

Tramadol: Detection and Confirmation

Developed By

The PA Equine Toxicology & Research Center
(Attn.: Dr. Uboh): (610) 436-3501; Fax (610) 436-3504)

Drug Administration Facility:
University of Pennsylvania, New Bolton Center
(Attn.: Dr. Soma): (610) 444-5800; Fax (610) 444-4724)

For Testing Integrity Program

General

Tramadol [Ultram®] (2-[(dimethylamino) methyl]-1-(3-methoxyphenyl)-cyclohexanol is a centrally acting opioid analgesic agent. Evidence of the presence of Tramadol metabolites was detected on TLC in samples collected 8 hours post administration, and confirmable from a crude extract by GC/MS to 24 hours. The parent, Tramadol, was detected on TLC by Enzyme Hydrolysis Extraction to 2 hours, and confirmable by GC/MS to 4 hours. Although there was same cross reaction with both the ketorolac and the amphetamine ELISA test kits, the response was not sufficient from any of the samples of the present administration for a “positive” designation by our criteria on these ELISA kits. Therefore, the primary method of detection proposed is by TLC.

Scope

The following SOP is proposed for TLC detection and GC/MS confirmation of Tramadol or its metabolites in equine urine to 4 hours post administration following an oral administration of Tramadol (650 mg).

Definitions

GC/MS: Gas Chromatography with Mass Spectra Detection

PETRL: PA Equine Toxicology & Research Laboratory

SOP: Standard Operating Procedure

TMS: Trimethylsilyl

BSTFA: bis(trimethylsilyl)trifluoroacetamide

ELISA: Enzyme Linked Immuno-Sorbent Assay

BU: Base Urine Extraction

EH: Enzyme Hydrolysis Extraction

SB: Special Base Extraction

PC: Positive Control

NC: Negative Control

Detection

By TLC: Tramadol and its metabolites were detected using the following TLC Screens: BU, EH, and SB extractions. However, the EH procedure is the method of choice due to the superior yield of metabolites, and the detailed procedure follows.

Enzyme Hydrolysis Extraction (EH)

Scope:

Method uses urine as the medium and the TLC method described can generally detect concentrations of analytes in the low $\mu\text{g/mL}$ range. Time for completion of analysis is approximately 5-6 hours.

Principle:

Specific enzyme used to cleave β -Glucuronide linkages. Liquid/liquid extraction at proper pH range to yield desired drug analytes. Back extraction to yield cleaner extract.

Reagents

- pH 4.5 Phosphate Buffer — Formula #59
- β -Glucuronidase Enzyme Solution — Formula #50
- Ascorbic Acid Solution (10%) — Formula #49
- Ammonium Hydroxide:DI Water (50:50) — Formula #48
- DCM:IPA (10:1) — Formula #52
- Sulfuric Acid 0.5M — Formula #69
- Sodium Nitrite (NaNO_2) 5% Overspray - Formula #43
- Dragendorff's Overspray — Formula #25
- Sodium Nitrite (NaNO_2) 5% Overspray — Formula #43
- Davidow TLC Solvent System — Formula #11
- 72:18:10 (Prop A) solvent system --- Formula #18

Safety Precautions:

Samples must be cooled when removed from 65°C water bath and prior to extraction. If not cooled, DCM will bubble vigorously and may be ejected from the hot tube on to the face of the analyst. All spray procedures should be performed under a fume hood. Safety goggles should be worn.

Extraction:

Note: Only urine samples will be used in this procedure.

1. Label sufficient number of 16x150 mm screw-top test tubes to accommodate the set plus QA samples (normally one PC and one NC).

2. Label for each tube in step #1 a corresponding 16x125 mm culture tube for sample transfer.
3. To each screw-top tube add 4 mL pH 4.5 phosphate buffer.
4. To each screw-top tube add 1.2 mL β -Glucuronidase enzyme solution.
5. Add 5 mL urine to corresponding screw-top tube from each urine sample and control.
6. Cap each tube and rock tube back and forth once to mix contents by inversion.
7. Place screw-top tubes in 65°C water bath for three hours.
8. Remove from water bath and cool by immersion in cool tap water in a sink.
9. Ensure that tubes are cool to touch before proceeding with extraction.
10. Uncap tubes and add 0.5 mL of 10% ascorbic acid solution to each tube.
11. Add that amount of ammonium hydroxide:water (50:50) to effect final pH of 10.0 \pm 1 pH unit. Use a pH meter to check.
12. Add 5 mL DCM:IPA (10:1) to each tube.
13. Cap tubes and rotorack for 5 minutes.
14. Centrifuge for 5 minutes at 3000 rpm.
15. Uncap tubes and aspirate upper, aqueous layer, to waste.
16. Add 3 mL 0.5 M (1 N) sulfuric acid (formula #69) solution to each tube.
17. Recap tubes and rotorack for 5 minutes.
18. Centrifuge for 5 minutes at 3000 rpm.
19. Uncap and working quickly, aspirate the lower, organic layer to waste.
20. Add 0.2 mL of 10% ascorbic acid solution to each tube.
21. Add that amount of ammonium hydroxide:DI water (50:50) to effect final pH of 10.0 \pm 1 pH unit. Use pH meter to check.
22. Add 5 mL DCM:IPA (10:1)
23. Recap all tubes and rotorack for 5 minutes.
24. Centrifuge for 5 minutes at 3000 rpm.
25. Uncap tubes and aspirate upper, aqueous layer to waste.
26. Pour remaining organic layer into corresponding 16X125 mm culture tube.
27. Evaporate to dryness in a 65°C water bath in a fume hood.

TLC Plates

1. ***Prepare two 1-Dimensional (1-D) TLC Plates as follows***
 - a) Using the plate cutter or scribing tool, cut one 20x20 cm Merck 60F-254 TLC plate in half, resulting in two 10 x 20 cm TLC plates.
 - b) Using a sharp point and a metal ruler, scribe a line through the silica to the glass **exactly** 5 cm from one of the long sides.
 - c) Using a soft (#2) lead pencil and the metal ruler, lightly draw a line 1 cm from the base of one of the long sides.
 - d) Store the cut and scored plates in a warm oven to prevent absorption of moisture.
2. ***Spot plates:***
 - a) Add 10 μ l of DCM to evaporated EH extracted tube contents and vortex.
 - b) Spot 1/3 of each sample on the plate.
 - c) Spot standards as required per QA as well as a Tramadol standard.

- d) Develop one plate in Davidow solvent system (85:10:5, ethyl acetate: methanol: ammonium hydroxide) to the ascribed 5 cm line in a fume hood.
- e) Develop one plate in Prop A solvent system (72:18:10 chloroform:methanol:propionic acid) to the ascribed 5 cm line in a fume hood.
- f) Dry plates in fume hood with hair dryer.

3. *Spray Plates*

- a) Spray plates with Dragendorff's Reagent.
- b) Record any Drag positive anomalies.
- c) Overspray the plates with sodium nitrite.
- d) Record any anomalies.

GC/MS Prep:

Crude EH Extraction Prep: Five mL aliquots of each administration urine sample are subjected to enzyme hydrolysis (EH) extraction as described above. The contents of the tubes (containing the dried extracts) are reconstituted with 10 µl of ethyl acetate. A 1 µl volume of the reconstituted extract is injected for GC/MS analysis. Parent Tramadol was detected and confirmed to 4 hr. post administration in samples prepped by this method.

TLC Scraping Prep: Samples may be further purified by spotting the crude extract on a TLC plate, scraping the plate and eluting the analyte(s) using methanol. Plate scraping is the preferred method for "dirty" samples especially if there is sufficient volume of sample or for samples with lower concentrations of analyte, since a larger volume of sample may be concentrated.

1. Do EH extraction as above using 4 tubes of 5 mL each for each sample
 - a) Mark place for standards, then spot entire contents of each tube on plate.
 - b) Spot Tramadol standard on each end of plate (leave 2 cm between standards and samples).
 - c) Develop plate to the ascribed 5 cm line in Davidow solvent system (85:10:5, ethyl acetate: methanol: ammonium hydroxide) in a fume hood.
 - d) Dry plate in fume hood with hair dryer.
 - e) Break off parts of plate with standards spotted and spray with Dragendorff's reagent. Dry with air hose.
 - f) Realign 2 parts of plate. With pencil and ruler, grid off part of plate containing spots of interest.
 - g) Using plugged fire drawn Pasteur pipette (from Frank Railing) attach vacuum line and vacuum off all silica from indicated area.
 - h) Break off the top of the Pasteur pipette tube and add MeOH equal in volume to the volume of the silica.
 - i) Elute under pressure with bulb or air line into screw top tube and dry under air pressure. The dried eluent is then analyzed by GC/MS.

Confirmation Of Tramadol by GC/MS:

General:

Enzyme Hydrolysis extraction produced three possible metabolites in addition to the parent, Tramadol. The presence of these metabolites in quantities greater than the parent compound, Tramadol, suggests that these metabolites should be the analytes of choice. Since pure and traceable standards for the metabolites are not available at this time, the A2LA amendments to ISO 25 guide allow isolates from an authenticated drug-administered urine to serve as reference standards (section 2.11, Animal Drug Testing Program Requirement, June 1, 1994). Therefore, both the parent Tramadol, and the metabolites will be targeted for GC/MS confirmation.

GC/MS Conditions:

Splitless mode at Head Pressure: 8 psi
Column: Capillary- 25M, BPX-5, 5 μ (SGE)
Initial Temp: 65°C 1 min. hold
Program Rate: 30°C/min.
Final Temp: 320°C 3.5 min. hold

GC-MS run was performed in full scan EI mode over the mass range of 46-446 amu
(Base Peak for Tramadol = 263)

Reagents for Tramadol SOP

EH Extraction

Formula #48. Ammonium Hydroxide (NH₄OH):DI H₂O (1:1) Reagent For Use in EH Urine Extraction

Procedure (perform under a fume hood):

Combine 500 mL concentrated NH₄OH and 500 mL DI H₂O in an Oxford re-pipettor bottle.

Formula #49. Ascorbic Acid solution 10% for Use in Enzyme Hydrolysis Urine Extraction

Procedure for 1 Liter:

Dissolve 100 gm of L-Ascorbic Acid (Fisher) into 1 liter of DI H₂O.

Formula #50. β -Glucuronidase (*Patella vulgata*) Enzyme Used for Enzyme Hydrolysis Urine Extraction

Procedure: For a minimum final concentration of 5000 AU/mL

1. 1 vial 500,000 units in 88 mL DI H₂O.
2. 1 vial 1,000,000 units in 175 mL DI H₂O
3. 1 vial 2,000,000 units in 350 mL DI H₂O
4. Use 1 1/2 mL per sample.
5. Store at 4° C, Maximum shelf life is 6 days if enzyme solution has been stored at 4°C (less, if left at room temperature for several hours).

Formula #52. DCM:IPA (10:1) Reagent For Use in EH Urine Extraction. Procedure for 4000 mL:

1. From a fresh 4000 mL bottle of dichloromethane (DCM) remove 500 mL DCM (can place the 500 mL DCM in DCM pipettor bottle).
2. Add 350 mL isopropanol (IPA) and **mix thoroughly**.

Formula #59. pH 4.5 Buffer For Use with Enzyme Hydrolysis Urine Extraction.

Procedure for 1 liter:

1. Prepare a saturated solution of monobasic potassium phosphate (KH₂PO₄) by adding KH₂PO₄ to 1 liter of DI H₂O while stirring until saturated (no more will go into solution) and a precipitate remains. The pH of this solution should be 4.5 if it is saturated.

2. Let stand a minimum of 12 hours and decant clear solution into a clean reagent bottle.

Formula #69. Sulfuric Acid (1N, 0.5M) H₂SO₄ For Use in EH Urine Extraction

Procedure for 3600 mL. (Wear goggles.):

1. Pour 2000 mL of DI H₂O into a 4000 mL flask.
2. Slowly add 100 mL of Conc. H₂SO₄ (36N)/mix thoroughly while adding.
3. Dilute to 3600 mL w/ DI H₂O.

Warning: Add acid to H₂O. **Never** add water to acid

TLC Analysis

Formula #11: 85:10:5 (Davidow) solvent system: ethyl acetate:methanol:ammonium hydroxide

Formula #18: 72:18:10 (Prop A) solvent system: chloroform:methanol:propionic acid

Formula #25 — Dragendorff's Overspray

Procedure for 140 mL spray:

NOTE: This mix will be a white suspension. Shake well before use.

1. Solution "A," combine:
 - 2 gm of bismuth subnitrate (or 2 gm bismuth subcarbonate)
 - 100 mL DI H₂O
 - 25 mL glacial acetic acid
2. Solution "B," combine:
 - 40 gm of potassium iodide (KI)
 - 100 mL DI H₂O
3. Combine:
 - 10 mL of Solution "A"
 - 10 mL of Solution "B"
 - 20 mL of Glacial Acetic Acid
 - 100 mL DI H₂O

NOTE: Solutions A & B can be saved for future use.

Formula #43 — Sodium Nitrite (NaNO₂) 5% Overspray

Procedure for 800 mL: Dissolve 40 gm of NaNO₂ in 800 mL of DI H₂O.