IDENTIFICATION AND CONFIRMATION OF SELECT ACIDIC AND NEUTRAL DRUGS BY GAS CHROMATOGRAPHY

17.1 POLICY

This test method may be used to identify and/or confirm the presence of select acidic and neutral drugs in biological samples. Quantitative results obtained through the use of this method will only be reported within the dynamic range. 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 either the State Toxicologist, a Manager, or a Supervisor, and appropriately documented in the batch file.

17.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and/or quantification of select acidic and neutral drugs 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.

17.3 PRINCIPLE

The targeted compounds and internal standard, cyclopentobarbital (cyclopal), 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 gas chromatograph (GC) where they are separated between a gaseous mobile and liquid stationary phase. Each compound exits the GC at a reproducible time which is termed its retention time.

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.

For secondary identification/confirmation, samples may be injected into a GC equipped with a mass spectrometer (MS) detector equipped with an electron ionization source. As each compound is ionized in the source, it measures the mass-to-charge ratios of each compound and its related fragments.

17.4 SPECIMENS

17.4.1 The specimen volume is 1 mL.

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

17.4.3 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.

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

17.5 REAGENTS, MATERIALS AND EQUIPMENT

17.5.1 REAGENTS

17.5.1.1 Acetone

17.5.1.2 Acetonitrile

17.5.1.3 Certified blank blood

17.5.1.4 Deionized water (DI H_2O)

17.5.1.5 Ethyl acetate

17.5.1.6 Heptanes

17.5.1.7 Methanol

17.5.1.8 Washed Amberlite XAD-2 resin (see Appendix B for preparation instructions)

17.5.2 MATERIALS

17.5.2.1 Autosampler vials, inserts and caps

17.5.2.2 Disposable 16 x 125 mm tubes with closures

17.5.2.3 Disposable screw-cap tubes or conical centrifuge tubes with closures

17.5.2.4 Disposable pipette tips

17.5.2.5 GC column (Agilent HP-5; 30 m x 0.250 mm i.d. x 0.250 μm film thickness, or equivalent)

17.5.2.6 Laboratory glassware (graduated cylinders, flasks)

17.5.2.7 pH test paper

17.5.2.8 Volumetric glassware (flasks)

17.5.3 EQUIPMENT

17.5.3.1 Agilent GC (6890 or equivalent)

17.5.3.2 Agilent MS (5973 or equivalent)

17.5.3.3 Calibrated, adjustable air-displacement pipettes

17.5.3.4 Centrifuge

17.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

17.5.3.6 Magnetic stir plate, magnetic stir bars

17.5.3.7 Vacuum aspirator

17.5.3.8 Vortex mixer

17.6 STANDARDS AND CONTROLS

17.6.1 STANDARDS

17.6.1.1 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, positive controls and the working internal standard.

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

a.	Acetaminophen	5.0 mg/mL
b.	Carbamazepine	1.0 mg/mL
c.	Carisoprodol:	1.0 mg/mL
d.	Cyclopal (IS)	0.1 mg/mL
e.	Ibuprofen:	5.0 mg/mL
f.	Lamotrigine:	1.0 mg/mL
g.	Meprobamate:	1.0 mg/mL
h.	Phenytoin:	1.0 mg/mL
i.	Topiramate:	1.0 mg/mL

NOTE: Working standard may be prepared using certified methanolic standards (as described in 17.6.1.4) or directly from powdered reference materials (as described in 17.6.1.3). Cyclopal working internal standard is prepared directly from powder.

17.6.1.3 Working standard (0.1 mg/mL, 0.5 mg/mL ibuprofen, acetaminophen)

a. Powdered reference materials are weighed using a calibrated balance, with amounts based on total volume of working standard prepared and the certificate of analysis for each compound (adjust weight for salt, purity). Transfer each to a 100 mL class-A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working control standard is 0.1 mg/mL. The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Adjustments to final volume are permitted provided that proportions are maintained.

17.6.1.4 Working control standard (0.1 mg/mL)

d. Using a calibrated pipette, measure 2.5 mL each of carbamazepine, lamotrigine, phenytoin and topiramate stock standards into a 25 mL class-A volumetric flask.
e. Add methanol to the flask to the designated volume.
f. The final concentration of the working control standard is 0.1 mg/mL. The working control standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Adjustments to final volume are permitted provided that proportions are maintained.

17.6.1.5 Working internal standard (0.1 mg/mL)

a. Using a calibrated balance, weigh 20 mg cyclopal and transfer to a 200 mL 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 mg/mL. The working internal standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Adjustments to final volume are permitted provided that proportions are maintained.

17.6.2 CALIBRATORS

17.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 17.7 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.

17.6.3 CONTROLS

17.6.3.1 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.)

17.6.3.2 Positive Controls

a. Two 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 or supplier must be used, the working control standard should be prepared by someone other than the person that prepared the working standard.
d. The working control standard (0.1 mg/mL) is prepared as described in 17.6.1.4.
e. The preparation of the positive whole blood controls is detailed in 17.7 SAMPLE PREPARATION. Alternatively, quality control 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.

17.7 SAMPLE PREPARATION

17.7.1 Label a clean 16 x 125 mm tube for each member of the test batch. (i.e. calibrator, control, case sample)

17.7.2 Add approximately 1 g washed XAD resin to each tube.

17.7.3 Add 5 mL DI H_2O to each tube.

17.7.4 Add 1 mL certified blank blood to each of the calibrator, positive control and negative control tubes.

17.7.5 Use the working standard to spike the calibrators according to the following table.

Calibrator Description	Volume (µL) Added
Calibrator 1 (5.0/25 mg/L)	50
Calibrator 2 (10/50 mg/L)	100
Calibrator 3 (20/100 mg/L)	200

17.7.6 Use the working control standard to spike the positive controls according to the following table.

Control Description	Volume (µL) Added
Low Control (7.5 mg/L)	75
High Control (15 mg/L)	150

17.7.7 Add 1 mL of each case sample into its respective tube.

17.7.8 Add 100 µL of the working internal standard to each tube. Final concentration in each tube is 10 mg/L.

17.7.9 Cap the tubes and thoroughly vortex mix for at least 60 seconds. Allow tubes to stand for 60 seconds.

17.7.10 If XAD is well settled, aspirate the blood from each tube using appropriate waste disposal procedure. If necessary, centrifuge tubes at 2500 rpm for 5 minutes to pack XAD at bottom of tubes.

17.7.11 Add 6 mL ethyl acetate to each tube, cap and vortex mix for at least 60 seconds.

17.7.12 Centrifuge at 2500 rpm for 5 minutes to pack XAD at bottom of tubes.

17.7.13 Pour over the ethyl acetate layer into clean, labeled conical or screw cap tubes.

17.7.14 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C.

17.7.15 Reconstitute extracts by the addition of 75 µL acetonitrile and wash with 500 µL heptanes. Cap tubes and briefly vortex mix.

17.7.16 Centrifuge tubes at 2000 rpm for 5 minutes to separate layers.

17.7.17 Aspirate the heptanes layer to chemical waste.

17.7.18 Transfer the acetonitrile layer to labeled autosampler vials and cap.

17.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence table by first setting the data path in ChemStation to the date of the test. After entering all vial locations, sample descriptions, comments and/or lot numbers in the sequence table,

ensure the method listing in the table is TOXAN.M for each line. For GC-MS analysis, ensure the method listing is TOX-MS3.M for each line.

17.9 DATA ANALYSIS

17.9.1 Analysis of the batch data is conducted using the instrumental data analysis software in ChemStation.

17.9.2 Quantitative calculations are generated by internal standard, multi-point, linear regression with equal weighting. The calibration curves are updated using the calibrator results for the batch; no historical calibration curves are permitted.

17.9.3 Printed reports for each vial in the batch are generated for review.

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

17.10 CRITERIA FOR BATCH ACCEPTANCE

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

17.10.1 Calibrators and calibration curves

17.10.1.1 Chromatographic peaks for target compounds and internal standard shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

17.10.1.2 Retention times shall be within ±2% of those in calibrator 3. These are inclusive ranges.

17.10.1.3 Quantitative results for target compounds 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. Result comparisons will use values truncated after the first decimal place in units of mg/L.

17.10.1.4 The calibration curves for target compounds shall have correlation coefficients ≥0.99.

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

17.10.2 Controls

17.10.2.1 The negative control(s) shall not identify any target compound. Identification is based on a) acceptable retention time matching, b) distinct peaks for prominent ions present in mass spectrum.

17.10.2.2 Positive controls

a. Chromatographic peaks for target compounds and internal standard shall appear symmetrical.
b. Retention times shall be within ±2% of those in calibrator 3 for each compound.
c. Quantitative results for target compounds in each control shall be within ±20% of their target values. These are inclusive ranges.

Result comparisons will use values truncated after the first decimal place in units of mg/L.

d. The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.
e. At least one positive control must meet these criteria for all compounds for the batch to be accepted.

17.11 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.

17.11.1 Quantitative acceptance

17.11.1.1 Any chromatographic peak for target compounds shall appear symmetrical.

17.11.1.2 The retention times for any reportable compounds are ±2% of those in calibrator 3.

17.11.1.3 The quantitative results for each identified compound must be within the dynamic range of the test method.

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

17.11.2 Qualitative acceptance

17.11.2.1 Any chromatographic peak for target compounds identified shall appear symmetrical.

17.11.2.2 The retention times for any identified compounds are ±2% of those in calibrator 3.

17.11.2.3 Mass spectra are generated for the target compound in both the case sample and calibrator 3 for spectral pattern comparison. A spectrum from an approved mass spectral library may also be used for comparison.

17.12 REPORTING

17.12.1 Quantitative reporting

17.12.1.1 Results are reported in units of milligrams per liter (mg/L).

17.12.1.2 Results are truncated to no more than two significant figures for reporting.

a. Example 1: carbamazepine is measured as 9.88 mg/L.

b. The result is truncated to 9.8 mg/L (two significant figures) and reported.

c. Example 2: phenytoin is measured at 13.91 mg/L.

d. The result is truncated to 13.9 mg/L, but reported as 13 mg/L (two significant figures).

e. Example 3: topiramate is measured at 10.03 mg/L.

f. The result is truncated to 10.0 mg/L (three significant figures), but reported as 10 mg/L (one significant figure).

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

17.12.1.4 Compounds contained in the positive controls (carbamazepine, phenytoin, topiramate and lamotrigine) may be quantified using this assay. All other compounds may be reported qualitatively (provided that criteria in 17.12.2 are met) or quantified with a separate method.

17.12.2 Qualitative reporting

17.12.2.1 To appropriately identify and report a compound as present in a case sample, the following must be demonstrated:

a. Chromatography and retention times for target compounds must meet criteria for acceptance found in 17.11.2.

b. When using a library match, spectrum agreement should be 75 or greater wherever possible, taking into consideration the appearance and abundance of ions specific to that compound (an extracted ion match may be necessary). A reference spectrum for the compound found in a published article, research paper or other reference material may be acceptable if an electronic library match is not feasible, provided the source is documented.

17.12.2.2 Any compounds initially identified using this method must be confirmed using this or another test method on a separate sampling before reporting.

17.13 METHOD PERFORMANCE

17.13.1 Lower limit of quantification: 5.0 mg/L

17.13.2 Dynamic range: 5.0 mg/L – 20 mg/L

17.13.3 Upper limit of quantification: 20 mg/L

17.14 TRACEABILITY

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

APPENDIX A
INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH – FLAME IONIZATION DETECTOR

Split/Splitless Inlet	
Mode	Split
Inlet Liner	4mm splitless w/glass wool plug
Split Ratio	60:1
Temperature	260°C
Gas Type	Helium
Gas Saver	On
Gas Saver Flow	20.0 mL/min
Gas Saver Time	2.0 min

Oven/Column	
Carrier Gas Mode	Constant Pressure
Inlet Pressure	16.9 psi
Initial Temperature	120°C
Initial Time	6.0 min
Ramp Rate	10°C/min
Final Temperature	220°C
Hold Time	0.0 min
Ramp Rate	20°C/min
Final Temperature	300°C
Hold Time	1.2 min
Run Time	21.2 min

Autosampler	
Injection Volume	2.0 µL
Solvent Wash A	6 (Methanol)
Solvent Wash B	6 (Methanol)
Sample Pumps	4

FID Parameters	
Heater	300°C
H_2 Flow	40.0 mL/min
Air Flow	300 mL/min
Makeup Flow	40.0 mL/min

GAS CHROMATOGRAPH - MASS SPECTROMETER

GAS CHROMATOGRAPH

Split/Splitless Inlet	
Mode	Split
Inlet Liner	4mm splitless w/glass wool plug
Split Ratio	40:1
Temperature	260°C
Gas Type	Helium
Gas Saver	On
Gas Saver Flow	20.0 mL/min
Gas Saver Time	2.0 min

Oven/Column	
Carrier Gas Mode	Constant Flow
Carrier Gas Flow	1.6 mL/min
Initial Temperature	60°C
Initial Time	0.0 min
Ramp Rate	10°C/min
Final Temperature	220°C
Hold Time	0.0 min
Ramp Rate	20°C/min
Final Temperature	300°C
Hold Time	1.0 min
Run Time	21.0 min

Autosampler	
Injection Volume	2.0 µL
Solvent Wash A	5 (Methanol)
Solvent Wash B	5 (Methanol)
Sample Pumps	4

MASS SPECTROMETER

Solvent Delay	11.0 min	MS Quad Temperature	150°C
EM Offset	Set in tune	MS Source Temperature	230°C
Scan Range	50 – 500	Threshold	100

APPENDIX B
AMBERLITE XAD-2 RESIN PREPARATION

1. Empty one container of XAD-2 resin into a 2 L glass beaker.
2. Add approximately 1600 mL DI H_2O and 100 mL methanol.
3. Place beaker on a magnetic stir plate and mix for about an hour with a magnetic stir bar.
   NOTE: As XAD is washed, the stir bar tends to stop and may need to be checked periodically.
4. Carefully pour off the DI H_2O/methanol mixture into chemical waste.
5. Wash with DI H_2O, repeating until pH neutral (test with pH paper), usually about three washes (water-only washes can be poured down the sink).
6. Add approximately 1600 mL acetone to the beaker and mix on the magnetic stir plate for about an hour, then carefully pour off the solvent into chemical waste.
7. Add approximately 1600 mL ethyl acetate to the beaker and mix on the magnetic stir plate for about an hour, then carefully pour off solvent into chemical waste.
8. Repeat step 7.
9. Allow to dry in a hood overnight (lightly cover with a watch glass).
10. If resin becomes too dry, add sufficient ethyl acetate until resin is damp.
11. Transfer resin to labeled amber glass containers with screw caps.

LIST OF CHANGES

Revision Date	Description	Page Number
04/22/13	Standard operating procedure reformatted and assigned document ID. See DRA dated 04/17/13.	All