CONFIRMATION OF BARBITURATES BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

8.1 METHOD

This test method may be used to confirm the presence of amobarbital (AMB), butalbital (BTB), pentobarbital (PTB), phenobarbital (PHB) and secobarbital (SCB) in biological specimens. The target compounds and hexobarbital internal standard are isolated from biological matrices by liquid-liquid extraction (LLE). The extracts are chemically derivatized and injected into a gas chromatograph (GC) coupled to a mass spectrometer (MS) detector equipped with an electron ionization source.

8.2 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.

NOTE: Method validation established that matrix-matching of the full calibration curve and all positive control levels is required for quantitation in serum/plasma or liver (tissue) homogenate specimens (see 8.4.2 and 8.4.3).

8.3 REAGENTS, MATERIALS AND EQUIPMENT

8.3.1 REAGENTS

NOTE: Organic solvents used are reagent grade.

- Acetonitrile (ACN)
- Certified blank blood and/or other biological matrices
- Deionized water (DI H_2O), laboratory general-use
- Ethyl acetate
- Extraction solvent

  Add 20 mL ethyl acetate to a glass flask. Add 20 mL hexanes and mix. Store the solvent in a glass flask/bottle at room temperature and use on date of preparation only.
- Hexanes
- Hydrochloric acid (HCl, concentrated)
- 0.1M HCl

  To 400 mL DI H_2O, 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.
- Methanol (MeOH)

- 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 pH6 ± 0.5 with concentrated NaOH or HCl. Store the buffer in a glass bottle at room temperature for up to one year.

- 0.1M Phosphate buffer (pH5):

  Add 32 mL 0.1M HCl to 300 mL 0.1M phosphate buffer pH6 and mix. Check the pH and, if necessary, adjust to pH5 ± 0.5 with concentrated NaOH or HCl. 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 ($NaH_2PO_4 \bullet H_2O$)
- Trimethylanilinium hydroxide (TMPAH) in methanol

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

8.3.2 MATERIALS

- Disposable extraction tubes (16 x 100mm recommended) and screw-cap or centrifuge tubes with closures
- GC column (Agilent HP-5MS; 30 m x 0.250 mm i.d. x 0.250 µm film thickness, or equivalent)
- Glass autosampler vials with inserts and caps
- Laboratory glassware (graduated cylinders, flasks)

8.3.3 EQUIPMENT

- Agilent GC (6890 or equivalent)
- Agilent MS (5973 or equivalent) with electron ionization source
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General-use equipment (centrifuge, evaporator, pH meter or paper, rotary mixer, vortex mixer)

8.4 STANDARDS, CALIBRATORS AND CONTROLS

8.4.1 STANDARDS

- Working standard: 0.1 mg/mL
- Working control standard: 0.1 mg/mL
- Working internal standard: 0.05 mg/mL

8.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 8.5 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 tissue homogenate specimens, a whole blood calibration curve is not required.

8.4.3 CONTROLS

8.4.3.1 At least one negative whole blood control and two positive whole blood controls are included in the batch, prepared as described in 8.5. For quantitative analysis of serum/plasma or liver (tissue) homogenate specimens only, whole blood controls are not required.

8.4.3.2 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.

8.4.3.3 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.

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

8.5 SAMPLE PREPARATION

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

8.5.2 Add 1 mL of 0.1M phosphate buffer (pH5) into each tube.

8.5.3 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).

8.5.4 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 ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after calibrator preparation.

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

Calibrator Description	Volume (µL) Added	Standard Concentration	Dilution of WS (or WS)
Calibrator 1 – 0.5 mg/L	25	0.01 mg/mL	1:10
Calibrator 2 – 2.0 mg/L	100	0.01 mg/mL	1:10
Calibrator 3 – 5.0 mg/L	25	0.1 mg/mL	WS
Calibrator 4 - 10 mg/L	50	0.1 mg/mL	WS
Calibrator 5 - 20 mg/L	100	0.1 mg/mL	WS

8.5.6 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 ACN or MeOH in a labeled tube.
b. Cap and vortex mix. This dilution shall be disposed of after control preparation.

8.5.7 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	Standard Concentration	Dilution of QC (or QC)
Control 1 – 3.0 mg/L	150	0.01 mg/mL	1:10
Control 2 - 15 mg/L	75	0.1 mg/mL	QC

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

8.5.9 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.

8.5.10 Cap the tubes and briefly vortex mix.

8.5.11 Add 3 mL extraction solvent to each tube.

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

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

8.5.14 Transfer the organic layer to a clean, labeled 10 mL centrifuge or screw cap tube.

8.5.15 Transfer the tubes to the evaporator and evaporate the extracts to dryness at 50°C. Extracts must be completely dry prior to reconstitution for efficient chemical derivatization.

8.5.16 In a fume hood, 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.

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

8.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

NOTE: Methanol must be used for solvent blanks included in the batch sequence.

- Acquisition method – BARB (instrumental parameters in Appendix B)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±2%, ion ratios ±20%) – Cal 3
- Result comparisons –

  Cal 1: truncated to two decimal places in units of mg/L (acceptable range 0.37 – 0.62 mg/L)

  Cals 2-5, Ctls 1-2: truncated to one decimal place in units of mg/L

8.7 REPORTING

Results are truncated to two significant figures for reporting, in units of milligrams per liter (mg/L).

8.8 METHOD PERFORMANCE

- Limit of detection: 0.05 mg/L
- Lower limit of quantification: 0.5 mg/L
- Dynamic range: 0.5 – 20 mg/L
- Upper limit of quantitation: 20 mg/L

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARD

Amobarbital
Butalbital
Hexobarbital (IS)
Pentobarbital
Phenobarbital
Secobarbital

APPENDIX B
INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH

NOTE: Methanol must be used for solvent blanks included in the batch sequence.

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
8/5/19	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 8.2 SPECIMENS, 8.4.2 CALIBRATORS and 8.4.3 CONTROLS. Edited STANDARDS section - this information is now included in the revised Standard Solution Preparation procedure. Criteria for batch acceptance (calibrators, controls) and specimen acceptability criteria, and specific data analysis and reporting information are now included in the General Requirements for Chromatographic Test Method Batch Analysis and Acceptance. Target compound/internal standard list added in APPENDIX A, with test method parameters moved to APPENDIX B. Formatting and minor edits throughout.	All