

Amoxapine Administration (Equine):

**Detection and Confirmation of the Amoxapine and its
metabolite (7-hydroxy amoxapine)**

A Procedure Developed

by

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SOP FOR DETECTION OF AMOXAPINE AND ITS METABOLITE IN EQUINE URINE

Scope

This SOP describes detection and confirmation of amoxapine and its metabolite, 7-hydroxy amoxapine in equine urine. Results are based on analysis of urine obtained 4 hours after oral administration of a single 600 mg dose of Asendin (Wyeth-Ayerst). Asendin is an antidepressant of the dibenzoxazepine class, with a mild sedative component to its action. The daily human effective dose ranges from 200 to 300 mg. The primary metabolite in humans is 8-hydroxyamoxapine.

Definitions

TLC: Thin Layer Chromatography
GC/MS : Gas Chromatography with Mass Spectra Detection
SOP: Standard Operating Procedure
EH: Enzyme Hydrolysis
EHUPC: Enzyme hydrolyzed urine positive control
L/L: liquid/liquid
TMS: Trimethylsilyl
BSTFA: bis(trimethylsilyl)triifluoroacetamide
PCU: Positive Control Urine
NCU: Negative Control Urine

BASIC PRINCIPLES:

A number of drugs and their metabolites are excreted in the urine as their β -glucuronide conjugates. A specific enzyme (β -glucuronidase) is used to cleave β -glucuronide linkages. Liquid/liquid extraction at the proper pH range yields desired drugs. Back extraction provides cleaner extracts. The extract is analyzed by TLC, and the drug and its metabolite are confirmed by GC/MS

REAGENTS:

CAUTION: IT IS SUGGESTED THAT ALL REAGENTS BE PREPARED UNDER THE HOOD WHILE WEARING GLOVES AND GOGGLES.

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

Extraction Reagents

1.0 M Acetate Buffer (pH 5.0)

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)

Combine 250 mL **ammonium hydroxide** (NH₄OH) and 250 mL **deionized water** in an Oxford pipettor.

10% Ascorbic Acid

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)

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

.-Glucuronidase "Patella Vulgata" Solution (5000 IU/ml)

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

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)

Pour 500 mL **deionized water** into a 1000 mL flask, slowly add 25 mL concentrated **sulfuric acid**. (**CAUTION: Add acid to water and cool the flask in ice-cold water if necessary!**) Dilute to 900 mL with **deionized water**.

TLC Solvent Systems

Prop-A: (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.

TLC SPRAY REAGENTS

NOTE: ALL SPRAY SOLUTIONS SHOULD BE PREPARED UNDER THE HOOD WHILE WEARING GLOVES.

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. Dragendorf'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 a reflux condenser with circulating cold water. Reflux for 2 hours, allow to cool to room temperature and 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.

EXTRACTION PROCEDURE:

NOTES:

1. pH meters must be standardized to a pH of 7.
2. The term "sample" refers to not only the urine received for testing, but also the negative urine control (NCU), the enzyme hydrolyzed urine control (EHUPC), and the open blind (### TAMU). The positive EHUPC obtained by equine administration contains O-desmethylpyrilamine glucuronide.

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(5000 units) -glucuronidase solution to each tube and vortex .
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°C ± 5°C).
4. Incubate tubes for 2-4 hrs. **DO NOT INCUBATE FOR MORE THAN 4 HOURS.**
 5. After incubation cool the tubes in ice-cold 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 (NH₄OH):dH₂O (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 NH₄OH:dH₂O (50:50). Use pH meter to measure pH. This step is **VERY** important.
 9. Add 5 mL dichloromethane):isopropanol (DCM:ISO) (10:1) to each tube.
 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 (H₂SO₄).
 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.

ANALYSIS:

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 plate (EH-1). Below the 2 cm line, label the negative urine control (NCU), the positive urine control (EHUPC), standards. 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°C - 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-micro-pipettor on the TLC plate. (If adding more solvent, then spot more sample, i.e., add 20 µL - 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 μ L of the appropriate standards.
2. Develop in Prop-A. allow to dry.
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. 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.
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 60
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 amoxapine (standard) appears as a brown spot with an Rf of approximately 0.55, and was detected in urine extracts in trace amounts. The predominant reaction (brown, $R_f \approx 0.40$) was attributable to a metabolite of amoxapine (either the 7- or 8-hydroxy compound). Reference standard 8-hydroxyamoxapine had an Rf of approximately 0.40.

LIMITS OF DETECTION

Limits of detection by TLC, as determined by spiking blank urine with reference standards prior to extraction, were 100 ng/ml for parent amoxapine (quench 254nm and NaNO_2) and 50 ng/ml and 200 ng/ml for 8-hydroxyamoxapine (quench 254nm vs. NaNO_2).

IMMUNOASSAY DETECTION

No immunoassay systems which are effective in screening urine samples for the presence of amoxapine and its metabolites have been found. This includes the ELISA kits available from ELISA Technologies, Inc.

GC/MS CONFIRMATION

Extraction: Ten ml urine were subjected to enzyme hydrolysis followed by L/L extraction (see above). After evaporation of the solvent, the residue was dissolved in 50 μ l ethyl acetate, 1 μ l was injected (derivatization not necessary). A sharper chromatographic peak with less tailing, however, is obtained for the primary metabolite after preparation of the TMS derivative (dissolve residue in 50 μ l BSTFA, heat for 5 min. at 80°C, inject 1 μ l).

SUMMARY

Parent amoxapine was detected and confirmed in equine urine extracts in trace amounts. The major metabolite which was readily detected by TLC and GC/MS appeared to be 7-hydroxyamoxapine for which no reference standard is available.

Trace amounts of unknown metabolites (possibly the N-methyl and hydroxy N-methyl) appeared to be present, but the 8-hydroxy metabolite was not seen. This is in direct contrast to human metabolism of amoxapine wherein 8-hydroxyamoxapine is predominant. The 7-hydroxy metabolite is also reported to be present in urine of human subjects in lesser amounts.

GC/MS conditions:

Varian Saturn ion trap/3400 GC

Column DB 1, 20 m (0.25 mm ID)
Septum-equipped programmable injector - 125°C (0.5 min) then ramp to 280°C @ 180°C/min. hold 6.0 min.
Initial oven temp - 80°C (1 min)
Ramp rate - 20°C/min
Oven temp - 130°C
Ramp rate - 30°C/min.
Final oven temp - 280°C
Mass range - 50-500 amu

HP 5972MSD/5890GC

Column HP5MS, 15 m (0.25 mm ID)
injection port - 200°C
Initial oven temp - 120°C (1 min)
Ramp rate - 20°C/min
Final oven temp - 280°C
Mass range - 50-500 amu

GC/MS DATA

<u>DRUG</u>	<u>RETENTION TIMES (mts)</u>	
	<u>HP</u>	<u>VARIAN</u>
Amoxapine	9.8	10.0
Amoxapine TMS	10.4	
8-Hydroxyamoxapine - mono TMS (reference standard)	11.5	11.7
8-Hydroxyamoxapine - di TMS (reference standard)	12.5	
7-Hydroxyamoxapine - mono TMS (tentatively identified)	11.7	12.1
7-Hydroxyamoxapine - diTMS (tentatively identified)	12.8	

MASS SPECTRAL PEAKS

Amoxapine:	245(100), 247(30), 257(41), 259(26), 313(6)(M ⁺), 314(9), 315(4).
Amoxapine TMS	128(100), 269(8), 270(7), 271(5), 385(2) (M ⁺),
8-Hydroxyamoxapine mono TMS	332(48), 333(100), 335(32), 401(6) (M ⁺), 402(9), 403(3).
8_hydroxyamoxapine diTMS	128(100), 332(9), 333(5), 334(3), 345(9), 473(2) (M ⁺),
7-Hydroxyamoxapine mono TMS	332(100), 333(96), 335(32), 401(19) (M ⁺), 402(17), 403(9).
7-Hydroxyamoxapine diTMS	128 (100), 332(79), 333(20), 334(30), 345(35), 473(11) (M ⁺)

References

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