

Methylphenidate Detection and Confirmation Procedures

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For Testing Integrity Program

General

Methylphenidate (Ritalin®) is a central nervous system stimulant. Base urine (BU) extraction effectively isolates the parent compound but confirmation is inhibited by co-extracted materials. These co-extracting compounds appear to be similar to atropine on a TLC plate and are therefore designated by the PETRL as “pseudo-atropine” and by other labs as “tri-spot”. These co-eluting compounds are assumed to be dietary in origin. The following procedure is a modification of the PETRL SOP #105 for BU extraction. By the acylation of the amino group, this method provides increased sensitivity for the analysis of sympaththomimetic amines on the GC/MS.

Safety Precautions

General Good Laboratory Practices

Scope

The following SOP is proposed for the detection by TLC and the subsequent confirmation by GC/MS of methylphenidate in equine urine, at an estimated concentration of 1-10 ng/mL.

Definitions

GC/MS: Gas Chromatography with Mass Spectrometry
PETRL: PA Equine Toxicology & Research Laboratory
SOP: Standard Operating Procedure
HFB: Heptafluorobutyric Anhydride
PC: Positive Control
NC: Negative Control
EtAc: Ethyl Acetate
BU: Base Urine
TLC: Thin-Layer Chromatography

Detection: Base Urine Extraction

Materials Required

1. 0.93 N NaOH Solution (pH 13-14, formula #64)
2. Dichloromethane (DCM)
3. "Davidow" Solvent System (85:10:5, formula #11)
4. Thin Layer Chromatography (TLC) Plates (Silica gel 60 F254, precoated 10 cm x 20 cm, 0.25 thickness - EM Science)
5. Fluram (formula #31) Overspray
6. Ninhydrin (formula #40) Overspray
7. Dragendorff's (formula #25) Overspray
8. CuCl_2 (formula #26)
9. Hot Plate
10. 10 ML pipetter with tips for every sample
11. Reference Drug Standards

Extraction Procedure

1. Add 2 ML of 0.93 N NaOH to each labeled screw capped tube.
2. Add 5 ML of DCM to each tube.
3. Transfer 10 ML of each urine sample into tube. Include a negative(NC) and positive(PC) urine control tube.
4. Cap (clean caps) each tube and rotorack for 5 minutes at slow speed (20 rpm).
5. Centrifuge ≈ 2500 rpm for 3-5 minutes.
6. Aspirate to waste the aqueous (top) layer and transfer (pour) the solvent (bottom) layer into a clean tube (16x150 or 16x125).
7. Evaporate each sample to dryness in a 65°C water bath.
8. Reconstitute each sample residue with 2-3 drops of DCM for spotting.

Spotting, Developing and Spraying Procedures

1. Label TLC plate.
2. Draw a baseline 1 cm from the bottom of the plate using a template. Identify each sample using the starting laboratory number and a pencil slash mark for each spot below the baseline. Score each plate 5 cm above the baseline with a diamond pencil.

3. Spot entire sample on the TLC plate using a drawn-out glass pipet. Make sure each spot does not exceed 2 mm in diameter.
4. Spot the extraction PC and NC on the plate.
5. Rinse the spotter using 15-30 μ l DCM between each sample.

"Davidow" Solvent System

1. Spot the QA standards: Acepromazine, caffeine, and meclufenamic acid on the plate baseline in the center channels of the TLC plate. Spot 1 μ l of the base standards and any other standard requested by the Laboratory Director or Supervisor.
2. Develop the plate in a *fresh* Davidow developing tank until the solvent reaches the 5 cm scored line. Do not overdevelop the plate. If a spot appears, make physical note of it using a #2 pencil.
Notify the Laboratory Director, Supervisor, or Chemist appointed by the supervisor to observe the remaining procedures.

3. Allow the plate to dry. Observe (***must wear goggles***) the plate under long wave (365 nm) wavelength ultraviolet (UV) light. Mark any spot that fluoresces at this point using (=) symbol.
5. Observe (***must wear goggles***) the plate using short-wave (254 nm) wavelength ultraviolet (UV) light. Mark all standards and spots that quench with (//).
6. Spray the plate with the Fluram Overspray (do not overspray) in a spray box in a fume hood.

Heat plate under hair dryer in fume hood for 15-20 seconds.

Place the plate under long (365 nm) wavelength ultraviolet (UV) light.

Mark all standards and spots that fluoresce after the Fluram was sprayed using (=) symbol and mark with an "F" to indicate reaction with Fluram spray.

7. Spray the plate with ninhydrin reagent in a spray box in a fume hood. After heating on hot plate on "low" setting in a fume hood, mark any spot with an "N" under the baseline if it turns pink, spray and heat the plate a second time with ninhydrin if the standards fail to turn pink.
8. Allow the plate to cool to room temperature.
9. Spray the plate with Dragendorff's Overspray in a spray box in a fume hood. An orange, pink-orange, or brown spot is considered to be a positive Dragendorff reaction. **The R_f value for Methylphenidate was \cong 0.78.** Identify any positive reaction with the letter "D."
10. Spray the plate with the CuCl_2 spray. Wait for a color change. Note the color.

The color reaction for Methyphenidate was brown.

11. Evaluate all results with the Laboratory Supervisor or chemist appointed by the supervisor.

Confirmation: GC/MS

Materials:

- DCM
- 0.93 N NaOH (formula #64)
- Heptafluorobutyric Anhydride (HFB, Sigma)
- Pyridine (*ibid.*)
- Saturated Sodium borate ($\text{Na}_2\text{B}_4\text{O}_7$; formula #51)
- 16 x 125 mm screw cap tube and 16 x 150 mm screw cap tube
- Ethyl Acetate (EtAc)

Procedure:

1. Combine the following in a 16 x 150 mm screw cap tube:
 - 10 mL urine
 - 2 mL NaOH (0.93 N)
 - 5 mL DCM
2. Rotorack for 5 min., centrifuge, and aspirate the aqueous (top) layer to waste.
3. Decant DCM into clean, dry 16 x 125 mm screw cap tube.
4. **Carefully** evaporate to \cong **50 μL of DCM remaining.**
5. Add 50 μL HFB and 15 μL pyridine and cap the tube.
6. Heat in 65°C water bath for 30 min.
7. Add 4 mL DCM and 4 mL $\text{Na}_2\text{B}_4\text{O}_7$ (saturated).
8. Cap, rotorack for 5 min., centrifuge.
9. Aspirate aqueous (top) to waste.
10. Transfer DCM to clean dry 16 x 125 mm screw cap tube.
11. Evaporate to dryness in a 65°C water bath.
12. Add 50 μL EtAc for GC/MS analysis.
13. Inject 2 μL sample reconstituted in EtAc

GC/MS analysis.

- 10 mL sample prepared as above, followed by HFB derivatization (as above).
Monitor ion 280 m/z.

Appendix: Reagent Formulas

Formula #11: 85:10:5 (Davidow) (For use with Base Urine, Enzyme Hydrolysis, and Neutral Urine extraction TLC plates.)

Procedure for 1000 ml :

- 850 ml (85%) Ethyl Acetate (EtAC)
- 100 ml (10%) Methanol (MeOH)
- 50 ml (5%) Concentrated Ammonium Hydroxide (NH₄OH)

Formula #25 — Dragendorff's Overspray

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

Procedure for 140 ml spray:

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 later use.

Formula #26 — Copper Chloride Overspray

Procedure for 800 ml spray:

1. Make 800 ml of Methanol (MeOH):DI H₂O (75:25)
 - 600 ml of MeOH
 - 200 ml DI H₂O
2. Make 5% Copper Chloride (CuCl₂)
 - Dissolve 40 gm CuCl₂ in 800 ml 75:25 MeOH:DI H₂O (above).

Formula #31 — Fluram (Fluorescamine) Overspray

Procedure for 800 ml:

Dissolve 156 mg of Fluram (4-Phenylspiro [furan-2(3H), 1'-phthalan]-3,3'-dione) (Sigma F-9878) in 800 ml of Acetone.

Formula #51 — Borate Buffer (Saturated) pH 9.5

Procedure for 1000 ml:

Pour enough sodium tetra borate into 1000 ml DI H₂O while stirring to ensure saturation. (The solution **must** have a precipitate.)

Formula #40 — Ninhydrin Overspray for TLC

Procedure for 800 ml:

1. Make 800 ml of methanol (MeOH):isopropanol (IPA) (30:70)
240 ml MeOH
560 ml IPA
2. Dissolve 1.6 gm of tri-Ketohydrindene hydrate (ninhydrin) in 800 ml of MeOH:IPA (30:70).

Formula #64 Sodium Hydroxide (NaOH)

0.93 N = 37.2 gm into 1000 ml H₂O