

Detection and Confirmation of Viloxazine

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Abstract

Traditional liquid/liquid extraction methods as well as solid phase extraction were explored to find the best practical method for detection and confirmation of viloxazine. As immunoassay kits are not yet available for this drug, thin-layer chromatography (TLC) is the method of choice for initial detection. For confirmation by GC/MS the solid phase extraction employed was found to give better recovery than the thin-layer chromatography extraction methods.

Complete procedures for detection and confirmation are included as well as a brief outline of some of the more significant TLC detection indicators.

Scope

The following SOP is proposed for TLC detection and GC/MS confirmation of Viloxazine in equine urine. TLC limit of detection for Viloxazine is approximately 50 ng/ml using EH (enzyme hydrolysis) or IP (ion pair) extraction's. Viloxazine was confirmable by GC/MS down to 50 ng/ml using SPE (solid phase extraction). Viloxazine was evident by GC/MS at 50 ng/ml using the EH extraction method, but below an acceptable level for confirmation.

References

Thin-layer chromatography (TLC) methods:

NASRC Quality Assurance Program (1982-1988)

ARCI Quality Assurance Program (1988-1995)

The TLC methods are modification (or verbatim) acquired from the above two programs.

Solid Phase Extraction (SPE) Method:

The solid phase extraction method used in this method development was derived from the application manual provided by United Chemical Technologies, Inc. for extraction of drugs using their Clean Screen® solid phase extraction columns.

Definitions

GC/MS: Gas Chromatography / Mass Spectroscopy

SOP: Standard Operating Procedure

TMS: Trimethylsilyl

BSTFA: bis(trimethylsilyl)trifluoroacetamide

EH: Enzyme Hydrolysis

IPU: Ion Pair Extraction

BU: Base Urine Extraction

Prop: Propionic acid development solvent

Dav: Davidow development solvent

T-1: T-1 development solvent

Drag: Dragendorff's overspray reagent

Nitrite: Sodium nitrite overspray reagent

Cupric: Cupric chloride overspray reagent

FES: FES overspray reagent

Mand: Mandelin's overspray reagent

Nin: Ninhydrin overspray reagent

HCl: Hydrochloric acid overspray reagent

Principle

Viloxazine is an alkaline drug and was extractable in all of the alkaline extracts examined. The enzyme hydrolysis extract is the extraction of choice for TLC detection, probably due to the cleaner residues obtained from this extract compared to the other alkaline extracts examined.

The enzyme hydrolysis method is essential for conjugated drugs and their conjugated metabolites. The hydrolysis step liberates the drug from its conjugate and therefore makes analysis of the drug more viable. The EH extraction also employs three partitioning steps, which yields a residue with a minimal amount of background material compared to the IPU and BU methods, which have only one partitioning step.

Standards

Viloxazine, 2-(2-ethoxyphenoxymethyl)tetrahydro-1,4-oxazine, a bicyclic antidepressant, was obtained from Imperial Chemical (lot # ADM 23364/81).

A 1.00 mg/ml stock solution was prepared by weighing out approximately 0.0100 grams of the viloxazine powder and dissolving it in the appropriate amount of methanol (approximately 10.0 mls, depending on actual weight of viloxazine) to yield a 1.00 mg/ml solution. A portion of this solution was diluted by a factor of ten (10) and appropriate amounts of this dilute solution were used to spike blank urine to yield urine concentrations of 0, 50, 100, 200, 500, and 1000 ng/ml.

Detection

By TLC: Viloxazine was detected using the following TLC Screens: EH, BU, and IPU extraction's.

1) **EH:** Three TLC plates were prepared for this extraction; Prop1, Prop2 and Dav.

TLC plate	Spray Reagent(s)	Lowest Spike Level Detected	Comments
Prop1	Drag	1000 ng/ml	faint orange
Prop1	Drag - Nitrite	50 ng/ml	brown (BEST)
Prop1	Drag - Nitrite - Cupric	100 ng/ml	brown
Prop2	FES + Heat	500 ng/ml	gray-blue or gray-purple
Prop2	FES + Heat - Mand	500 ng/ml	gray-purple
Dav	Drag	1000 ng/ml	faint orange
Dav	Drag - Nitrite	50 ng/ml	brown, aligns w/ common background spot
Dav	Drag - Nitrite - Cupric	50 ng/ml	brown, aligns w/ common background spot

2) **IPU:** Two TLC plates were prepared for this extraction; T-1 and Dav.

TLC plate	Spray Reagent(s)	Lowest Spike Level Detected	Comments
T-1	Nin + Heat	50 ng/ml	very faint pink
T-1	Nin + Heat - Drag	100 ng/ml	light brown
T-1	Nin + Heat - Drag - Cupric	200 ng/ml	brown
T-1	Nin + Heat - Drag - Cupric - Nitrite	200 ng/ml	brown
Dav	Drag	500 ng/ml	faint orange
Dav	Drag - Cupric	100 ng/ml	brown
Dav	Drag - Cupric - Nitrite	100 ng/ml	brown
Dav	Drag - Cupric - Nitrite- HCl - Drag	200 ng/ml	brown

3) **BU:** Two TLC plates were prepared for this extraction; Dav and T-1.

TLC plate	Spray Reagent(s)	Lowest Spike Level Detected	Comments
Dav	Drag	500 ng/ml	faint orange
Dav	Drag - Nitrite	100 ng/ml	brown, aligns w/ common background spot
Dav	Drag - Nitrite - Cupric	100 ng/ml	brown
T-1	FES + Heat	100 ng/ml	gray-purple
T-1	FES + Heat - Mand	500 ng/ml	gray-purple

Procedure

TLC Screening Procedure: Enzyme hydrolysis Extraction for Thin layer Chromatography

REAGENTS AND SOLUTIONS

- pH 5 acetate buffer
- *Patella vulgata* enzyme solution
- 10% ascorbic acid solution
- 1:1 ammonium hydroxide/water
- 10:1 dichloromethane/isopropyl alcohol
- 1.0 N sulfuric acid solution
- 9:1 dichloromethane/methyl alcohol
- FES reagent
- Mandelin's reagent
- Propionic acid solvent
- Dragendorff's solvent
- Sodium nitrite solution 5%
- Cupric chloride solution 25%
- Davidow's solvent
- Fluram reagent

APPARATUS

- E.M. Science, silica gel, Fluorescent, Thin Layer Chromatography Plates
- Capillary plate spotter
- Hot plate with small fan
- 16 x 125 mm screw top test tubes with caps
- Vortex mixer
- Automatic pipettor with 10 ml graduated pipettes
- Rotorack mixer for test tubes
- Centrifuge
- Disposable Pasteur pipettes
- Vacuum aspiration apparatus
- Disposable 15 x 85 mm tubes
- Light box with 254 nm and 365 nm UV light
- Water bath
- Hot Plate

Sample prep

1. Add 5.0 ml urine to a 16 x 125 mm screw top tube.
2. Add 2.0 ml pH 5 acetate buffer and 1.0 ml. *Patella vulgata* enzyme solution. Vortex until homogeneous. Incubate in 65 ° C water bath for 3 hours. Cool.
3. Make entry into "Reagent Addition" log book.
4. Add 0.5 ml 10% ascorbic acid. Adjust pH to 9.0 with 1:1 NH₄OH:H₂O. This requires approximately 0.75 ml.
5. Add 5.0 ml 10:1 DCM:IPA. Cap tube and rotorack for 5 minutes. Centrifuge for 5 minutes.
6. Aspirate aqueous (upper) layer and transfer organic layer to a clean 16 x 125 mm screw top tube.
7. Add 3.0 ml 1.0 N H₂SO₄. Cap tube and rotorack for 5 minutes. Centrifuge for 5 minutes.
8. Transfer acid aqueous (upper) layer to a clean 16 x 125 mm screw top tube with a disposable pipette.
9. Add 0.5 ml 10% ascorbic acid. Adjust pH to 9.0 with 1:1 NH₄OH:H₂O. This requires approximately 0.75 ml.
10. Add 5.0 ml 10:1 DCM:IPA. Cap tube and rotorack for 5 minutes. Centrifuge for 5 minutes.
11. Aspirate aqueous (upper) layer and transfer organic layer to a clean 15 x 85 mm tube. Concentrate to dryness in 60 ° C water bath.
12. Spot the entire residue equally on 3 TLC plates using 9:1 DCM:MeOH. Cool plates prior to development.

Notes on Procedure

- 1 Steps 5-7 and 10-12 should be carried out in a minimum amount of time (Degradation can occur when 1:1 NH₄OH:H₂O has been added. Samples should not be allowed to stand for a long period of time in this alkaline state).

Plate #1 - Propionic Acid

- 1 Spot acepromazine and methocarbamol as standards.
- 2 Develop in propionic acid (use 40-50 ml/tank) for 5 cm. Dry plate well.
- 3 Observe using 365 nm UV light. Indicate fluorescence with =.
- 4 Observe using 254 nm UV light. Indicate quenching with ||. Spray with FES. Record colors and Rf's.
- 5 Heat lightly on hot plate. Note colors or color changes and Rf's. Plate should be both front and back lit for maximum visual sensitivity.
- 6 Allow plate to cool. Spray with Mandelin's. Record colors and Rf's.

Plate #2 - Propionic Acid

- 1 Spot nalbuphine, oxymorphone and pentazocine as standards.
- 2 Develop in Propionic Acid solvent for 5 cm. Dry plate well.
- 3 Observe using 365 nm UV light. Indicate fluorescence with =.
- 4 Observe using 254 nm UV light. Indicate quenching with | |.
- 5 Spray with Dragendorff's. Record colors and Rf's.
- 6 Spray several times with sodium nitrite (wait between sprays for color change to develop). Record colors and Rf's.
- 7 Spray with cupric chloride. Record colors and Rf's.

Plate #3 - Davidow

- 1 Spot oxymorphone, hordenine and nalbuphine as standards.
- 2 Develop in Davidow for 5 cm. Dry plate well.
- 3 Observe using 365 nm UV light. Indicate fluorescence with =.
- 4 Observe using 254 nm UV light. Indicate quenching with | |.
- 5 Spray lightly with 4:4:2.
- 6 Spray with Dragendorff's. Record colors and Rf's.
- 7 Spray several times with sodium nitrite (wait between sprays for color change to develop). Record colors and Rf's.
- 8 Spray with cupric chloride. Record colors and Rf's.

GC/MS Confirmation Procedure: Solid Phase Extraction

REAGENTS

- Water (deionized)
- Methanol (HPLC grade)
- Hydrochloric acid (ACS reagent grade)
- Methylene chloride (HPLC grade)
- Ethyl acetate (HPLC grade)
- Acetic acid (glacial) (ACS reagent grade)
- Ammonium hydroxide (ACS reagent grade)
- Sodium phosphate monobasic (NaH_2PO_4) (ACS reagent grade)
- Sodium phosphate dibasic (Na_2HPO_4) (ACS reagent grade)
- Isopropyl alcohol (HPLC grade)
- BSTFA (with 1% TMCS)

SOLUTIONS

- A. 0.1M Phosphate Buffer, pH 6 (100 ml)
- B. 1.0M Acetic Acid (50 ml)
- C. Methylene Chloride:Isopropyl Alcohol (80:20) with 2% Ammonium Hydroxide (100 ml)
- D. 2 N NaOH
- E. 1 N HCl

APPARATUS

Solid Phase Extraction (SPE) Columns

Vacuum Manifold with stopcocks, variable vacuum control, gauge and collection rack for test tubes

Adjustable volume Automatic pipettor with disposable tips (100 ul tips)

pH paper (broad range 0 to 14 and narrow range 6 +/- 0.5)

Vortex mixer

Heating bath

Vials

Centrifuge

Test tubes

Horn sonicator

Sample prep

1. MANIFOLD PREPARATION

Wipe the tips on the ports of the extraction manifold lid with methanol. The same port must be used for all stages of preparation of a given urine aliquot. Plug all unused ports.

2. SPECIMEN PREPARATION

A. SHORT ENZYME HYDROLYSIS AND SONICATION

Add 20 ml urine to a clean 100 ml beaker.

Add 8.0 ml pH 5 acetate buffer and 4.0 ml. *Patella vulgata* enzyme solution.

Sonicate urine with horn sonicator for 1.5 min. at full power. Turn up power slowly over the first 10 to 15 sec.

Incubate in 65°C water bath for 3 hours. Cool.

B. ADJUST PH AND CENTRIFUGE

Check the pH of the specimen with pH paper. Adjust pH to 6.0 +/-0.5 using drop-wise addition of 2 N NaOH (D) or 1 N HCl (E) as needed.

Centrifuge as needed: precipitates, urine still viscous, too much foam, suspended particulates, etc. will clog SPE column. Unless urine is very clean and clear it is a good idea to centrifuge.

3. SPE COLUMN PREPARATION

Place a Worldwide Monitoring Clean Screen[®] solid phase extraction cartridge, Catalog ZSDUA020 or equivalent, into the vacuum manifold port referred to in step 1 above. Pass through the column sequentially:

- 3 ml methanol
- 3 ml deionized water
- 2 ml pH 6 phosphate buffer (A).

Important: Turn off vacuum as soon as water reaches the top of the sorbent bed to prevent column drying.

4. SPECIMEN APPLICATION

Pour the specimen into the column reservoir. Loosen the flow valve to reduce vacuum. Draw the specimen slowly through the column.

Important: It should take at least two (2) minutes for the specimen to pass through the SPE column.

5. COLUMN RINSE

Pass through the column sequentially:

- 3 ml deionized water
- 2 ml 1.0 M acetic acid (B)
- 3 ml methanol

6. ELUTE ALKALINE DRUGS

Place a rack with new labeled tubes into the vacuum manifold. Pass through the column and collect:

5 ml methylene chloride:isopropyl alcohol (80:20) with 2% ammonium hydroxide (C)

7. RECONSTITUTE AND INJECT

Evaporate the eluant under nitrogen 40°C. Reconstitute with 40 ul ethyl acetate, heat gently and vortex to dissolve residue.

Inject 2 ul of the solution into GC/MS.

Note: Derivatization procedures may be substituted if desired. Viloxazine does not derivatize, however, research in the future may reveal metabolites which may require derivatization.

Confirmation Of Viloxazine by GC/MS:

Detection:

Viloxazine was confirmable by GC/MS down to 50 ng/ml using the above SPE (solid phase extraction) method. Viloxazine was evident by GC/MS at 50 ng/ml using the EH extraction method, but below an acceptable level for confirmation.

GC/MS Conditions:

Splitless for 0.8 min. at a Head Pressure of 15 psi
Column: 25 meter HP-5, 0.33 um film, 0.2 mm ID
Initial Temp: 80°C 1.51 min. hold
Program Rate: 20°C/min.
Final Temp: 280°C 13.74 min. hold

GC/MS was performed in full scan EI mode over the mass range of 40-550 amu.
Some significant ions for Viloxazine in order of abundance: 100, 56, 138, 110,
237, 70

Retention time: ~11 minutes

Reagents for Viloxazine SOP

EH Extraction

- **Acetate Buffer pH 5**

Dissolve 328 gm of sodium acetate in 3 liters of distilled or deionized water. Add 66 ml of glacial acetic acid. Dilute to 4 liters with water.

- ***Patella Vulgata* Enzyme Solution**

Dissolve one bottle (2 million units) of *patella vulgata* B-glucuronidase in 400 ml distilled or deionized water.

- **Ascorbic Acid**

Dissolve 90 gm ascorbic acid (reagent) in 900 ml of distilled or deionized water.

- **Ammonium Hydroxide : DI water (1:1)**

Add equal parts NH₄OH and DI water in fume hood.

- **Dichloromethane/Isopropanol**

Mix ten parts distilled dichloromethane to 1 part isopropanol (reagent).

- **1.0N Sulfuric Acid**

Dilute 111 ml concentrated H₂SO₄ to 4 liters with distilled or deionized water.
ALWAYS ADD ACID TO WATER never add water to acid..

TLC Analysis

Developing Solvents

Davidow: Ethyl acetate 3400 ml
Methanol 400 ml
Ammonium hydroxide 200 ml

T-1: Methanol 4000 ml
Ammonium hydroxide 60 ml

(Easiest to add 60 ml ammonium hydroxide to a new 4 liter reagent bottle of methanol.)

Propionic Acid: Chloroform 2880 ml
Methanol 720 ml
Propionic acid 400 ml

Spray Reagents

- **Dragendorff's Spray**

Mix equal amounts of Solution A and Solution B.

Solution A: 9.4 g Bismuth Subnitrate dissolved in approximately 600 ml DI-H₂O. Add 306 ml Glacial Acetic Acid. Bring to 1 liter with DI-H₂O. Mix for several minutes and filter.

Solution B: 112.1 g Potassium Iodide in 1 liter DI-H₂O.

- **Sodium Nitrite Spray**

5% solution of sodium nitrite in DI-H₂O.

- **Cupric Chloride**

200 g cupric chloride in 600 ml DI-H₂O. Add 200 ml methanol.

- **Floram**

0.25 g fluorescamine in 1 liter methanol.

- **Mandelin's**

Heat 800 ml concentrated sulfuric acid in an Erlenmeyer flask in a 60°C water bath about 1 hour. Add 4.0 g Ammonium Metavanadate, slowly. Heat for an additional hour.

- **Ninhydrin**

5% solution of ninhydrin in methanol.

- **FES - Phenothiazine Spray**

Dissolve 2.0 g ferric chloride in 600 ml ethanol. Slowly add 200 ml concentrated sulfuric acid. (Danger: Exothermic reaction! Add acid to ethanol with cooling and stirring. Use proper face and eye protection.)

Solid Phase Extraction

A. 0.1M Phosphate Buffer, pH 6 (100 ml)

Weigh 0.17g of Na_2HPO_4 and 1.21g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ in 80 mls of deionized (DI) water. Dilute to 100 mls and mix. Adjust pH to 6.0 \pm 0.1 with saturated monobasic sodium phosphate (lowers pH) or saturated dibasic sodium phosphate (raises pH). Store in glass at 5° C. Stable for 1 month.

B. 1.0M Acetic Acid (50 ml)

To 40 mls of DI water pipette 2.86 mls of glacial acetic acid. Dilute to 50 mls and mix. Store in glass or plastic at 25° C. Stable for 6 months.

C. Methylene Chloride:Isopropyl Alcohol (80:20) with 2% Ammonium Hydroxide (100 ml)

Into a 100 ml reagent bottle add 80 ml of methylene chloride and 20 ml of isopropyl alcohol. Mix. Remove 2 ml of this solution and add 2 ml of ammonium hydroxide. Mix well. Transfer to a capped container. Prepare fresh daily.

D. 2 N NaOH

Dissolve 80 grams of sodium hydroxide into 1 liter of DI water.

Use caution: dissolution is exothermic.

E. 1 N HCl

In a 1000 ml volumetric flask containing approximately 800 mls of DI water add 83.3 mls of concentrated HCl. Add distilled water to 1000 mark and mix.