solvent	75:25 MeOH: DI H_2O
Cooler Temperature	25°C

MASS SPECTROMETER

Scan Mode	(+) sMRM	Curtain/collision gas	Nitrogen
Ion Mode	ESI	Curtain Gas flow	30.0 L/min
Peak width	0.05 min	Collision gas flow	6.00 L/min
Resolution (MS1)	Unit	Gas 1 Temp	60°C
Resolution (MS2)	Unit	Gas 2 Temp	50°C
MRM detection window	30 sec	Ion Voltage	2.0kV
Target Scan Time	1.0 sec	Interface Temp	550°C
Time Segment 1	To Waste		
Time Segment 2	To MS		
Time Segment 3	To Waste		

Compound	MRM Transitions
bupropion	240.0 → 184.2, 131.2
bupropion-d_9	249.1 → 185.1, 131.2
citalopram	325.0 → 262.2, 109.2
cyclobenzaprine	276.0 → 216.1, 189.2
cyclobenzaprine-d_3	279.0 → 231.2, 189.2
dextromethorphan	272.1 → 128.1, 171.2
diphenhydramine	256.1 → 152.2, 167.2
O-desmethylvenlafaxine	264.1 → 107.2, 246.3
O-desmethylvenlafaxine-d_6	270.2 → 252.3, 107.2
venlafaxine	278.1 → 260.2, 121.2
venlafaxine-d_6	284.1 → 266.3, 78.2

LIST OF CHANGES

Revision Date	Description	Page Number
8/7/18	Method approved by Washington State Toxicologist. See DRA dated 8/3/18. Method released for use in evidentiary testing as of 8/7/18.	All