CONFIRMATION OF CARISOPRODOL AND MEPROBAMATE BY GAS CHROMATOGRAPHY – MASS SPECTROMETRY

12.1 POLICY

This test method may be used to confirm the presence of carisoprodol (CAR) and meprobamate (MEP) 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.

12.2 PURPOSE

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

12.3 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 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.

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 carisoprodol or meprobamate identified in a sample is determined from its calibration curve.

12.4 SPECIMENS

12.4.1 The specimen volume is 0.5 mL.

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

12.4.3 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.

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

12.5 REAGENTS, MATERIALS AND EQUIPMENT

12.5.1 REAGENTS

12.5.1.1 Acetonitrile

12.5.1.2 Ammonium hydroxide (concentrated)

12.5.1.3 Certified blank blood

12.5.1.4 Deionized water (DI H_2O)

12.5.1.5 Ethyl acetate

12.5.1.6 Elution solvent

To 20 mL isopropanol, add 2 mL concentrated ammonium hydroxide and mix. Add 78 mL methylene chloride and mix. Store in 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 elution solvent are maintained.

12.5.1.7 Hexanes

12.5.1.8 Hydrochloric acid (concentrated)

12.5.1.9 0.1M Hydrochloric acid

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

12.5.1.10 Iso-octane

12.5.1.11 Isopropanol (IPA)

12.5.1.12 Methanol

12.5.1.13 Methylene chloride (dichloromethane, CH_2Cl_2)

12.5.1.14 0.1M phosphate buffer (pH3)

Add 100 mL 0.1M HCl to 600 mL 0.1M phosphate buffer pH6 and mix. Adjust pH if necessary. Store the solution 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.

12.5.1.15 0.1M phosphate buffer (pH6)

Dissolve 1.7 g Na_2HPO_4 and 12.14 g NaH_2PO_4 in 800 mL DI H_2O. 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.

12.5.1.16 Sodium phosphate, dibasic anhydrous (Na_2HPO_4)

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

12.5.2 MATERIALS

12.5.2.1 Autosampler vials, inserts and caps

12.5.2.2 Disposable 16 x 100mm tubes

12.5.2.3 Disposable screw-cap tubes or centrifuge tubes with closures

12.5.2.4 Disposable pipette tips

12.5.2.5 Disposable safety closures for 16 x 100mm tubes

12.5.2.6 Extraction column: United Chemical Technologies' Clean Screen SPE cartridge (CSDAU206, 200mg/6mL), or equivalent

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

12.5.2.8 Laboratory glassware (graduated cylinders, flasks)

12.5.2.9 Volumetric glassware (flasks)

12.5.3 EQUIPMENT

12.5.3.1 Agilent GC (6890 or equivalent)

12.5.3.2 Agilent MS (5973 or equivalent)

12.5.3.3 Calibrated, adjustable piston pipettes

12.5.3.4 Centrifuge

12.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

12.5.3.6 pH Meter and/or indicating pH paper

12.5.3.8 Vortex mixer

12.5.3.9 Vacuum manifold

12.6 STANDARDS, CALIBRATORS AND CONTROLS

12.6.1 STANDARDS

12.6.1.1 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.

12.6.1.2 Stock standards and stock internal standard (IS) are purchased from an approved reference material supplier and include the following:

a. Carisoprodol: 1.0 mg/mL
b. Meprobamate: 1.0 mg/mL
c. Hexobarbital (IS): 1.0 mg/mL

12.6.1.3 Working standard (0.1 mg/mL)

a. Using a calibrated pipette, measure 2.5mL each of CAR and MEP 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.

12.6.1.4 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.

12.6.2 CALIBRATORS

12.6.2.1 Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 12.7 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.

12.6.3 CONTROLS

12.6.3.1 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.)

12.6.3.2 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 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 12.6.1.3.
e. The preparation of the positive whole blood controls is detailed in 12.7 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.

12.7 SAMPLE PREPARATION

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

12.7.2 Place 2 mL of 0.1M phosphate buffer pH3 into each tube.

12.7.3 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).

12.7.4 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.

12.7.5 Using the working standard and the prepared dilution, spike the calibrators according to the following table.

Calibrator Description	Volume (µL) Added	Working Standard
Calibrator 1 (1.0 mg/L)	50	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

12.7.6 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.

12.7.7 Using the control working standard and the prepared dilution, spike the positive controls according to the following table.

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

12.7.8 If in-house positive controls are being used, transfer 0.5 mL of each into their labeled tubes.

12.7.9 Sample 0.5 mL of each case sample into its respective tube.

12.7.10 Add 20 µL of the working internal standard solution to each tube. Final concentration of the internal standard is 2.0 mg/L.

12.7.11 Cap the tubes and briefly vortex mix. Centrifuge the tubes for 10 minutes at 3500rpm.

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

12.7.13 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.

12.7.14 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.)

12.7.15 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

12.7.16 Dry the columns for 5 minutes under vacuum.

12.7.17 Wash each column with 2 mL hexane.

12.7.18 Dry the columns for 5 minutes under vacuum.

12.7.19 Place clean, labeled centrifuge tubes in the collection rack underneath their corresponding SPE columns.

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

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

12.7.22 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.

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

12.8 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 that the method listing in the table is CARMEP.M for each line.

12.9 DATA ANALYSIS

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

12.9.2 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.

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

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

12.10 CRITERIA FOR BATCH ACCEPTANCE

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

12.10.1 Calibrators and calibration curves

12.10.1.1 Chromatographic peaks for carisoprodol, meprobamate and internal standard shall appear symmetrical (i.e. no co-elution, split peaks, or shoulders).

12.10.1.2 Retention times shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4. These are inclusive ranges.

12.10.1.3 Quantitative results for carisoprodol and meprobamate 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.

12.10.1.4 The calibration curves for carisoprodol and meprobamate shall have correlation coefficients ≥0.99.

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

12.10.2 Controls

12.10.2.1 The negative control(s) shall not identify carisoprodol or meprobamate 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.

12.10.2.2 Positive controls

a. Chromatographic peaks for carisoprodol, meprobamate and internal standard shall appear symmetrical.
b. Retention times shall be within ±2% and ion ratios shall be within ±20% of those in calibrator 4 for each compound in the positive control. These are inclusive ranges.
c. Quantitative results for carisoprodol and meprobamate 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.

12.11 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.

12.11.1 Any chromatographic peak for carisoprodol or meprobamate shall appear symmetrical.

12.11.2 The retention times for carisoprodol and meprobamate are ±2% and the ion ratios are within ±20% of those in calibrator 4. These are inclusive ranges.

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

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

12.12 REPORTING

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

12.12.2 Results are truncated to two significant figures for reporting.

a. Example 1: meprobamate is measured as 8.75 mg/L.

b. The result is truncated to 8.7 mg/L (two significant figures) and reported.

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

12.13 METHOD PERFORMANCE

12.13.1 Limit of detection: 0.25 mg/L

12.13.2 Lower limit of quantification: 1.0 mg/L

12.13.3 Dynamic range: 1.0 mg/L – 20 mg/L

12.13.4 Upper limit of quantitation: 20 mg/L

12.14 TRACEABILITY

12.14.1 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

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	Ion	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