

SOP FOR
DETECTION AND CONFIRMATION OF
VENLAFAXINE AND METABOLITES
Texas Veterinary Medical Diagnostic Laboratory
College Station, Texas
VENLAFAXINE DETECTION AND CONFIRMATION

Safety Precautions

TLC procedures must be performed in a fume hood. Protective clothing (safety goggles, lab coats, gloves) should be worn.

Scope

This SOP describes detection and confirmation of venlafaxine metabolites in equine urine.

DETECTION: TLC

Five ml urine subjected to enzyme hydrolysis followed by liquid/liquid extraction:

Basic Principles: Specific enzyme used to cleave β -Glucuronide linkages. Liquid/liquid extraction at proper pH range to yield desired drugs. Back extraction to yield cleaner extract.

Extraction

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-desmethylypyrilamine glucuronide. Throw away the extraction tube used for the EH-PCU.

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 2000-2500 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 NH_4OH : dH_2O (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 NH_4OH : dH_2O (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 $45^{\circ}\text{C} \pm 5^{\circ}\text{C}$ using N_2 .

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 (EH-1 and EH-2). Include date, tech's initials, race track, race date, solvent system, UV and spray sequences. Below the 2 cm line, label the negative urine control (NCU), the positive urine control (EHUPC), standards, and lab sample 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 $113^{\circ} - 130^{\circ}\text{F}$) 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 μ L 3 times) or discard the used pipette tip.

TLC ANALYSIS

1. In the middle of the plate, spot 2 μ L 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. (Won't take long, 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. Parent venlafaxine (standard) appears as a brown spot with an Rf of approximately 0.24. Venlafaxine metabolites appear as brown spots at approximately Rf 0.08 and 0.12.

REAGENTS FOR VENLAFAXINE SOP

- 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 NH₄OH:dH₂O (50:50) [EH]

Combine 250 mL **ammonium hydroxide** (NH₄OH) 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 (H₂SO₄) [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.

- 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 Dragendorff'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.

Dragendorff'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 by TLC, as determined by spiking blank urine with reference standards prior to extraction, were 50 ng/ml for venlafaxine and 100 ng/ml for O-desmethylvenlafaxine.

GC/MS CONFIRMATION

Extraction: 5 ml urine subjected to enzyme hydrolysis followed by LL extraction (see above).

Residue dissolved in 20 µl **ethyl acetate**, 1 µl injected (derivatization not necessary).

GC/MS conditions:

Column HP-5 MS, 30 m (0.25 mm ID)
Port temp - 200°C
Initial oven temp - 80° (1 min)
Ramp rate - 10°/min
Oven temp - 130°C (0 min)
Ramp rate - 20°/min
Final oven temp - 280°C

MS conditions:

Mass range - 50-400 amu

GC/MS Results

Two metabolites - O-desmethylvenlafaxine (major metabolite) and a hydroxyvenlafaxine (minor metabolite) - have been tentatively identified. Parent may be present in trace amounts.

Approximate retention times -

Venlafaxine (MW 277)	12.7 min
O-desmethylvenlafaxine (MW 263)	13.0 min
Hydroxyvenlafaxine (MW 293)	13.8 min

Mass spectra -

Venlafaxine - 58(100), 134(40), 179(10), 277(1)

O-desmethylvenlafaxine - 58(100), 120(40), 165(10), 263(1)

Hydroxyvenlafaxine - 58(100), 134(70), 179(10), 293(1)

References

AT Parsons, Anthony RM, Meeker JE: Two fatal cases of venlafaxine poisoning. *J Analyt Tox* 20, 266-268 (1996).