CONFIRMATION OF BARBITURATES BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY

8.1 POLICY

This test method may be used to confirm the presence of amobarbital (AMB), butalbital (BTB), pentobarbital (PTB), phenobarbital (PHB) and secobarbital (SCB) in biological samples. Quantitative results obtained through the use of this method will only be reported within the validated 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 a member of TLD Management, and appropriately documented in the batch file.

8.2 PURPOSE

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

8.3 PRINCIPLE

The targeted compounds and internal standard, hexobarbital (HXB), are isolated from whole blood, serum, plasma, urine or other submitted biological samples by the use of liquid-liquid extraction (LLE). Following LLE, 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.

The GC is coupled to a mass spectrometer (MS) detector equipped with an electron ionization source. As each compound is ionized in the source, selected-ion-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.

8.4 SPECIMENS

8.4.1 The specimen volume is 0.5 mL.

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

8.4.3 Dilutions of specimens may be analyzed at the Forensic Scientist's discretion; in addition, the specimen may be analyzed at standard volume, as dictated by screening results, to ensure that concentrations of all target compounds present are within the dynamic range of the test method.

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

8.5 REAGENTS, MATERIALS AND EQUIPMENT

8.5.1 REAGENTS

8.5.1.1 Acetonitrile (ACN)

8.5.1.2 Certified blank blood

8.5.1.3 Deionized water (DI H_2O)

8.5.1.4 Ethyl acetate

8.5.1.5 Extraction solvent

Add 20 mL ethyl acetate to a glass flask. Add 20 mL hexanes and mix. Store in a 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 extraction solvent are maintained.

8.5.1.6 Hexanes

8.5.1.7 Hydrochloric acid (HCl, concentrated)

8.5.1.8 0.1M Hydrochloric acid

To 400 mL DI H2O, add 4.2 mL concentrated HCl. Dilute to 500 mL with DI H_2O. Store the acid 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.

8.5.1.9 Methanol (MeOH)

8.5.1.10 0.1M phosphate buffer (pH6)

Dissolve 1.7 g Na_2HPO_4 and 12.14 g NaH_2PO_4 in 800 mL DI H2O. Dilute to 1 L with DI H_2O and mix. Check the pH and, if necessary, adjust to 6 ±0.5. Store the buffer 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.

8.5.1.11 0.1M phosphate buffer (pH5)

Add 32 mL 0.1M HCl to 300 mL 0.1M phosphate buffer pH6 and mix. Adjust pH as necessary. Store the buffer in a glass bottle at room temperature for up to 1 year. Adjustments to final volume are permitted as long as the proportions are maintained.

8.5.1.12 Sodium phosphate, dibasic anhydrous (Na_2HPO_4)

8.5.1.13 Sodium phosphate, monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$)

8.5.1.14 Trimethylanilinium hydroxide (TMPAH) in methanol

8.5.2 MATERIALS

8.5.2.1 Autosampler vials (glass), inserts and caps

8.5.2.2 Disposable 16 x 100mm tubes

8.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

8.5.2.4 Disposable pipette tips

8.5.2.5 Disposable safety closures for 16 x 100mm tubes

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

8.5.2.7 Laboratory glassware (graduated cylinders, flasks)

8.5.2.8 Volumetric glassware (flasks)

8.5.3 EQUIPMENT

8.5.3.1 Agilent GC (6890 or equivalent)

8.5.3.2 Agilent MS (5973 or equivalent)

8.5.3.3 Calibrated, adjustable piston pipettes

8.5.3.4 Centrifuge

8.5.3.5 Evaporator (Caliper LS, formerly Zymark TurboVap)

8.5.3.6 pH Meter and/or indicating pH paper

8.5.3.7 Rotary mixer

8.5.3.8 Verified, adjustable repeater-pipettes

8.5.3.9 Vortex mixer

8.6 STANDARDS, CALIBRATORS AND CONTROLS

8.6.1 STANDARDS

8.6.1.1 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.

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

a.	Amobarbital:	1.0 mg/mL
b.	Butalbital:	1.0 mg/mL
c.	Hexobarbital (IS):	1.0 mg/mL
d.	Pentobarbital:	1.0 mg/mL
e.	Phenobarbital:	1.0 mg/mL
f.	Secobarbital:	1.0 mg/mL

8.6.1.3 Working standard (0.1 mg/mL)

a. Using a calibrated pipette, measure 2.5 mL each of AMB, BTB, PTB, PHB and SCB stock standards 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 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 (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.

8.6.1.4 Working internal standard (0.05 mg/mL)

a. Using a calibrated pipette, measure 2.5 mL HXB stock internal standard into a 50 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 0.05 mg/mL. 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.

8.6.2 CALIBRATORS

8.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 8.7 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. If the matrix has not been verified as negative, a matrix blank must be included in the batch.

8.6.3 CONTROLS

8.6.3.1 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.)

8.6.3.2 Positive Controls

a. At least 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 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 (0.1 mg/mL) is prepared as described in 8.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 8.7 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.

8.7 SAMPLE PREPARATION

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

8.7.2 Place 1 mL of 0.1M phosphate buffer pH5 into each tube.

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

8.7.4 Prepare a 1:10 dilution of the working standard. (0.01 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.

8.7.5 Using a calibrated pipette, spike the calibrators according to the following table, using the working standard and prepared dilution.

Calibrator Description	Volume (µL) Added	Working Standard
Calibrator 1 (0.5 mg/L)	25	0.01 mg/mL
Calibrator 2 (2.0 mg/L)	100	0.01 mg/mL
Calibrator 3 (5.0 mg/L)	25	0.1 mg/mL
Calibrator 4 (10 mg/L)	50	0.1 mg/mL
Calibrator 5 (20 mg/L)	100	0.1 mg/mL

8.7.6 Prepare a 1:10 dilution of the control working standard. (0.01 mg/mL)

a. Using a calibrated pipette, combine 0.1 mL of the control 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 control preparation.

8.7.7 Using a calibrated pipette, spike the positive controls according to the following table, using the control working standard and prepared dilution.

Control Description	Volume (µL) Added	Control Working Standard
Control 1 (3.0 mg/L)	150	0.01 mg/mL
Control 2 (15 mg/L)	75	0.1 mg/mL

8.7.8 If in-house positive controls are being used, transfer 0.5 mL of each into their labeled tubes, using a calibrated pipette.

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

8.7.10 Using a calibrated pipette or verified repeater-pipette, add 50 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 5 mg/L.

8.7.11 Cap the tubes and briefly vortex mix.

8.7.12 Add 3 mL extraction solvent to each tube.

8.7.13 Cap the tubes and place on a rotary mixer for 20 minutes.

8.7.14 Centrifuge the tubes for 10 minutes at 3500rpm.

8.7.15 Transfer the organic layer to clean, labeled 10 mL centrifuge or screw cap tubes.

8.7.16 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C. Make sure the extracts are evaporated to dryness before reconstitution.

8.7.17 Reconstitute the extracts by the addition of 100 µL TMPAH in methanol to each tube and cap. 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.

8.7.18 Transfer the extracts to labeled glass autosampler vials with inserts and cap.

8.8 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 and sample descriptions in the sequence table, ensure that the method listing in the table is BARB.M for each line.

8.9 SPECIAL INSTRUCTIONS

Methanol is to be used when solvent blanks are included in the test sequence.

8.10 DATA ANALYSIS

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

8.10.2 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.

8.10.3 Printed reports for each vial in the batch are generated for review along with the updated calibration curves and data analysis parameters (calibration report).

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

8.11 CRITERIA FOR BATCH ACCEPTANCE

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

8.11.1 Calibrators and calibration curves

8.11.1.1 Chromatographic peaks for AMB, BTB, PTB, PHB, SCB and internal standard shall appear symmetrical (i.e., no co-elution, split peaks, or shoulders).

8.11.1.2 Retention times for target compounds and internal standard shall be within ±2%, and ion ratios shall be within ±20%, of those in calibrator 3. These are inclusive ranges.

8.11.1.3 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.

For calibrator 1 (target concentration 0.5 mg/L), result comparisons will use values truncated after the second decimal place in units of mg/L (acceptable range 0.37 – 0.62 mg/L).

For target concentrations ≥1.0 mg/L, result comparisons will use values truncated after the first decimal place in units of mg/L.

8.11.1.4 The calibration curves for AMB, BTB, PTB, PHB and SCB shall have correlation coefficients ≥0.99.

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

8.11.2 Controls

8.11.2.1 The negative control(s) shall not identify AMB, BTB, PTB, PHB or SCB 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.

8.11.2.2 Positive controls

a. Chromatographic peaks for AMB, BTB, PTB, PHB and SCB and internal standard shall appear symmetrical.
b. Retention times for target compounds and internal standard shall be within ±2%, and ion ratios shall be within ±20%, of those in calibrator 3. These are inclusive ranges.
c. Quantitative results for AMB, BTB, PTB, PHB and SCB 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. All positive controls in the batch must meet acceptability criteria for a target compound in order to report quantitative results for that compound in a case specimen.
e. The failure to meet any of these criteria for one compound does not invalidate the acceptability of another compound.

8.12 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.

8.12.1 Chromatographic peaks for AMB, BTB, PTB, PHB, SCB and internal standard shall appear symmetrical.

8.12.2 The retention times for target compounds and internal standard are within ±2%, and the ion ratios are within ±20%, of those in calibrator 3. These are inclusive ranges.

8.12.3 The quantitative results for each identified compound must be within the dynamic range of the test method. Results greater than the upper limit of quantitation may be reported qualitatively, provided that all other criteria for acceptance are met.

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

8.13 REPORTING

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

8.13.2 Results are truncated to two significant figures for reporting.

a. Example 1: amobarbital is measured as 6.38 mg/L.

b. The result is truncated to 6.3 mg/L (two significant figures) and reported.

c. Example 2: phenobarbital is measured at 15.92 mg/L.

d. The result is truncated to 15.9 mg/L but reported as 15 mg/L (two significant figures).

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

8.14 METHOD PERFORMANCE

8.14.1 Limit of detection: 0.05 mg/L

8.14.2 Lower limit of quantification: 0.5 mg/L

8.14.3 Dynamic range: 0.5 mg/L – 20 mg/L

8.14.4 Upper limit of quantitation: 20 mg/L

8.15 TRACEABILITY

8.15.1 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

Split/Splitless Inlet	
Mode	Split
Inlet Liner	4mm splitless w/glass wool plug
Temperature	280°C
Split Ratio	40:1
Gas Type	Helium
Gas Saver	On
Gas Saver Flow	15.0 mL/min
Gas Saver Time	2.00 min

Autosampler	
Injection Volume	2.0 µL
Solvent Wash A	4 (Acetonitrile)
Solvent Wash B	4 (Methanol)
Sample Pumps	2

Oven/Column	
Carrier Gas Mode	Constant Flow
Carrier Gas Flow	2.0 mL/min
Initial Temperature	110° C
Initial Time	1.00 min
Ramp Rate	15° C/min
Final Temperature	300° C
Final Time	0.33 min

MASS SPECTROMETER

Solvent Delay	5.00 min	MS Quad Temperature	150°C
EM Offset	Set in tune	MS Source Temperature	230°C
Resolution	Low	Dwell Time	50 msec

Signals	Ions	Ion Ratios
Amobarbital	169, 184, 185	184/169, 185/169
Butalbital	196, 195, 209	195/196, 209/196
Hexobarbital (IS)	235, 169	169/235
Pentobarbital	169, 184, 112	184/169, 112/169
Phenobarbital	232, 175, 146	175/232, 146/232
Secobarbital	196, 195, 181	195/196, 181/196

LIST OF CHANGES

Revision Date	Description	Page Number
11/01/11	Method approved by Washington State Toxicologist. See DRA dated 10/20/11. Method released for use in evidentiary testing on 11/01/11.	All
2/4/16	Added wording for adjustment of prepared volumes in 8.5.1.4, 8.5.1.7, 8.5.1.9, 8.5.1.10, 8.6.1.3 and 8.6.1.4 and added clarification to 8.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Added note regarding CRM expiration dates to 8.6.1.3 and 8.6.1.4. Edited 8.13.2 to reflect that only two significant figures are used for reporting and removed example in 8.13.2.e-f. Added "Printed Copies are Uncontrolled" to footer. Other minor edits throughout.	All
5/8/17	Wording added to 8.4.3 regarding dilution and standard volume testing. Specified use of calibrated pipettes for measurement of blank blood, specimens and standards throughout sample preparation in 8.7. Specified calibrator concentration criteria/ranges in 8.11.1.3. Edited 8.11.2.2.d to indicate all positive controls must pass for a target compound to report quantitative results. Other minor edits throughout.	1-8