IDENTIFICATION/CONFIRMATION OF VALPROIC ACID IN BIOLOGICAL SPECIMENS BY GAS CHROMATOGRAPHY

20.1 POLICY

This test method may be used to identify and/or confirm the presence of valproic acid (VPA) 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.

20.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the identification and quantitation of valproic acid 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 compound.

20.3 PRINCIPLE

The targeted compound and internal standard, 2-cyclohexylacetic acid (CHX), 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 valproic acid 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 and 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.

20.4 SPECIMEN

20.4.1 The specimen volume is 1 mL.

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

20.4.3 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.

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

20.5 REAGENTS, MATERIALS AND EQUIPMENT

20.5.1 REAGENTS

20.5.1.1 Acetate buffer (pH 4.8)

Dissolve 6 g sodium acetate trihydrate in 400 mL DI H_2O. Add 1.7 mL glacial acetic acid. Dilute to 500 mL with DI H_2O and mix. Check pH and, if necessary, adjust to 4.8 ±0.2. Store the buffer in glass or plastic bottle at room temperature for up to one year. Volumes may be adjusted, provided that proportions remain constant.

20.5.1.2 Acetic acid (Glacial)

20.5.1.3 Acetone

20.5.1.4 Amberlite XAD-2 resin (see appendix B for preparation instructions)

20.5.1.5 Certified blank blood

20.5.1.6 Chloroform

20.5.1.7 Ethyl acetate

20.5.1.8 Deionized water (DI H_2O)

20.5.1.9 Hydrochloric acid (HCl, concentrated)

20.5.1.10 3N Hydrochloric acid

Add 600 mL DI H_2O to a 1 L flask. Add 250 mL concentrated HCl and dilute to 1 L with DI H_2O. Store in a glass container at room temperature for up to one year. Adjustments to final volume are permitted as long as proportions are maintained.

20.5.1.11 Methanol

20.5.1.12 Sodium acetate trihydrate

20.5.1.13 Sodium hydroxide (NaOH, 10N, concentrated)

20.5.1.14 0.5N Sodium hydroxide

Add 800 mL DI H_2O to a glass flask. Add 50 mL concentrated NaOH (10N). Dilute to 1 L with DI H_2O and mix. Store the solution in a glass or plastic bottle at room temperature for up to one year. Adjustments to final volume are permitted as long as proportions are maintained.

20.5.1.15 UTAK Bi-Level Anticonvulsants mid-range serum toxicology control or serum toxicology control UTAK Lamotrigine Plus

20.5.2 MATERIALS

20.5.2.1 Autosampler vials, inserts and caps

20.5.2.2 Disposable 16 x 125mm tubes with closures

20.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

20.5.2.4 Disposable pipette tips

20.5.2.5 GC column (Agilent HP-5; 30 m x 0.32 mm i.d. x 0.25 μm film thickness, or equivalent)

20.5.2.6 GC column (Agilent HP-5MS; 30 m x 0.25 mm x 0.25 film thickness, or equivalent)

20.5.2.7 Laboratory glassware (graduated cylinders, flasks)

20.5.2.8 Volumetric glassware (flasks)

20.5.3 EQUIPMENT

20.5.3.1 Agilent GC (6890 series or equivalent)

20.5.3.2 Agilent MS (5973 series or equivalent)

20.5.3.3 Calibrated, adjustable piston pipettes

20.3.3.4 Centrifuge

20.3.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

20.5.3.6 pH Meter and/or indicating pH paper

20.5.3.7 Vacuum aspirator

20.5.3.8 Vortex mixer

20.6 STANDARDS, CALIBRATORS AND CONTROLS

20.6.1 STANDARDS

20.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of the calibrators.

20.6.1.2 Stock standards are purchased from an approved reference material supplier and include the following:

a. Valproic acid: 1.0 mg/mL
b. 2-cyclohexylacetic acid

NOTE: 2-cyclohexylacetic acid internal standard is purchased as a solid reference material and weighed at the time of the working internal standard preparation. Valproic acid may be purchased as a solid reference material and weighed at time of the working control standard preparation (alternatively, a UTAK serum control is used).

20.6.1.3 Working internal standard (1.0 mg/mL)

a. Using a calibrated balance, weigh 10 mg of 2-cyclohexylacetic acid and transfer to a 10mL class A volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working internal standard is 1.0 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.

20.6.1.4 Working control standard (1.0 mg/mL)

In lieu of use of a UTAK serum positive control, a working control standard may be prepared and used in preparation of a whole blood positive control.

a. Using a calibrated balance, weigh 25 mg of valproic acid and add to a class-A 25 mL volumetric flask.
b. Add methanol to the flask to the designated volume.
c. The final concentration of the working control standard is 1.0 mg/mL. The working control standard is stored in the freezer in an amber bottle and expires one year from the date of preparation. Volumes may be adjusted provided that proportions remain constant.

20.6.2 CALIBRATORS

20.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the stock standard. The preparation of the calibrators is detailed in 20.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been determined to not contain any of the compounds tested for by this procedure.

20.6.3 CONTROLS

20.6.3.1 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.)

20.6.3.2 Positive Controls

a. At least one positive control is tested with every batch. The positive control may be purchased from an approved supplier (UTAK serum) or may be prepared using certified blank blood to which the designated volume of control working standard has been added.
b. The UTAK serum positive control is reconstituted with 5 mL DI H_2O (Bi-Level Anticonvulsants mid-range) or 3 mL DI H_2O (Lamotrigine Plus) using a volumetric pipette. Must be used within 30 days of reconstitution.
c. The preparation of the positive whole blood control is detailed in 20.7 SAMPLE PREPARATION.
d. When testing different sample types, wherever possible, include at least one positive control prepared from that matrix. For example, if testing urine samples, a positive urine control may be prepared by addition of working standard solution to blank urine to achieve the chosen concentration.

20.7 SAMPLE PREPARATION

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

20.7.2 Add approximately 1 g washed XAD-2 resin to each tube.

20.7.3 Add 5 mL DI H_2O to each tube.

20.7.4 Add 1 mL of acetate buffer pH 4.8 into each tube.

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

20.7.6 Prepare a 1:10 dilution of the working standard. (0.1 mg/mL)

a. Using a calibrated pipette, combine 0.1 mL of the working standard with 0.9 mL of methanol or acetonitrile in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

20.7.7 Using the stock standard and prepared dilution, spike the calibrators according to the following table.

Calibrator Description	Volume (µL) Added	Standard Solution Conc. (mg/mL)
Calibrator 1 (10 mg/L)	100	0.1
Calibrator 2 (25 mg/L)	25	1.0
Calibrator 3 (50 mg/L)	50	1.0
Calibrator 4 (100 mg/L)	100	1.0

20.7.8 If a reconstituted serum control is used, transfer 1 mL of the positive serum control to the positive control tube. If a spiked whole blood control is used, add 70 µL of the working control standard to the positive control tube. The control target concentration is 70 mg/L.

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

20.7.10 Add 50 µL of the CHX working internal standard solution to each tube. Final concentration of the internal standard is 50 mg/L.

20.7.11 Cap the tubes and thoroughly vortex mix. Allow tubes to stand for 60 seconds.

20.7.12 If XAD is well-settled, aspirate the liquid from each tube using appropriate waste disposal procedures. If necessary, centrifuge the tubes before aspiration for 5 minutes at 2000 rpm to pack XAD at bottom of tubes.

20.7.13 Add 6 mL ethyl acetate to each tube and thoroughly vortex mix. Centrifuge for 5 minutes at 2000 rpm to pack XAD at bottom of tubes.

20.7.14 Transfer, by pouring, the ethyl acetate to an appropriately labeled, clean centrifuge tube.

20.7.15 Evaporate the extracts at 50°C to approximately 1 mL volume.

20.7.16 Add 200 µL 0.5N NaOH to each tube and vortex mix.

20.7.17 Centrifuge tubes for 5 minutes at 2000 rpm.

20.7.18 Aspirate the ethyl acetate layer using appropriate chemical waste procedures.

20.7.19 Add 50 µL 3N HCl to each tube.

20.7.20 Add 100 µL chloroform to each tube and thoroughly vortex mix.

20.7.21 Centrifuge the tubes for 5 minutes at 2000 rpm.

20.7.22 Transfer the chloroform (bottom) layer to labeled autosampler vials.

20.8 INSTRUMENTAL PARAMETERS

The instrumental parameters can be found in Appendix A. Prepare a sequence table and set 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 that the method listing in the table is VPA for each line. For GC-MS confirmation analysis, ensure the method listing is VPA for each line. [Note: The method name may contain a numeric suffix to differentiate between instruments; for example VPA-MS3.]

20.9 DATA ANALYSIS

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

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

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

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

20.10 CRITERIA FOR BATCH ACCEPTANCE

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

20.10.1 Calibrators and calibration curves

20.10.1.1 Chromatographic peaks for valproic acid and internal standard shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

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

20.10.1.3 Quantitative results for valproic acid in each calibrator shall be within ±20% of the target value with the exception of calibrator 1 which shall be within ±25% of the target. These are inclusive ranges. Result comparisons will use values truncated after the first decimal place in units of mg/L.

20.10.1.4 The calibration curve for valproic acid shall have a correlation coefficient ≥0.99.

20.10.2 Controls

20.10.2.1 The negative control(s) shall not identify valproic acid. Identification is based on a) acceptable retention time matching, b) confirmation with mass spectrum match to library, calibrator or control.

20.10.2.2 Positive controls

a. Chromatographic peaks for valproic acid and internal standard shall appear symmetrical.
b. Retention times shall be within ±2% of those in calibrator 3. These are inclusive ranges.
c. Quantitative results for valproic acid shall be within ±20% of the target value of 70 mg/dL. This is an inclusive range. Result comparison will use values truncated after the first decimal place in units of mg/L.
d. The positive control must meet these criteria for the batch to be accepted.

20.11 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.

20.11.1 Any chromatographic peak for valproic acid and internal standard shall appear symmetrical.

20.11.2 The retention times for valproic acid and internal standard are ±2% of those in calibrator 3. These are inclusive ranges.

20.11.3 The quantitative results for valproic acid must be within the dynamic range of the test method.

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

20.12 REPORTING

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

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

a. Example 1: valproic acid is measured at 13.91 mg/L.

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

c. Example 2: valproic acid is measured at 40.04 mg/L.

d. The result is truncated to 40.0 mg/L, but reported as 40 mg/L (one significant figure).

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

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

20.13 METHOD PERFORMANCE

20.13.1 Lower limit of quantification: 10 mg/L

20.13.2 Dynamic range: 10 mg/L – 100 mg/L

20.13.3 Upper limit of quantitation: 100 mg/L

20.14 TRACEABILITY

20.14.1 Traceability of the reference materials 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	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 Pressure
Inlet Pressure	16.9 psi
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	290°C
Hold Time	1.5 min
Run Time	21.0 min

Autosampler	
Injection Volume	1.0 µL
Solvent Wash A	6 (Methanol)
Solvent Wash B	6 (Methanol)
Sample Pumps	4

FID Parameters	
Heater	300°C
H_2 Flow	30.0 mL/min
Air Flow	400 mL/min
Const Column + Makeup Flow	30.0 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	3.0 min	MS Quad Temperature	150°C
EM Offset	Set in tune	MS Source Temperature	230°C
Scan Range	50 – 175	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
06/26/13	Method approved by Washington State Toxicologist. See DRA dated 06/24/13. Method released for use in evidentiary testing on 06/26/13.	All
10/01/15	Calibrated pipettes changed to “piston” type in equipment list in 20.5.3. Added description of use of UTAK serum control or spiked whole blood control to 20.6.3.2 and 27.8. Positive control target concentration added to 20.7.8 and 20.10.2.2.c. Added 20.6.1.4 for working control standard preparation. Note added to 20.8 to indicate the instrument acquisition method name may include suffix specific to the instrument.	1-7