



TOX 10-21: Carisoprodol, Meprobamate, and Carbamazepine Quantitation By LC-MS/MS

Purpose

This procedure specifies the required elements for the extraction and quantitation of Carbamazepine, Carisoprodol and its metabolite, Meprobamate, from biological specimens using the principles of solid phase extraction (SPE) technology. This specific procedure uses the UCT Clean Screen® Solid Phase Extraction Column, taking advantage of hydrophobic exchange properties with Neutral/Acidic drugs.

Principle

Carbamazepine, Carisoprodol, and Meprobamate are extracted by SPE extraction and injected into a LC-MS/MS and drug specific ions are detected by multiple reaction monitoring (MRM). MRM is very selective for the drug of interest. The precursor or parent ion is selected in the first quadrupole (Q1). A collision is induced between the precursor and a molecule of argon gas in Q2. The collision fragments the precursor ion and those fragments or daughter ions are then selected Q3. Each drug fragments in a specific and known way which results in a high level of drug identification. The ratio of sample peak area to internal standard peak area is compared to the calibration curve to provide a quantitation of Carbamazepine, Carisoprodol, and Meprobamate.

Scope

D ₇ -Carisoprodol (IS)	Carisoprodol
D ₇ -Meprobamate (IS)	Meprobamate
D ₁₀ -Carbamazepine (IS)	Carbamazepine

Specimen Criteria

1. This procedure is appropriate for
 - a. Blood specimens collected in gray top evacuated tubes (containing 100 mg sodium fluoride and 20 mg potassium oxalate) or similar.
 - b. Urine specimens collected in a container that may or may not have preservative.
 - c. Solid specimens that have been homogenized into a tissue slurry that may or may not have preservative.
 - d. Other types of liquids or solids dissolved or reconstituted in a liquid media.
2. The minimum volume required to perform the analysis is 0.05 mL, any quantity less will be reported as QNS or "Quantity not sufficient to perform the analysis".
3. Specimens will be maintained at a refrigerated temperature of 0° C to 8° C until analyzed.
 - a. Specimens will be allowed to equilibrate at room temperature before analysis.

Reference Material and Reagents

1. Commercially prepared stock standards of Carisoprodol, Meprobamate, Carbamazepine. Supplied by Cerilliant or similar.
2. Commercially prepared stock internal standards of D₇-Carisoprodol, D₇-Meprobamate, D₁₀-Carbamazepine. Supplied by Cerilliant or similar.
3. Sodium Acetate (ACS grade preferred)

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4. Methanol (LCMS grade preferred)
5. Glacial Acetic Acid (GMP grade preferred)
6. Hexane (HPLC grade preferred)
7. Ethyl Acetate (ACS grade preferred)
8. Isopropanol
9. Formic Acid (High Purity grade preferred)
10. Acetonitrile (LCMS grade preferred)
11. Ultrapure water
12. Drug free matrix

Supplies and Equipment

1. Water Aquity UPLC and Xevo TQD MS/MS
2. Waters MassLynx™ Software
3. Cerex® System 48 Solid-Phase Extraction Positive Pressure Manifold or equivalent
4. Evaporator
5. Centrifuge
6. Vortex mixer
7. 16 x 100 mm culture tubes
8. 13 x 100 mm culture tubes
9. Volumetric Flasks
10. Graduated Cylinders
11. Media Bottles with caps
12. LC autosampler vials (without inserts) and pre-slit caps
13. 2-20 µL, 20-200 µL, 100-1000 µL adjustable pipetters
14. Disposable transfer pipettes
15. Repeater pipette
16. UCT Clean Screen® Solid Phase Extraction Columns - Size 6CC/200 mg – Part #CSDAU206 or equivalent

The following preparations are recommended, however the specific preparation will ultimately be at the analyst’s discretion and expertise.

Internal Standard Working Stock Preparation

Drug	External Provider Ampule []	Caris/Mepro/Carba ISTD Mix 10 ug/mL
D₇-Carisoprodol	100 ug/mL	1 mL each 100 ug/mL external provider ampule



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D₇-Meprobamate	100 ug/mL	QS to 10 mL with methanol in 10 mL volumetric flask
D₁₀-Carbamazepine	100 ug/mL	Final [] 10 ug/mL

Calibrator Working Stock Preparation

Drug	External Provider Ampule []	Caris/Mepro/Carba Calibration Mix 100 ug/mL	Caris/Mepro/Carba Calibration Mix 10 ug/mL
Carisprodol	1 mg/mL	1 mL each 1.0 mg/mL external provider ampule	1 mL of Caris/Mepro/Carba 100 ug/mL Calibration Mix
Meprobamate	1 mg/mL	QS to 10 mL with methanol in 10 mL volumetric flask	QS to 10 mL with methanol in 10 mL volumetric flask
Carbamazepine	1 mg/mL	Final [] 100 ug/mL	Final [] 10 ug/mL

Control Working Stock Preparation

Drug	External Provider Ampule []	Carbamazepine Control WS 1 mg/mL	Caris/Mepro/Carba Control Mix 100 ug/mL	Caris/Mepro/Carba Control Mix 10 ug/mL
Carisoprodol	1 mg/mL		1 mL each 1.0 mg/mL external provider	1 mL of Caris/Mepro/Carba 100

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Meprobamate	1 mg/mL		ampule/control WS QS to 10 mL with methanol in 10 mL volumetric flask Final [] 100 ug/mL	ug/mL Control Mix QS to 10 mL with methanol in 10 mL volumetric flask Final [] 10 ug/mL
Carbamazepine		10 mg of external provider powder QS to 10 mL with methanol in 10 mL volumetric flask Final [] 1 mg/mL		

1. All external provider ampules, working stocks, calibrators, controls, and internal standards shall be stored at a freezer temperature.
 2. Working controls shall be made from a different external provider than working calibrators.
 3. Expiration: 1 year from date of preparation
 - a. Ideally, the Calibration mix and the Control mix should be staggered by 6 months
- QC check: All newly prepared working stocks shall be analyzed at the end of a batch, compared against a previous run, and pass all QC requirements prior to implementation of the working stocks into production for casework analysis.

Calibrators and Controls (Alternate WS [] may be used and volumes adjusted)

To be created with each analytical batch

Blood Calibrators (0.50 mL sample size) ng/mL	Caris/Mepro/Carba 10 ug/mL Calibration Mix uL to add	Caris/Mepro/Carba 100 ug/mL Calibration Mix uL to add
250	12.5	---
500	25	---
1,000	50	---
5,000	---	25
10,000	---	50
20,000	---	100



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Blood Controls (0.50 mL sample size) ng/mL	Caris/Mepro/Carba 10 ug/mL Control Mix uL to add	Caris/Mepro/Carba 100 ug/mL Control Mix uL to add
Control 1 or 350	17.5	---
Control 2 or 15,000	---	75

Reagent Preparation

- Dilution and SPE Wash Buffer, 0.10 M Sodium Acetate Buffer
 - To make 4 L, add 32.8 g sodium acetate in 4.0 L ultrapure water
 - pH with glacial acetic acid to ~6
 - Store at refrigeration temperature for up to 2 years
- SPE Wash Buffer, 1 M Acetic Acid
 - To make 1 L, add 57 mL glacial acetic acid into a 1 L volumetric flask
 - QS to 1 L with ultrapure water
 - Store at room temperature for up to 2 years
- SPE Elution Solvent, Hexane:Ethyl Acetate (50:50)
 - To make 1 L, add 500 mL hexane and 500 mL ethyl acetate
 - Store at room temperature for up to 2 years
- Reconstitution Solvent, Water:Acetonitrile (80:20)
 - To make 250 mL, add 200 mL ultrapure water and 50 mL acetonitrile
 - Store at room temperature for up to 2 months
- Aqueous Mobile Phase, 0.1% Formic Acid in Water
 - To make 1 L, add 1 mL formic acid to 1 L ultrapure water
 - Store at room temperature for up to 2 months
- Organic Mobile Phase, 0.1% Formic Acid in Acetonitrile
 - To make 1 L, add 1mL formic acid to 1L acetonitrile
 - Store at room temperature for up to 6 months.
- Needle Wash, Acetonitrile:Methanol:Isopropanol:Water (1:1:1:1)
 - To make 1 L, add 250 mL acetonitrile, 250mL methanol, 250mL isopropanol, and 250mL ultrapure water
 - Store at room temperature for up to 6 months
- Purge, Water:Acetonitrile (90:10) with 0.01% Formic Acid
 - To make 1 L, add 100 uL formic acid to 900 mL ultrapure water and 100mL acetonitrile
 - Store at room temperature for up to 2 months
- Seal Wash, Water:Methanol (60:40)
 - To make 1 L, add 600 mL ultrapure water and 400 mL methanol
 - Store at room temperature for up to 6 months



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Procedure

1. Accurately measure 0.50 mL of blank blood or appropriate matrix for the calibrators, blank control, and positive controls as well as 0.50 mL of case samples to appropriately labeled 16 x 125 mm or 16 x 100 mm test tubes.
 - a. If a matrix other than blood is analyzed, a positive matrix matched control should be run.
2. Supplement the calibrators and positive controls with the appropriate volume of Calibration and Control mixes, respectively, as specified in the above tables.
3. With a repeater pipet, add 50 μ L of the Caris/Mepro/Carba Internal Standard Mix (10 μ g/mL) to each tube and vortex. Final concentration of internal standard, 1000 ng/mL.
4. Add 2 mL 0.10 M sodium acetate buffer and vortex.
5. Centrifuge at approximately 3000-3400 rpms for 10 minutes.
6. Assemble the positive pressure manifold with the appropriate number of solid phase extraction columns and waste container.
7. With minimal airflow, condition the columns with 2 mL methanol and 2 mL 0.10 M sodium acetate buffer, allowing each to pass through the column before the addition of the other. **Also, be careful not to let the columns dry out.**
8. Before applying the samples, change SPE waste container to receive the biological material and acetate buffer.
9. Pour the samples onto the columns and allow to slowly pass through.
10. Wash the columns with 2 mL 0.10 M sodium acetate buffer, 2 mL 1 M acetic acid. Switch back to the original SPE waste container and wash with 2 mL hexane, allowing each to pass before the addition of the other. The biological aqueous waste can be discarded in the sink.
11. Dry the columns under full pressure for approximately 15-20 minutes.
12. Turn off the airflow and assemble the manifold with 13 x 100 mm culture tubes for collection of the eluent.
13. Add 2 mL of elution solvent, 50:50 hexane:ethyl acetate, to each column and allow to pass through the column by gravity only.
14. Evaporate the eluent to dryness.
15. Reconstitute the samples in 1 mL of reconstitution solvent. Vortex.
16. Transfer the liquid to labeled LC autosampler vials (without inserts) and cap with pre-slit caps for LC-MS/MS analysis.
17. Instrumentation preparation includes the following:
 - a. Check solvent expiration date and refill solvents as needed.
 - b. Prime mobile phases and washes.
 - c. Download inlet method and allow instrument initial conditions until pressure delta \sim 50 psi.
18. Load samples onto LC-MS/MS and analyze samples using the Carisoprodol method in the following manner:
 - a. Negative
 - b. Calibrators
 - c. Negative
 - d. Controls
 - e. Casework
 - f. Negative-end
 - g. Middle calibrator as QC-end
 - h. Controls 1,2, etc as QC-end

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19. A sequence check shall be conducted, either pre or post injection, by another individual.

Instrumentation

Waters Acquity UPLC H-Class coupled with a Xevo TQD

Column

Waters Acquity UPLC BEH C18 1.7 μ m VanGuard™ 2.1 x 5mm Pre-Column
 Waters Acquity UPLC BEH C18 1.7 μ m 2.1 x 50mm Column

LC parameters:

Sample Manager Temperature: 10°C

Column Temperature: 50°C

Injection Volume: 10 μ L

Flow Rate: 0.400 mL/min

Gradient:

Time	% Aqueous <i>0.1% formic acid in water</i>	% Organic <i>0.1% formic acid in acetonitrile</i>
Initial	80	20
3.00	30	70
3.01	5	95
4.00	5	95
4.01	80	20
5.50	80	20

Note: Slight variations in gradient may exist due to instrument capabilities, column properties, etc.

MS/MS parameters:

Capillary Voltage: 2.0 kV

Ionization: ESI, positive

Source Temperature: 150°C

Desolvation Temperature: 500°C

Cone Gas Flow: 0 L/Hr

Desolvation Gas Flow: 1000 L/Hr

Ion Transitions (Quantitation Ions in Bold):

Compound	Precursor Ion (M+1)	Product Ions	Cone (V)	Collision (eV)
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D7-Meprobamate	226	165	14	8
		104		14
Meprobamate	219	158	14	8
		97		14
D7-Carisoprodol	268	183	18	8
		165		10
Carisoprodol	261	176	18	8
		158		10
D10-Carbamazepine	247	204	38	20
		202		35
Carbamazepine	237	194	38	20
		179		34

Note: Precursor ions, product ions, cone voltages, and collision energies may vary slightly between instruments.

Instrument Standardization

The following concentrations shall be used to create a quadratic calibration curve for meprobamate, carisoprodol, and carbamazepine (ignore origin and 1/x weight):

250 ng/mL
 500 ng/mL
 1,000 ng/mL
 5,000 ng/mL
 10,000 ng/mL
 20,000 ng/mL

Quality Control and Acceptance Criteria

See TOX DOM 10-06

Limitations of Procedure

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1. Lower quantitation limit = Lowest Calibrator = 250 ng/mL
 - a. Trace amounts of drug or below the LOQ may be reported as “present < LOQ” if the drug meets the identification criteria, has acceptable chromatography, and has a retention time comparable to the lowest calibrator or drug of interest.
2. Upper quantitation limit = 20,000 ng/mL

References

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