CONFIRMATION OF CARISOPRODOL AND MEPROBAMATE BY GAS CHROMATOGRAPHY - MASS SPECTROMETRY

12.1 METHOD

This test method may be used to confirm the presence of carisoprodol (CAR) and meprobamate (MEP) in biological specimens. The target compounds and hexobarbital internal standard are isolated from biological matrices by solid phase extraction (SPE). The extracts are injected into a gas chromatograph (GC) coupled to a mass spectrometer (MS) detector equipped with an electron ionization source.

12.2 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 12.4.2 and 12.4.3).

12.3 REAGENTS, MATERIALS AND EQUIPMENT

12.3.1 REAGENTS

NOTE: Organic solvents used are reagent grade.

- Acetonitrile (ACN)
- Ammonium hydroxide (NH_4OH), concentrated
- Certified blank blood and/or other biological matrices
- Deionized water (DI H_2O), laboratory general-use
- Ethyl acetate
- 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.
- 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.
- Iso-octane
- Isopropanol (IPA)

- Methanol (MeOH)
- Methylene chloride (dichloromethane, CH_2Cl_2)
- 0.1M Phosphate buffer (pH3):

  Add 100 mL 0.1M HCl to 600 mL 0.1M phosphate buffer pH6 and mix. Check the pH and, if necessary, adjust to pH3 ± 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 (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.
- 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.

12.3.2 MATERIALS

- Disposable extraction tubes (16 x 100 mm 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
- 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)

12.3.3 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, vacuum manifold, vortex mixer)

12.4 STANDARDS, CALIBRATORS AND CONTROLS

12.4.1 STANDARDS

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

12.4.2 CALIBRATORS

Calibrators are prepared in certified blank blood at the time of analysis, as detailed in 12.5 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.

12.4.3 CONTROLS

12.4.3.1 At least one negative whole blood control and two positive whole blood controls are included in the batch, prepared as described in 12.5. For quantitative analysis of serum/plasma or liver (tissue) homogenate specimens only, whole blood controls are not required.

12.4.3.2 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.

12.4.3.3 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.

12.4.3.4 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.

12.5 SAMPLE PREPARATION

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

12.5.2 Add 2 mL of 0.1M phosphate buffer (pH3) into each tube.

12.5.3 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).

12.5.4 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.

12.5.5 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 – 1.0 mg/L	50	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

12.5.6 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.

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

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	WS

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

12.5.9 Using a calibrated pipette or verified repeater-pipette, add 20 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 2.0 mg/L.

12.5.10 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500 rpm (recommended for 16 x 100 mm tubes).

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

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

a. 3 mL methanol
b. 3 mL DI H_2O
c. 1 mL 0.1M phosphate buffer (pH3)

Do not let columns dry out between each conditioning step.

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

12.5.14 Wash the SPE columns by passing each of the following 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. 1 mL 0.1M HCl

12.5.15 Dry the columns for 5 minutes under vacuum.

12.5.16 Wash each column with 2 mL hexanes.

12.5.17 Dry the columns for 5 minutes under vacuum.

12.5.18 Place clean, labeled centrifuge or screw-cap tubes in the collection rack underneath their corresponding SPE columns.

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

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

12.5.21 Reconstitute the extracts by the addition of 100 µL ethyl acetate to each tube. 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.

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

12.6 INSTRUMENTAL PARAMETERS/DATA ANALYSIS

- Acquisition method – CARMEP (instrumental parameters in Appendix B)
- Calibration curve – linear, 1/a weighting factor
- Updating calibrator (retention times ±2%, ion ratios ±20%) – Cal 4
- Result comparisons –

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

12.7 REPORTING

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

12.8 METHOD PERFORMANCE

- Limit of detection: 0.25 mg/L
- Lower limit of quantification: 1.0 mg/L
- Dynamic range: 1.0 – 20 mg/L
- Upper limit of quantitation: 20 mg/L

APPENDIX A
TARGET COMPOUNDS AND INTERNAL STANDARD

Carisoprodol
Meprobamate
Hexobarbital (IS)

APPENDIX B
INSTRUMENTAL PARAMETERS

GAS CHROMATOGRAPH

Split/Splitless Inlet	
Mode	Split
Inlet Liner	4mm splitless w/glass wool plug
Temperature	270° C
Split Ratio	15:1
Gas Type	Helium
Gas Saver	On
Gas Saver Flow	15.0 mL/min
Gas Saver Time	40 min
Autosampler	
Injection Volume	2.0 μL
Solvent Wash A	4 (Iso-octane)
Solvent Wash B	4 (Ethyl acetate)
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	250° C
Final Time	0.67 min

MASS SPECTROMETER

Solvent Delay	6.00 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	
Carisoprodol	158, 104, 245	104/158, 245/158	
Meprobamate	83, 114, 144	114/83, 144/83	
Hexobarbital (IS)	221, 155	155/221	

LIST OF CHANGES

Revision Date	Description	Page Number
03/01/12	Method approved by Washington State Toxicologist. See DRA dated 02/14/12 . Method released for use in evidentiary testing on 03/01/12.	All
2/4/16	Added wording for adjustment of prepared volumes in 12.5.1.8, 12.5.1.13, 12.5.1.14, 12.6.1.3 and 12.6.1.4 and added clarification to 12.6.3.2.c for use of same CRM in preparation of working standard and working control standard. Added note regarding CRM expiration dates to 12.6.1.3 and 12.6.1.4. Edited 12.12.2 to reflect that only two significant figures are used for reporting and removed example in 12.12.2.c-d. Added "Printed Copies are Uncontrolled" to footer. Other minor edits throughout.	All
5/8/17	Wording added to 12.4.3 regarding dilution and standard volume testing. Specified use of calibrated pipettes for measurement of blank blood, specimens and standards throughout sample preparation in 12.7. Edited 12.10.2.2.d to indicate all positive controls must pass for a target compound to report quantitative results. Other minor edits throughout.	1, 3-8
8/8/19	Removed policy, purpose and principle sections, summarizing under new section METHOD. Added specific wording regarding matrix-matching in 12.2 SPECIMENS, 12.4.2 CALIBRATORS and 12.4.3 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