

TIP Approved SOP

DETECTION AND CONFIRMATION OF TRAMADOL IN GREYHOUND URINE

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General

Tramadol (Ultram) is a centrally acting analgesic agent. Tramadol and metabolites can be detected by TLC after EH liquid/liquid and SPE protocols. Confirmation is by GC/MS.

Scope

This SOP is proposed for TLC detection and GC/MS confirmation of Tramadol and metabolites in greyhound urine following oral administration of 30 mg.

Safety Precautions

TLC spray procedures should be performed under a fume hood. Protective clothing and goggles should be worn.

**Extraction Procedure I: Enzyme Hydrolysis Followed by
Liquid/Liquid Extraction**

A specific enzyme is used to cleave β -glucuronide linkages. Liquid/liquid extraction at proper pH range yields desired drugs. Back extraction yields cleaner extract.

NOTE: pH meters must be standardized to a pH of 7. Record the values in the pH Meter Logbook (see **QA/QC LOGS - pH Meters**). The term "sample" refers to not only the urine received for testing, but also the negative urine control (NCU), the enzyme hydrolyzed urine positive control (EHUPC), and the open blind (### TAMU). The EHUPC obtained by equine administration contains O-desmethylpyrilamine glucuronide. Throw away the extraction tube used for the EHUPC.

1. To 5 mL of sample in a 16 x 125 mm screw-top glass tube, add 2 mL of 1M acetate buffer (pH 5).
2. Add 1 mL β -glucuronidase to each tube. Vortex each tube.
3. Cover tubes with foil (or loosely cap), place in a small test tube rack and set in the water bath or incubating oven (approximately $60^{\circ}\text{C} \pm 5^{\circ}$).
4. Incubate tubes for 2-4 hrs. **DO NOT INCUBATE MORE THAN 4 HOURS.**
5. At end of heating period, place tubes in cool water for minimum of 5 minutes.
6. Add 0.5 mL ascorbic acid (10%) solution to each tube.
7. Add 0.3 mL of ammonium hydroxide (NH_4OH): dH_2O (50:50) to each tube. Vortex 3 seconds.
8. Adjust each tube to pH 8.5-9.2 using 6N HCl (1 drop at a time) or 0.1-0.2 mL NH_4OH : dH_2O (50:50). Use pH meter to measure pH. This step is **VERY** important.
9. Add 5 mL dichloromethane (DCM):isopropanol (ISO) (10:1) to each tube. (Sample will bubble if not completely cooled)
10. Cap tightly and rotorack slowly for 5 minutes.
11. Centrifuge at approximately 2000-25000 rpm for 5 minutes.
12. Aspirate aqueous (top) layer and carefully transfer solvent (bottom) layer to clean screw-top tube.
13. Add 3.0 mL 0.5 M sulfuric acid (H_2SO_4).

14. Cap tightly and rotorack slowly for 5 minutes.
15. Aspirate bottom layer. **SAVE TOP LAYER!**
16. Add 0.2 mL ascorbic acid (10%) solution to each tube.
17. Add 0.6 mL NH₄OH:dH₂O (50:50) to each tube.
18. Vortex each tube for three seconds.
19. Adjust each tube to pH 8.5-9.2 using 6N HCl (1 drop at a time) or 0.1-0.2 mL NH₄OH:dH₂O (50:50). Use pH meter to measure pH. This step is **VERY** important.
20. Add 5 mL DCM:ISO (10:1) to each tube.
21. Cap tightly and rotorack slowly for 5 minutes.
22. Aspirate aqueous (top) layer and carefully transfer solvent (bottom) layer to clean 13 x 100 mm disposable glass test tube.
23. Evaporate at 45°C ± 5°C using N₂.

**Extraction Procedure II: Enzyme Hydrolysis Followed by
Solid Phase Extraction**

After incubation with β-glucuronidase enzyme, urine samples are loaded onto mixed bed SPE columns and drugs are sequentially eluted. This generic protocol gives details for recovery of acidic/neutral, steroid class, and basic drugs, even though Tramadol and metabolites are only recovered in the basic fraction. For this specific application, steps (4) and (5) under Sample Preparation and steps (7-12) under SPE Sample Extraction may be omitted.

The columns used in this protocol are from United Chemical Technologies, Inc. (XTRACT, 500 mg, # XRDAF515).

NOTE: The term "sample" refers to not only the urine received for testing, but also the solid phase extraction positive control urine (SPCU), open blind (### TAMU) and an optional negative control (limited space for columns on the manifold).

NOTE: The pipetting syringe must be primed with at least 20 ml of the new solution prior to application to the column.

SAMPLE PREPARATION

1. To 5 ml of sample in a numbered 20 x 125 mm glass tube, add 2 ml of 1M acetate buffer (pH 5) for the basic fraction - enzyme hydrolysis.

2. Add 1 ml β -glucuronidase to each tube. Mix well.
3. Place tubes in test tube rack and cover loosely with foil. Set into 65°C (\pm 5°C) water bath. Set timer for 2-4 hrs.
4. After 1 hr., 45 min. (or within 15 mins of timer from above), add 1 ml of 0.1N NaOH (sodium hydroxide) to 2 ml of sample in a numbered 16 x 100 mm disposable culture tube for the acid fraction - basic hydrolysis. Mix well and let stand for 10-15 min.
5. After the 15 min. interval (2-4 hr. interval for the basic fraction), add the acid fraction sample to the corresponding basic fraction sample; i.e., acid fraction #552 is poured into the basic fraction #552 tube.
6. Add 5 ml 0.2 M phosphate buffer (pH 6.5) into the combined sample tube and mix.
7. Check the pH and, if necessary, add acid (6N HCl) or base ($\text{NH}_4\text{OH}:\text{dH}_2\text{O}$ 50:50) until the pH is between 5.5 and 6.5. (**NOTE:** After each addition of acid or base, the sample must be mixed)

COLUMN PREPARATION

All solid phase extraction manifolds are used according to manufacturer's instructions. Traps must be installed between manifold and vacuum source. Empty when half full.

1. Prepare numbered 16 x 100 mm disposable glass tubes labeled **A** (Acid/Neutral drugs) and 16 x 125 disposable screw-top glass tubes labeled **B** (Basic drugs) for sample collection.
2. Place the appropriate number of stopcocks on the manifold.
3. Insert the appropriate number of SPE columns (numbered accordingly) on stopcocks.
4. Add 5 ml methanol to each SPE syringe bed and aspirate. Do not allow the column to go dry during steps 4-6. Use minimum vacuum.
5. Immediately add 5 ml dH_2O to each column and aspirate.
6. Add 3 ml 1.0 N acetic acid (HOAc) to each column and aspirate. At this time the column is activated.

SPE SAMPLE WASHES AND EXTRACTION

NOTE: If a sample does not flow through the column, the sample is to be worked up individually. More vacuum may be applied or liquid/liquid protocols may be followed.

1. Apply the sample to the activated column at a rate of approximately 1-3 ml/min.
2. Add 5 ml 0.2 M phosphate buffer (pH 6.5) to each column and aspirate. (Wash)
3. Add 2 ml 1.0 N HOAc to each column and aspirate. (Wash)
Dispose of waste.
4. Dry the column under strong vacuum (e.g., 12-15 in) for •10-40 min.
5. Release vacuum.
6. Add 5 ml hexanes to each column and aspirate. (Wash)
Dispose of waste.
7. Place numbered glass tubes labeled **A** in appropriate collection positions.
8. Elute the Acid/Neutral drugs with 5 ml DCM containing 1% methanol. Collect eluate at •5 ml/min. Use vacuum as necessary to recover residual solvent.
9. Remove the **A** tubes and place them in a test tube rack.

STEPS 10-12 ARE OPTIONAL. (Steroid fraction)

10. Insert numbered 16 x 100 mm glass tubes labeled with **S** (Steroid).
11. Elute the Steroid drugs with 7 ml Ethyl Acetate (EtOAc). Collect eluate at •5 ml/min.
12. Remove the **S** tubes and place them in a test tube.
13. Add 7 ml methanol to each SPE column and aspirate. (Wash)
14. Insert numbered 16 x 125 mm screw-top glass tubes labeled with **B** (Basic/Enzyme hydrolyzed) into the manifold.
15. Mix thoroughly 80:20:2 DCM:ISO:NH₄OH (ISO is isopropanol) prior to use.

NOTE: (Mixture should not be allowed to sit in an open container and is to be prepared fresh daily) Elute the Basic/Enzyme hydrolyzed drugs with 10 ml 80:20:2 DCM:ISO:NH₄OH. Flow rate should not exceed 1 to 3 ml/min.

16. Apply vacuum as necessary to recover residual solvent from the column.
17. Remove the **B** tubes and place them in a test tube rack. Place columns in a bag and dispose at supervisor's discretion (usually after 1-2 days). Clean manifold.
18. Add 4 ml distilled or deionized H₂O to each **B** tube. Cap and rotorack for 5 minutes. Aspirate off the aqueous (top) phase. Transfer the organic phase into a properly labeled disposable 16 x 100 mm tube.
19. Evaporate **A**, **B** and **S** tubes at 40°C ± 5°C with nitrogen (N₂).
(**NOTE:** If using N-Evap, before loading sample tubes clean N-Evap needles as follows: Dip needle in a beaker filled with methanol, then vent with N₂). Remove tubes as soon as they are dry. Drugs may be lost if tubes are left too long in water bath.

TLC SAMPLE APPLICATION

1. Using a #1 pencil, lightly mark a line at 2 cm across two 10 x 20 cm TLC plates and heavily score a line at 7 cm. Above the 7 cm line, label the plates with date, tech's initials, race track, race date, solvent system, UV and spray sequences. Below the 2 cm line, label the negative urine control, the positive urine control, standards, and lab samples numbers at application spots. Place standards in the middle of the plate. Allow the plates to activate for at least 10 minutes on a hot plate (approximate temperature 113° - 130°F) before spotting.
2. Dissolve each sample in 10 µL of ethyl acetate (EtOAc) immediately before spotting.
3. Spot 2 µL of each sample using either a 10 µL Hamilton syringe or an Eppendorf Ultra-micropipettor on the TLC plate. (If adding more solvent, then spot more sample, i.e., add 20 µL - spot 4 µL)
4. Rinse the syringe with 30 µL of EtOAc between samples, (pull up and dispense 10 µL 3 times) or discard the used pipette tip.

TLC ANALYSIS

1. In the middle of the plate, spot 2 µL of the appropriate standards.
2. Develop in Prop-A while still warm, allow to dry. Record in

Solvent Tank Logbook. After 4 plates are developed, make fresh Prop-A.

3. Observe the plate under SUV and LUV. Mark any quenching or fluorescence and record results.
4. Place dry plate in iodine vapor chamber for 2 minutes. (Apomorphine should turn green) Record the Rf value and color for any other spot that appears after exposure to these vapors. Spots fade quickly.
5. Heat plate for 4 minutes under fume hood. Green color of apomorphine is enhanced.
6. Let plate cool. Spray lightly with Folin Denis until blue-grey color appears.
7. Expose plate to ammonia vapors only until standards turn a darker blue-grey (approximately 30-60 seconds). Circle any spot that turns grey at this point. This color generally indicates the presence of a phenolic group.
8. Heat plate for at least 5 minutes so ammonia won't interfere with Drag.
9. Let plate cool and spray with Drag., cover with a glass plate, observe on light box, and mark any orange spots. Record colors and Rf values for spots observed.
10. Spray with NaNO_2 , cover with a glass plate and observe on light box. Record colors and Rf values. Record Rf values for standards on Standards Rf Logsheet.
11. After completion of spray sequence, parent Tramadol appears as a brown spot at approximately Rf 0.36. Tramadol metabolites appear as brown spots at approximately Rf 0.12 and 0.40.

REAGENTS

- Extraction Reagents and TLC solvent Systems -

NOTE: It is suggested that all reagents be prepared under the hood while wearing gloves and goggles. Reagents should be labeled with their identifying name, date of preparation and initials of tech preparing the reagent. Expiration dates are not applicable except for β -glucuronidase solution which should have an expiration date of one month after the date of preparation on the label. Observe all reagents in their bottles or pipettors and if particulate matter or cloudiness is apparent, make fresh reagent. Record the components and amounts used in preparing all reagents in the Reagents and Sprays Log.

NOTE: Many chemicals (e.g., phosphate salts) come in a hydrated form. Check the label of the chemical versus the recipe. Adjust the quantity required as necessary.

NOTE: FOR YOUR SAFETY AND OTHERS IN THE LAB WHEN PREPARING CAUSTIC MATERIALS, PLEASE TAKE PRECAUTIONS. ALWAYS ADD ACIDS TO WATER.

1.0 M Acetate Buffer (pH 5.0) [EH]

Place 164.0 g **sodium acetate** or 272.0 g **sodium acetate trihydrate** in a 2 L flask. Add 66.0 mL **acetic acid**. Dilute to volume (2000 mL) with **deionized water**. Check pH (5.0 ± 0.1). Pour part of solution in Oxford pipettor. Store the remainder.

50% Ammonium Hydroxide $\text{NH}_4\text{OH}:\text{dH}_2\text{O}$ (50:50) [EH]

Combine 250 mL **ammonium hydroxide** (NH_4OH) and 250 mL **deionized water** in a small Oxford pipettor.

10% Ascorbic Acid [EH]

Dissolve 25 g of **ascorbic acid** in 250 mL **deionized water**. Pour into an Oxford pipettor. Store in refrigerator.

Dichloromethane:Isopropanol DCM:ISO (10:1) [EH]

Combine 900 mL **DCM** and 90 mL **isopropanol** in an Oxford pipettor.

β -Glucuronidase "Patella vulgata" Solution (5000 IU/ml [EH]

Dissolve one bottle (1 million units) of **β -glucuronidase** from Patella vulgata (e.g., Sigma) in 200 mL **deionized water**. Pour into a small Oxford pipettor. Store in the refrigerator.

NOTE: The quantity of raw material may vary due to differences in specific activity. When preparing the solution, make sure you use 1 million units.

6 N Hydrochloric Acid (HCl) [EH]

Slowly add 258 mL concentrated **hydrochloric acid** (HCl) to 242 mL **deionized water** in a 1000 mL beaker. (**CAUTION: Add acid to water! Wear goggles!**). Using a stir bar, slowly mix the solution, then allow to cool. Pour into an Oxford pipettor.

0.5 M (1.0 N) Sulfuric Acid (H_2SO_4) [EH]

Pour 500 mL **deionized water** into a 1000 mL flask, slowly add 25 mL concentrated **sulfuric acid**. (**CAUTION: Add acid to water!**) Dilute to 900 mL with **deionized water**.

Prop-A [BU] & [EH]

Solvent: chloroform 72 mL : methanol 18 mL : propionic acid 10 mL

Stock: chloroform : methanol (72:18)

Daily: **Mix stock solution well.** Dispense 90 mL stock into a 100 mL graduated cylinder. Add 10 mL propionic acid. Pour into tank, cover, mix thoroughly by tilting tank, and let equilibrate for 15 minutes.

NOTE: Make fresh solvent after 4 TLC plates are developed.

Dichloromethane:Isopropanol:Ammonium Hydroxide DCM:ISO:NH₄OH (80:20:2) [SPE]

Combine 2 mL **NH₄OH** to 20 mL **isopropanol** in a graduated cylinder (mix well). Add mixture to 80 ml **DCM**.

0.2 M Phosphate Buffer pH 6.5 [SPE]

Dissolve 87.04 g **potassium phosphate monobasic** (KH₂PO₄) in 3200 mL **deionized water**. Mix well. Adjust pH to 6.5 with **sodium hydroxide** pellets (approximately 11 g) or 5-10N Na or KOH.

- TLC Spray Solutions -

NOTE: All spray solutions should be prepared under the hood while wearing gloves. Sprays should be labeled with their identifying name, date of preparation and initials of tech preparing the solution. Record the components and amounts used in preparing all sprays in the Reagents and Sprays Log. Expiration dates are generally not applicable except for Dragendorf's (2 days) and mercuric chloride:DPC (1 week). Appropriate expiration dates should be recorded on the labels of these solutions. Store extra spray solution in the refrigerator.

NOTE: FOR YOUR SAFETY AND OTHERS IN THE LAB WHEN PREPARING CAUSTIC MATERIALS, PLEASE TAKE PRECAUTIONS. ALWAYS ADD ACIDS SLOWLY TO WATER OR SOLUTION.

Ammonia Vapors

Under the fume hood, pour enough **ammonium hydroxide** to cover the bottom of a 50 mL beaker. Place the beaker in the developing tank labeled "ammonia vapors" under the fume hood. Close the lid, making sure a tight seal is formed. Allow vapors to form (approximately 30 minutes) before use.

Dragendorf's Spray

Reagent A: Add 2.0 g **bismuth subnitrate** to 25 mL **glacial acetic acid**. Dilute to 100 mL with **deionized water**.

Reagent B: Dissolve 40.0 g **potassium iodide** in **deionized water**; dilute to 100 mL with **deionized water**.

Spray: Mix 10 mL **Reagent A** and 10 mL **Reagent B**, add 20 mL **glacial acetic acid**; dilute to 100 mL with **deionized water**.

Unused A and B are stored at room temperature. Dragendorff's is stable for two days.

Folin Denis Spray

In a 250 mL round-bottom flask, combine 10 g **sodium tungstate**; 2 g **12-molybdosilicic acid**; 5 mL of concentrated **phosphoric acid**; 50 mL **deionized water**; and 5-10 boiling chips.

Place flask in heating mantle.

Attach condenser to the flask.

Clamp the condenser to a ring stand so it is in a vertical position fitting snugly into the flask.

Attach one piece of rubber tubing to the water spigot. Attach the other end of this tubing to the lower spigot on the condenser.

Attach a second piece of tubing to the upper spigot and put the other end in the sink.

Turn the water on **slowly** (low flow).

Plug in the heating mantle to start refluxing.

Allow the mixture to reflux for 2 hours.

Allow the mixture to cool at room temperature.

Dilute the mixture to 100 mL with deionized water.

Iodine (I₂) Vapors

Pour enough **iodine crystals** to cover the bottom of a large desiccator. Cover with the lid, making sure a tight seal is formed. Allow vapors to form (approximately 30 minutes) before use.

Sodium Nitrite Spray (NaNO₂)

Dissolve 5.0 g **sodium nitrite** in deionized water. Dilute to 100 mL with **deionized water**.

LIMITS OF DETECTION

Limits of detection of tramadol in greyhound urine as determined by TLC were 200 ng/ml by liquid/liquid extraction and 600 ng/ml by solid phase extraction. Reference standards for metabolites were not available.

GC/MS CONFIRMATION

Extraction: 5 ml urine subjected to enzyme hydrolysis followed by LL extraction or SPE.

Add 20 to 40 μ l commercially available BSTFA to the appropriate extraction residue in a small test tube or glass insert for an autosampler vial. Heat for 1 to 2 min. at 60°-80°C in a heating block (or equivalent). Longer heating times of up to 1 hour may be used without detriment. One μ l is injected for GC/MS analysis.

NOTE: As TMS derivatives are very sensitive to H₂O, residues should be completely dry prior to the addition of BSTFA.

Alternately, the residue may be dissolved in 20 μ l **ethyl acetate**, and 1 μ l is analyzed. Derivatization is not necessary for GC/MS analysis; however, resolution and peak confirmation are improved for TMS derivatives of Tramadol metabolites.

GC/MS RESULTS

Parent Tramadol is the major component in urine. Three metabolites -- hydroxytramadol (major metabolite), O-desmethyltramadol (minor metabolite), and N-desmethyltramadol (minor metabolite) -- have been tentatively identified. Chromatograms were similar qualitatively whether extraction was by SPE or liquid/liquid protocol.

Mass spectra of TMS derivatives --

Tramadol - 58 (100), 73 (20), 335 (mono-TMS, 5)

Hydroxytramadol - 58 (100), 73 (10), 423 (di-TMS, 1)

O-desmethyltramadol - 58 (100), 73 (20), 393 (di-TMS, 2)

N-desmethyltramadol - 73 (25), 116 (100), molecular ion not seen.