

APOMORPHINE

DETECTION AND CONFIRMATION

IN EQUINE URINE

by

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For The

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**Method review provided by the Pennsylvania Equine
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Drug administrations provided by Dr. Thomas Tobin
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Abstract

Apomorphine (5,6,6a,7-Tetrahydro-6-methyl-4H-diabenzo[de,g]quinoline-10,11-diol) is listed in the Merck index as an emetic, it is also used as an expectorant for dogs. From *Drugs and The Performance Horse*, by Dr. Thomas Tobin, chapter 13, apomorphine is described as a powerful locomotor stimulant for horses.

The screening technique to be described is enzyme hydrolysis followed by alkaline extraction into organic solvent, followed by thin-layer chromatography (TLC). The propionic solvent system is our system of choice for TLC development. Iodine vapors are used for visualization of the apomorphine.

Scope

The following method is proposed for thin-layer chromatography (TLC) detection and gas chromatography / mass spectroscopy (GC/MS) confirmation of apomorphine. The thin-layer chromatography (TLC) limit of detection for apomorphine in equine urine is approximately 300 ng/ml (sometimes less in clean urine). GC/MS detection limits for apomorphine are about 50 ng/ml (full scan, clean spectrum), however this varies with the type of instrument and handling of the extraction process.

An alternate screening method to TLC has recently become available (January 2001), Neogen's apomorphine ELISA kit. We have tested this kit down to 50 ng/ml. It appears the kit could detect apomorphine well below that level (see page 5).

Principle

The enzyme hydrolysis (EH) method is essential for conjugated drugs and their conjugated metabolites. The hydrolysis step liberates the drug from its conjugate and therefore makes chromatography of the drug more viable. The EH extraction also employs three partitioning steps, which yields a residue with a minimal amount of background material. Apomorphine is excreted from the equine conjugated.

Standards

Apomorphine is available from Sigma, Catalog # A 4393.

Limitations

Beware of aporphine. Aporphine has the same (or very similar) mass spectral fingerprint as apomorphine using standard electron impact mass spectroscopy. The gas chromatographic retention time may also be nearly identical depending on your particular chromatographic conditions.

There are two main ways we have used to differentiate between apomorphine and aporphine: (1) Aporphine (apparently) cross-react well with Neogen's opiate group kit; apomorphine does not. (2) Apomorphine shows up well by TLC (enzyme hydrolysis

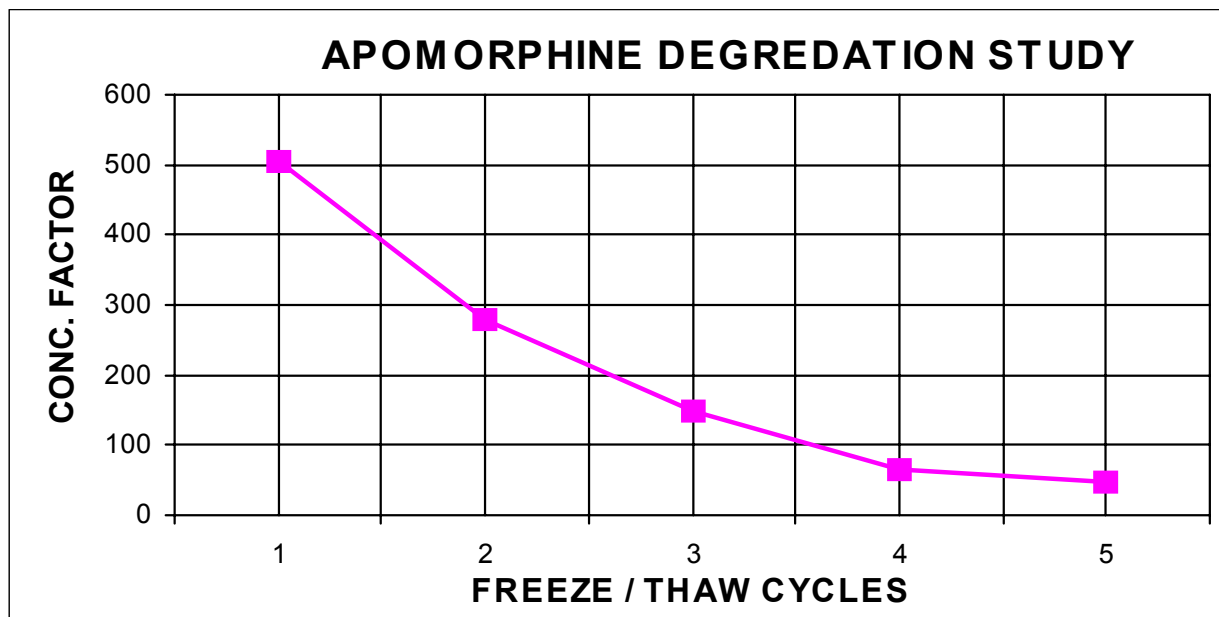
method, propionic development, visualize with I₂ vapors) yielding a characteristic green spot; aporphine does not show up this way (no green spot).

Apomorphine's characteristic green spot can show up with exposure to air, before exposure to I₂ vapors. Please note that Dr. Steve Barker of Louisiana State University and Dr. Allen Ray of Texas A & M have observed aporphine by TLC. Apparently if the concentration is high enough it can be observed the same way as apomorphine, but has a grayer color to it.

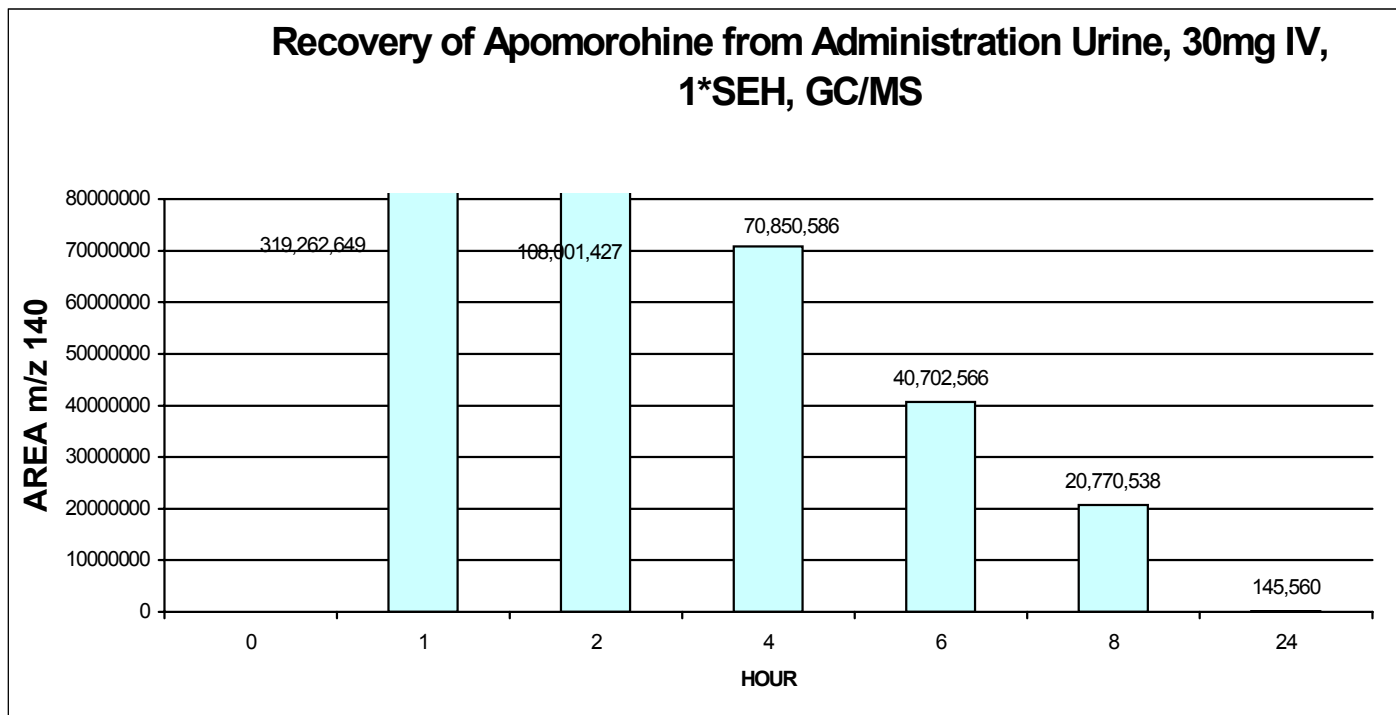
The way we have typically uncovered aporphine is a positive reaction from the Neogen Opiates Group kit. We then performed a solid phase extraction in preparation for GC/MS analysis. The mass spectral analysis revealed aporphine as well as two other alkaloids. We named the alkaloids *aporphine marker #1* and *aporphine marker #2*. Their GC retention times were approximately 14.5 and 14.7 minutes using the GC method that will be described later. The mass spectra of these compounds are also included later in this SOP (page 10).

Beware that apomorphine degrades quickly in spiked urine samples.

Stability



After five freeze-thaw cycles, apomorphine was nearly undetectable. Starting concentration was 500 ng/ml. Morphine was used as internal standard.



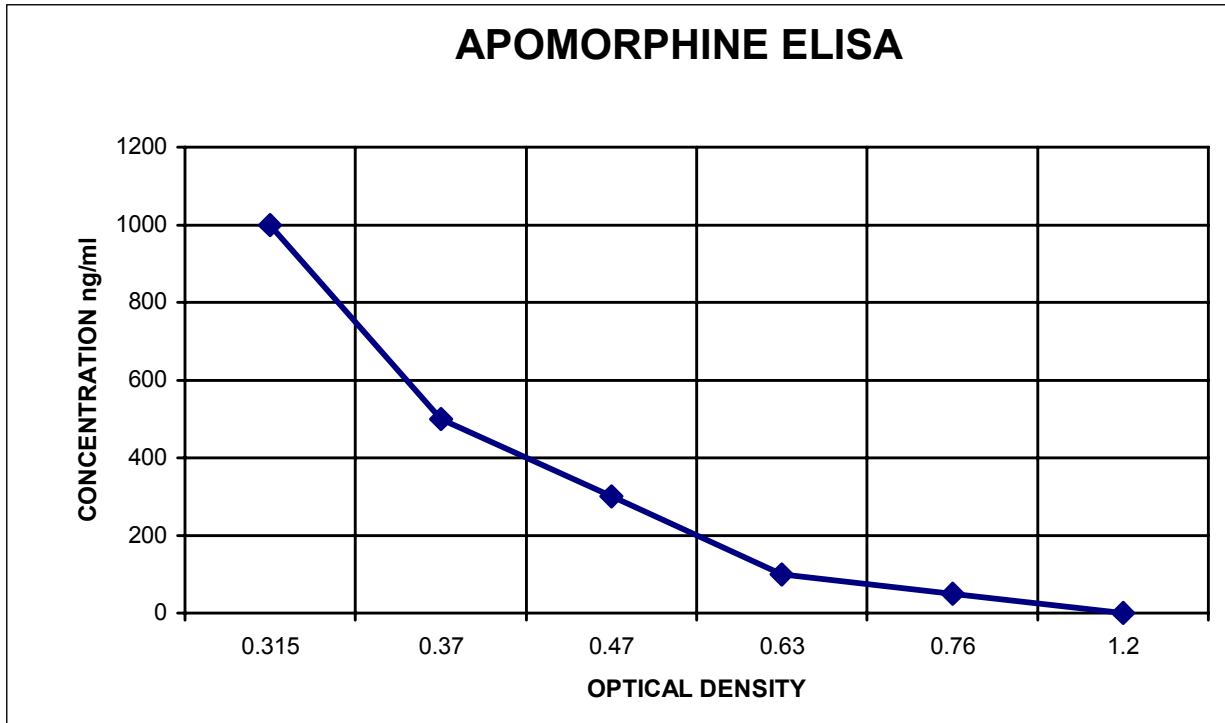
Administration Study (GC/MS analysis)

5 mls of urine for each time point was extracted in preparation for GC/MS analysis. The GC/MS analysis was performed using the instrument parameters described in the confirmation section of this SOP. The top of the graph is truncated to more clearly show the relative concentrations at the later collection times. Only a trace amount of apomorphine was recovered from the 24-hour administration urine.

Administration Study (ELISA analysis)

Using the Neogen Apomorphine kit, we analyzed the same samples that were used for the GC/MS study above. All time points beyond zero gave reactions that indicate concentrations greater than 500 ng/ml except for the 24-hour urine which was ~400 ng/ml. As has been seen before with other drugs, the ELISA readings do not always agree with the concentrations indicated by GC/MS. In this case, the ELISA readings are higher. This is possibly due to other alkaloids or metabolites cross reacting with the kit or a higher cross reactivity with the conjugated apomorphine.

Apomorphine ELISA (Neogen)



Using Neogen's Apomorphine ELISA kit concentrations of 1000, 500, 300, 100, 50 and 0 ng/ml were evaluated and plotted against optical density.

METHODOLOGY

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.

Definitions

GC/MS:	Gas Chromatography / Mass Spectroscopy
SOP:	Standard Operating Procedure
TMS	Trimethylsilyl
BSTFA	bis(trimethylsilyl)trifluoroacetamide
SEH	Short Enzyme Hydrolysis
Dav	Davidow development solvent
Prop	Propionic development solvent
Drag	Dragendorff's overspray reagent
Nitrite	Sodium nitrite overspray reagent
Cupric	Cupric chloride overspray reagent
DCM	dichloromethane
IPA	isopropyl alcohol
10:1	10:1 dichloromethane:isopropyl alcohol

Procedures

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

Apparatus

- E.M. Science, silica gel, Fluorescent, Thin Layer Chromatography Plates
- Capillary plate spotter
- Warming tray 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
- TLC development tanks

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 (at ~600 g,s) 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 (at ~600 g's) 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 nalbuphine, apomorphine 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 Place plate in I₂ tank for 5 minutes, remove, record colors and Rf's.

GC/MS CONFIRMATION OF APOMORPHINE

Use 5 mls of urine to prepare for GC/MS confirmation using the above enzyme hydrolysis method, but do not transfer the residue to a TLC plate. Reconstituted the raw residue in 10 µl ethyl acetate plus 10 µl BSTFA and heat for 20 minutes at 70°C in a sealed vial.

Inject 1 or 2 ul of the solution into GC/MS.

GC/MS Conditions:

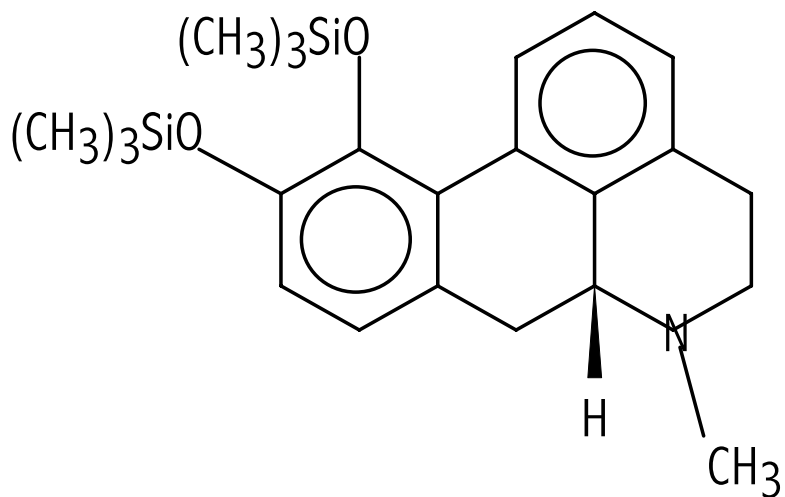
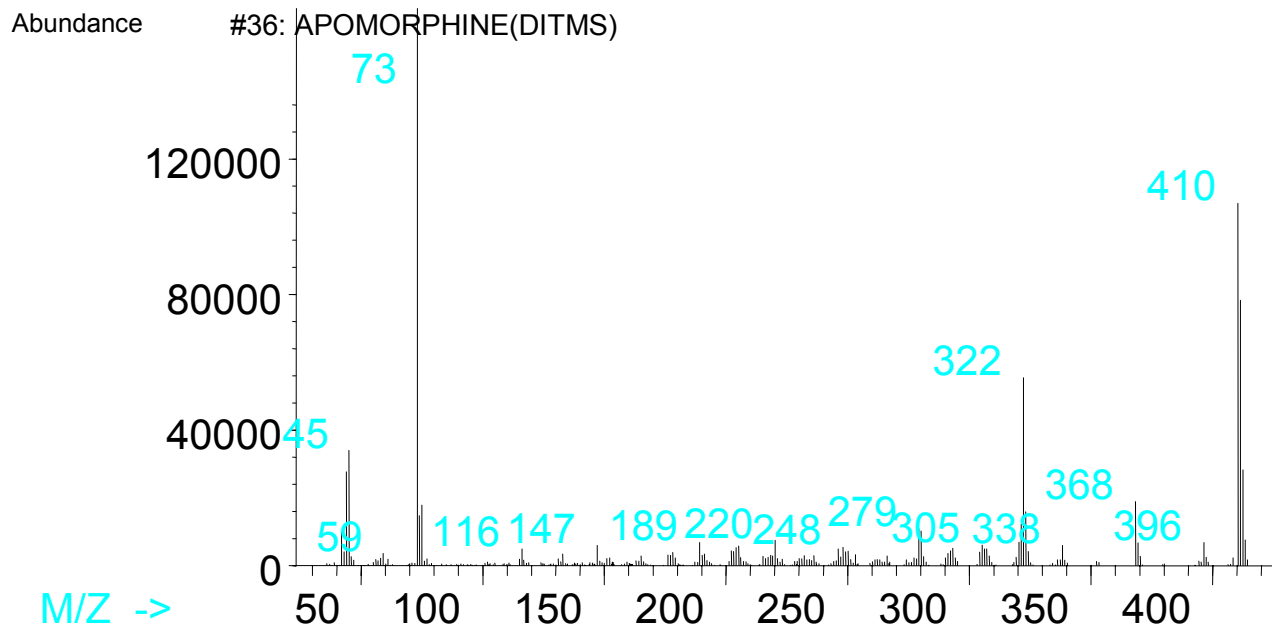
Splitless for 0.8 min. at a Head Pressure of 15 psi
Column: 25 meter HP-5, 0.33 um film, 0.2 mm ID
Initial Temp: 80°C, 1.51 min. hold
Program Rate: 20°C/min.
Final Temp: 275°C, 13.74 min. hold

GC/MS was performed in full scan EI mode over the mass range of 40-450 amu.

Some significant ions in order of abundance:

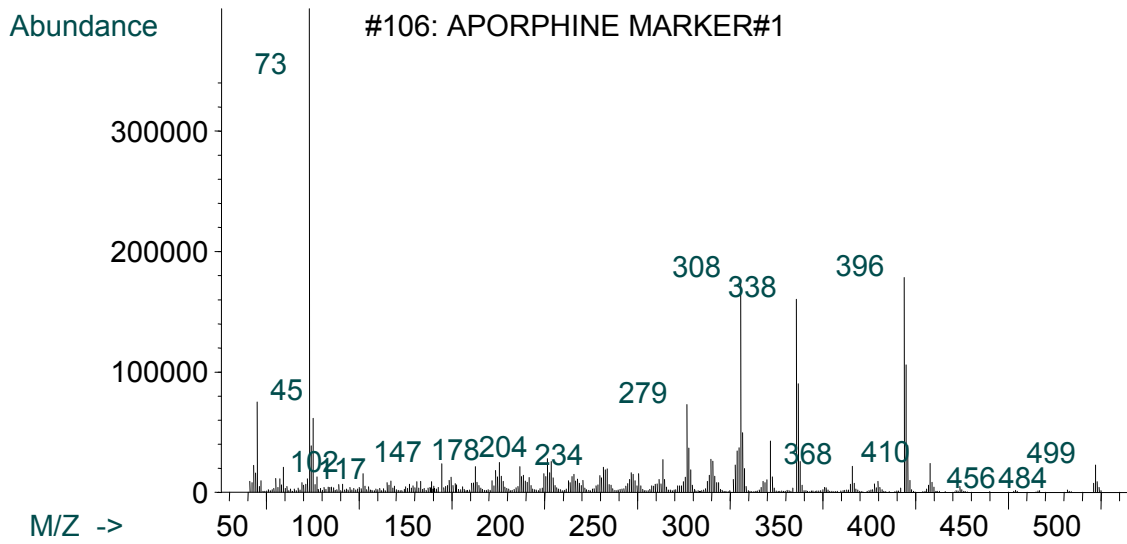
Apomorphine diTMS 410, 411, 322, 412, 368, 279, 280, 396, 338

Retention time: ~14.5 min.

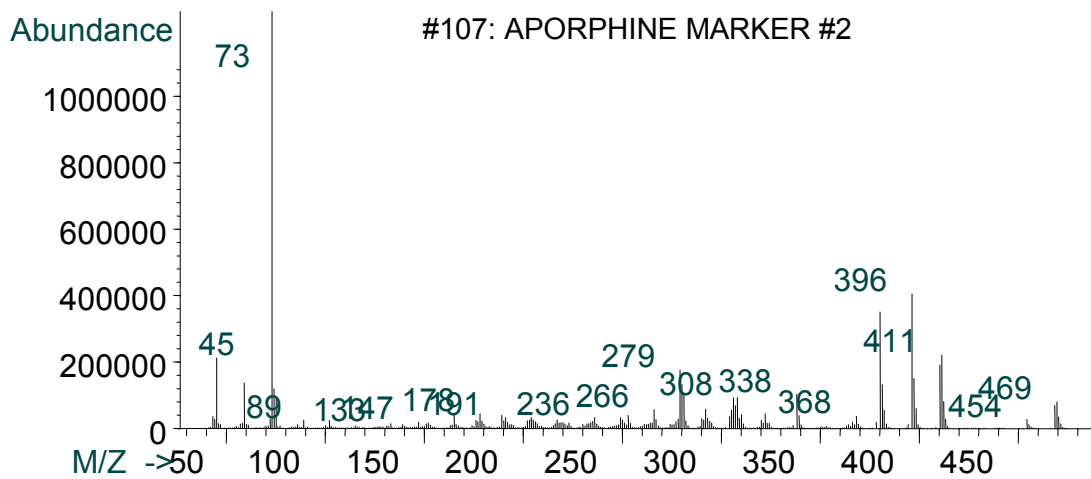


$C_{23}H_{33}NO_2Si_2$
mol wt. = 411

These compounds were detected along with aporphine (not apomorphine).



Retention time 14.5 minutes ↑ and 14.7 minutes ↓.



Reagents for APOMORPHINE SOP

TLC Analysis

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.

- **10% Ascorbic Acid Solution**

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.

- **10:1 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..

- **1:1 Ammonium Hydroxide/Water**

Mix equal parts NH₄OH with deionized water.

TLC Analysis

Developing Solvent

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.

- **I₂ vapors**

I₂ vapors are produced by allowing I₂ crystals to sublime at room temperature in a dry TLC tank.