

Flupirtine

DETECTION AND CONFIRMATION

IN EQUINE URINE

by

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For The

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Abstract

Flupirtine [2-Amino-6-[[4-fluorophenyl)methylamino]-3-pyridinyl]carbamic acid ethyl ester is a substituted pyridine with analgesic properties. Flupirtine has been compared in studies to morphine because of its analgesic effect without morphine's addictive characteristics and is noted as a centrally acting non-opioid analgesic (1). It should be noted that this drug is not approved for use in the United States.

The screening technique to be described is enzyme hydrolysis followed by alkaline extraction into organic solvent, followed by thin-layer chromatography (TLC). TLC development is performed utilizing propionic acid and Davidow solvent systems. Several different spray reagents can be used for visualization. Confirmation is by gas chromatography / mass spectroscopy using the same extraction as was used for screening, but without the TLC step.

Scope

The following method is proposed for thin-layer chromatography (TLC) detection and gas chromatography / mass spectroscopy (GC/MS) confirmation of flupirtine. The thin-layer chromatography (TLC) limit of detection for flupirtine in horse urine is 200 to 500 ng/ml. The GC/MS detection limit for flupirtine is about 50 ng/ml (full scan), however this varies greatly with the age of the GC column, sample matrix, and handling of the extraction process.

Principle

The enzyme hydrolysis (EH) method is essential for recovering drugs and metabolites that are excreted in urine as conjugates. Hydrolysis with glucuronic acid liberates the drug or metabolite from its conjugate and therefore makes isolation of the drug or metabolite from urine possible. The EH extraction procedure employs three partitioning steps, which yields a residue with a minimal amount of background material.

Standards

Flupirtine is not commercially available in the United States. However, with a schedule 2 Drug Enforcement Administration (DEA) certificate, and a veterinarian's prescription, flupirtine can be ordered online at www.globalRX.com.

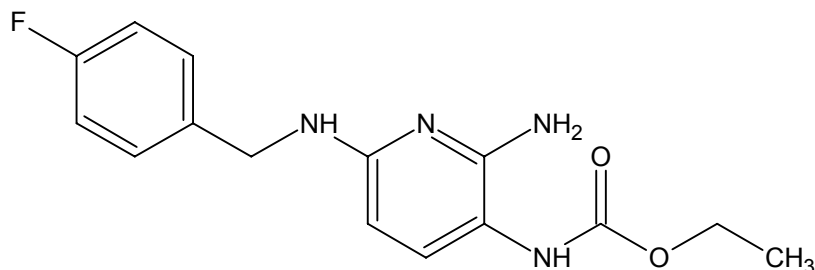
A 1.0 mg/ml stock solution is used in this standard operating procedure and is prepared by weighing out an appropriate amount of flupirtine and dissolving it in methanol.

Note: Prepare stock solution fresh if quantitation is necessary or if significant breakdown is suspected. See *Limitations* and *Stability*.

(1) Flupirtine: A review of Its Neuroprotective and Behavioral Properties (G. Schuster, M. Schwarz, F. Block, G. Pergande, and W. J. Schmidt)

Limitations

Flupirtine oxidizes with exposure to air. Store stock methanolic solutions under nitrogen to reduce oxidation. Exposure to light may also cause degradation.

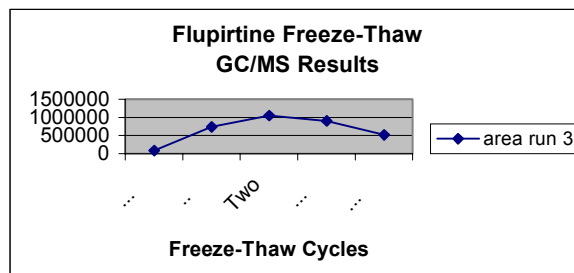
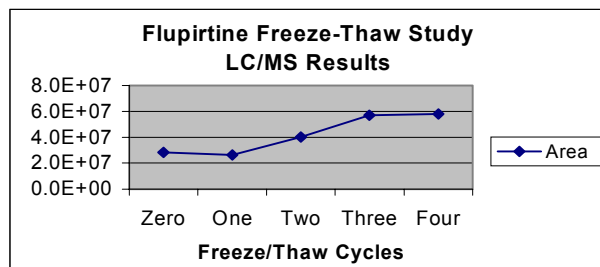


Stability

A freeze-thaw stability study was performed by extracting spiked samples, using the EH procedure described in this SOP, after consecutive freeze-thaw cycles. Residues from the extracts were stored at -15°C in methanol. After all methanolic extracts were collected, they were analyzed by gas chromatography / mass spectroscopy (GC/MS) or by liquid chromatography / mass spectroscopy (LC/MS).

This study was repeated several times with varying results. Flupirtine appears to be degrading in the stored methanol as well as from repeated freeze-thaw cycles. Further evidence of flupirtine degradation in methanol is seen from the greenish color that methanolic solutions acquire with time.

Although obtaining a clear degradation pattern from this study was not accomplished even after several attempts; it can certainly be concluded that flupirtine is not a stable compound. Three studies were performed using GC/MS to analyze the extracts, and one study was performed using both GC/MS and LC/MS. A graph of the LC/MS results and one of the results from the GC/MS studies follows.

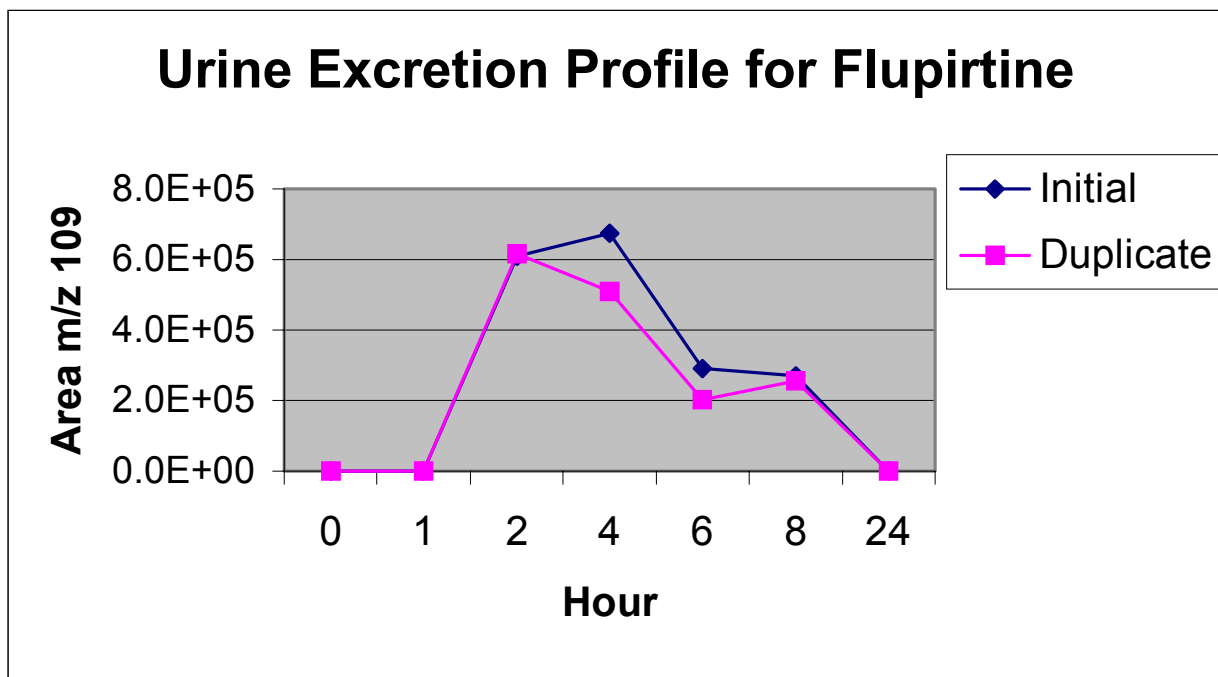


It should also be noted that flupirtine degrades in gas chromatograph injection ports. Repeated injections of flupirtine show the first injection of flupirtine to be severely degraded with relatively good stability thereafter. The first injection effect will vary depending on the initial condition of the injection port.

Administration Study (GC/MS analysis)

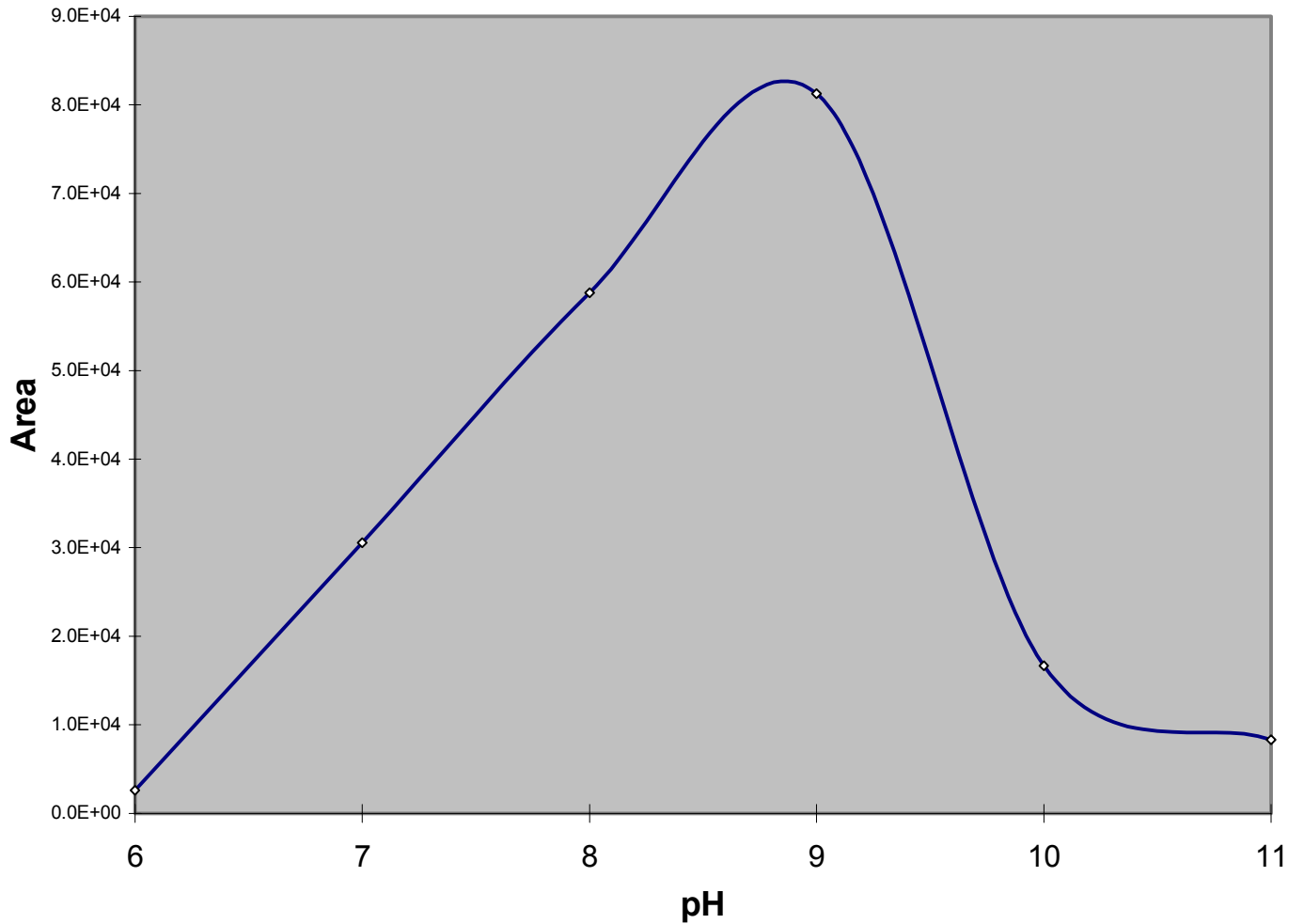
Recovery of flupirtine from administration urine is plotted below. Duplicate 5 ml portions of urine for each time point were extracted in preparation for GC/MS analysis. The GC/MS analysis was performed using the instrument parameters described in the confirmation section of this SOP.

Dr. Thomas Tobin from the University of Kentucky provided the administration samples.



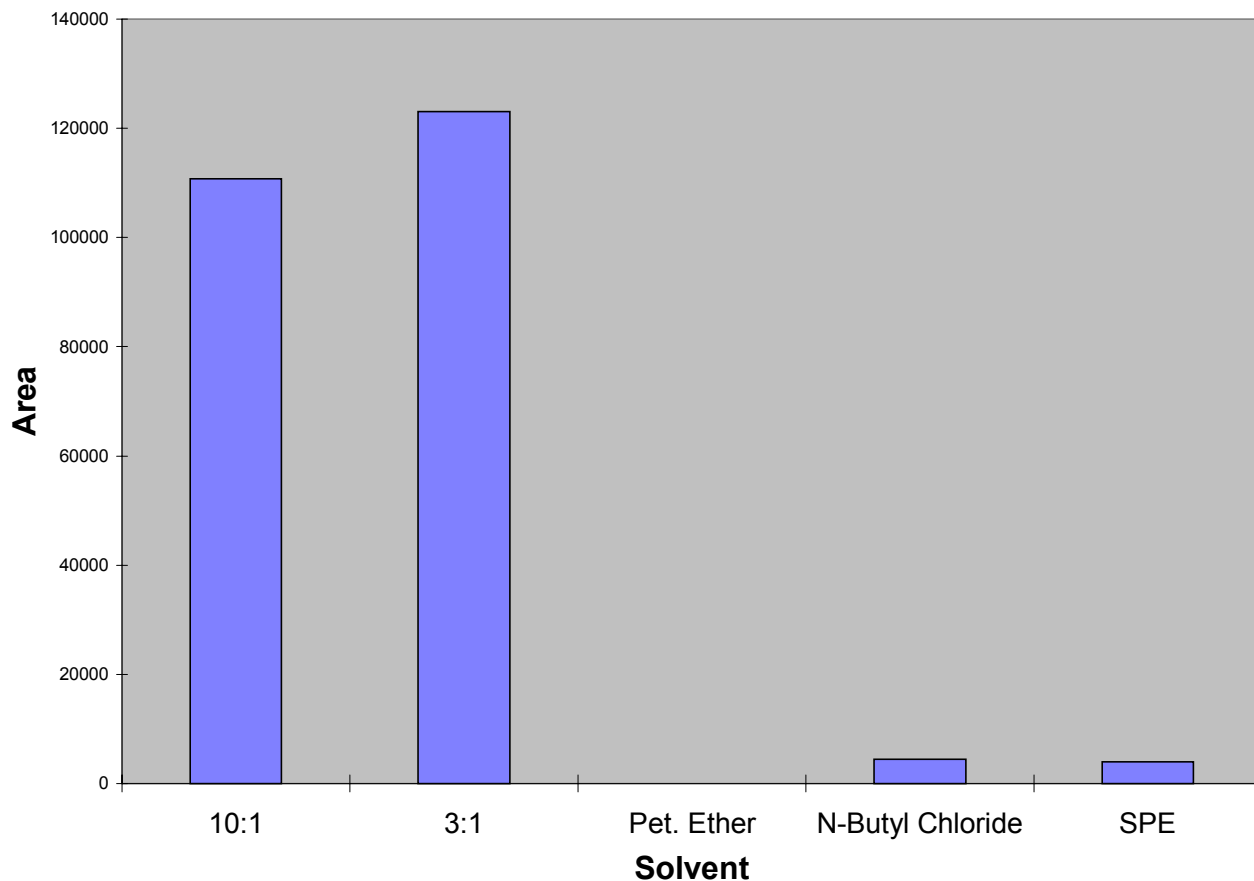
The amounts recovered were only slightly above our GC/MS detection limit, making integration difficult. Two other attempts were made within a week's time with no recovery. Apparently, the flupirtine concentration had already degraded below our GC/MS detection limit.

pH Effect on Recovery of Flupirtine



Above figure depicts the recovery of flupirtine from buffered urine verses pH (extracted from urine in carbonate buffer; the solvent was 3:1 dichloromethane-: isopropanol). The “raw residue” from each extract was analyzed by GC/MS.

Effect of Solvent on Recovery of Flupirtine



3:1 = 3:1 dichloromethane:isopropanol (DCM:IPA)

10:1 = 10:1 DCM:IPA

Above figure shows recovery of flupirtine from buffered urine as a function of different solvent systems (extracted from urine in carbonate buffer at pH 8). The figure also compares the this liquid-liquid method to that of a solid phase extraction (SPE) method for drugs of abuse (United Chemical Technologies method using ZSDAU020 columns). In each extraction, equal volumes of urine were used; the “raw residue” from each extract was analyzed by GC/MS.

METHODOLOGY

References

Thin-layer chromatography (TLC) methods:

NASRC Quality Assurance Program (1982-1988)

ARCI Quality Assurance Program (1988-1995)

The TLC method is a modification acquired from the above two programs.

Definitions

GC/MS:	Gas Chromatography / Mass Spectroscopy
SOP:	Standard Operating Procedure
UV	Ultra Violet
EH	Enzyme Hydrolysis
Dav	Davidow development solvent
Prop	Propionic development solvent
Drag	Dragendorff's overspray reagent
Nitrite	Sodium nitrite overspray reagent
DCM	dichloromethane
IPA	isopropyl alcohol
10:1	10:1 dichloromethane:isopropanol

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
- 1.0 N hydrochloric acid
- 1.0 N sulfuric acid
- 10:1 dichloromethane/isopropanol
- 1.0 N sulfuric acid solution
- 9:1 dichloromethane:methanol
- FES reagent
- Dragendorff's reagent
- Sodium nitrite solution 5%

- Davidow solution
- Propionic acid solution
- 4:4:2 solution

Apparatus

- Thin Layer Chromatography Plates, E.M. Science, Fluorescent silica gel or equivalent
- Capillary plate spotter
- Warming tray with small fan
- 16 x 125 mm screw top test tubes with caps
- Vortex mixer
- pH paper, 8.0 to 9.5 range or similar.
- 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
- Reagent spray bottles with air pressure source (or equivalent)
- Fume hood

Sample Preparation

(Below is a simple per sample procedure. Add internal standards and prepare spiked calibrators, controls, etc. appropriate for your particular analytical needs. Promazine was used as the internal standard for some of the above studies.)

1. Add 5.0 ml of urine to a 16 x 125 mm screw top tube.
2. Add 2.0 ml of pH 5 acetate buffer and 1.0 ml of *Patella vulgata* enzyme solution. Vortex until homogeneous. Incubate in 65 ° C water bath for 3 hours. Cool.
3. Add 0.5 ml of 10% ascorbic acid and 0.75 mls of 1:1 NH₄OH:H₂O. Adjust to pH 8.5 ± 0.5 with 1:1 NH₄OH:H₂O and 1.0 N HCl.

4. Add 5.0 ml of 10:1 DCM:IPA. Cap tube and rotorack for 5 minutes. Centrifuge for 5 minutes.
5. Aspirate aqueous (upper) layer and transfer organic layer to a clean 16 x 125 mm screw top tube.
6. Add 3.0 ml of 1.0 N H₂SO₄. Cap tube and rotorack for 5 minutes. Centrifuge (at ~600 g's) for 5 minutes.
7. Transfer acid aqueous (upper) layer to a clean 16 x 125 mm screw top tube with a disposable pipette.
8. Add 0.5 ml of 10% ascorbic acid and 0.75 mls of 1:1 NH₄OH:H₂O. Adjust to pH 8.5 ± 0.5 with 1:1 NH₄OH:H₂O and 1.0 N HCl.
9. Add 5.0 ml of 10:1 DCM:IPA. Cap tube and rotorack for 5 minutes. Centrifuge (at ~600 g's) for 5 minutes.
10. Aspirate aqueous (upper) layer and transfer organic layer to a clean 15 x 85 mm tube. Concentrate to dryness in 60 ° C water bath.
11. Spot the entire residue equally on 2 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 an alkaline state or as a dried residue).

Plate #1 - Propionic Acid

- 1 Spot flupirtine as standard using approximately 2µl of the 1.0 mg/ml methanolic solution.
- 2 Develop in Propionic Acid solvent (use 40 to 50 mls per tank to obtain ~ 5mm depth) for 5 cm. Dry plate well.
- 3 Observe colors and Rf's in visible light (blue-green).
- 4 Observe using 365 nm UV light. Indicate fluorescence (blue with yellow outline) with =.
- 5 Observe using 254 nm UV light. Indicate quenching with | |.
- 6 Spray with Dragendorff's. Record colors and Rf's.
- 7 Spray several times with 5% sodium nitrite solution (wait between sprays for color change to develop). Record colors and Rf's.

Plate #2 - Davidow

- 8 Spot flupirtine as standard using approximately 2 μ l of the 1.0 mg/ml methanolic solution.
- 9 Develop in Davidow solvent (use 40 to 50 mls per tank to obtain ~ 5mm depth) for 5 cm. Dry plate well.
- 10 Observe colors and Rf's in visible light (blue-green).
- 11 Observe using 365 nm UV light. Indicate fluorescence (blue with yellow outline) with =.
- 12 Observe using 254 nm UV light. Indicate quenching with ||.
- 13 Spray lightly with 4:4:2. Observe under 365 nm UV light (light blue).
- 14 Spray with Dragendorff's. Record colors and Rf's.
- 15 Spray several times with 5% sodium nitrite solution (wait between sprays for color change to develop). Record colors and Rf's.

Note: Other visualization reagent that are useful include: Ninhydrin Δ , Modified Erlic's (purple), and Fearon's (light blue).

GC/MS CONFIRMATION OF FLUPIRTINE

Use 5 ml of urine to prepare for GC/MS confirmation using the above enzyme hydrolysis method, but do not transfer the residue to a TLC plate. Reconstitute the raw residue in 40 μ l of ethyl acetate.

Inject 1 or 2 μ l 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 μm film, 0.2 mm ID

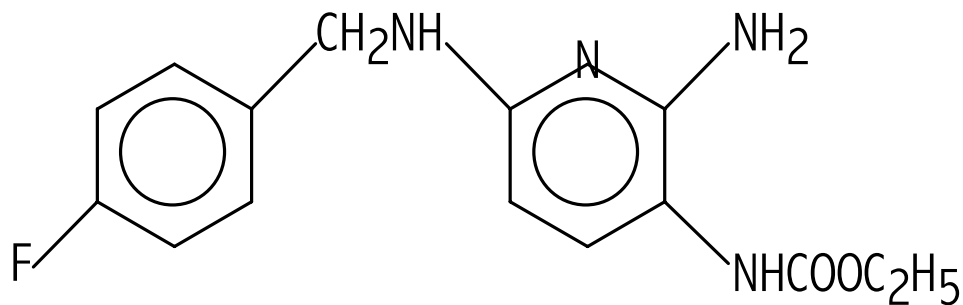
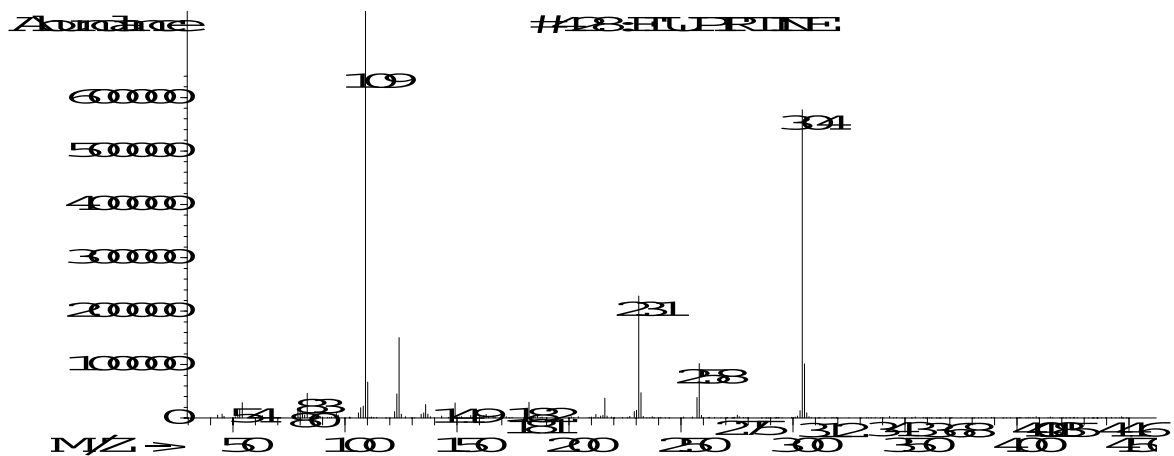
Initial Temp: 80°C, 1.51 min. hold

Program Rate: 20°C/min.

Final Temp: 275°C, 13.74 min. hold

Retention time: 15 to 16 minutes

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



MW = 304.32

C₁₅H₁₇FN₄O₂

Reagents for FLUPIRTINE SOP

TLC Analysis

Extraction Solutions

- **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**

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

- **10% Ascorbic Acid Solution**

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

- **10:1 Dichloromethane/Isopropanol**

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

- **9:1 Dichloromethane/Methanol**

Mix nine parts of distilled dichloromethane to one part of methanol (reagent).

- **1.0 N Sulfuric Acid**

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

- **1.0 N Hydrochloric acid**

Add 50 mls of concentrated HCl to 550 mls of deionized water.

ALWAYS ADD ACID TO WATER never add water to acid.

- **4:4:2 solution**

Combine 400 mls of chloroform, 400 mls of cyclohexane, and 200mls of glacial acetic acid.

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

Mix equal parts NH₄OH and deionized water.

TLC Analysis

Developing Solvents

- **Davidow:** Ethyl acetate 3400 ml
Methanol 400 ml
Ammonium hydroxide 200 ml
- **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 of Bismuth Subnitrate dissolved in approximately 600 ml of DI-H₂O.
Add 306 ml of Glacial Acetic Acid. Bring to 1 liter with DI-H₂O. Mix for several minutes and filter.

Solution B:

112.1 g of Potassium Iodide in 1 liter of DI-H₂O.

- **Sodium Nitrite Spray**

5% solution of sodium nitrite in DI-H₂O.

- **4:4:2 solution**

Combine 400 mls of chloroform, 400 mls of cyclohexane, and 200mls of glacial acetic acid.

- **Ninhydrin solution**

5% solution of ninhydrin in methanol.

- **Fearon's Reagent**

Dissolve 10 grams of NaOH into 250 mls of DI water. Dissolve 2.5 grams of pentacyanoamine ferroate (ammonium disodium salt) into the 250 mls of NaOH solution.

- **Modified Ehrlich's Reagent**

Dissolve 5 grams of p-dimethylaminocinnamaldehyde in 475 mls of DI water. Add 25 mls of concentrated HCl.