

IPRATROPIUM: SCREENING, QUANTIFICATION AND CONFIRMATION BY LC/MS/MS

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Liquid-Chromatography Mass Spectrometric Method For Screening, Quantification and Confirmation of Ipratropium in Equine Urine Samples

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Introduction

Respiratory disease conditions are one of the most common conditions that affect racehorses. Besides musculoskeletal problems, treatment of respiratory diseases in racehorses is ranked among the largest number of reported clinical cases per year. One mode of treatment and maintenance of horses with these conditions is the use of bronchodilators such as β_2 -agonists and anticholinergic drugs. Examples of β_2 -agonists and anticholinergic-bronchodilatory drugs include albuterol, clenbuterol; and, atropine, glycopyrrolate, and ipratropium, respectively. Many anticholinergic drugs (such as glycopyrrolate and ipratropium) are quaternary amine compounds. Quaternary amine compounds are not easily extracted and analyzed by traditional methodologies geared towards more lipophilic substances.

Background

Glycopyrrolate has traditionally been screened by using enzyme-linked immunosorbent assay (ELISA). Hydrolysis of the isolated quaternary compound to a unique fragment (cyclopentyl mandelic acid) is then amenable to detection and confirmation following derivatization and analysis by gas-chromatography-mass spectrometry (GC-MS)^(See figure #1). Recently, we noted that presumptive identifications of the presence of glycopyrrolate by ELISA were not confirmed using this approach. In addition, quantities of the benzyl analog of cyclopentyl mandelic acid were identified in the samples. This observation suggested the possibility that a drug other than glycopyrrolate was responsible for the initial ELISA indications for the presumptive glycopyrrolate positive results^(See figure #1). The challenge one faces is that the benzyl analog of cyclopentyl mandelic acid fragment is common to an entire group of drugs and is therefore not unique to a single compound.^(See figure 2) To address this challenge, it therefore became necessary to develop a method to isolate, screen and confirm a group of drugs, which included anticholinergics cross-reacting on glycopyrrolate ELISA test kit, as well as other related anticholinergic quaternary compounds, such as ipratropium.

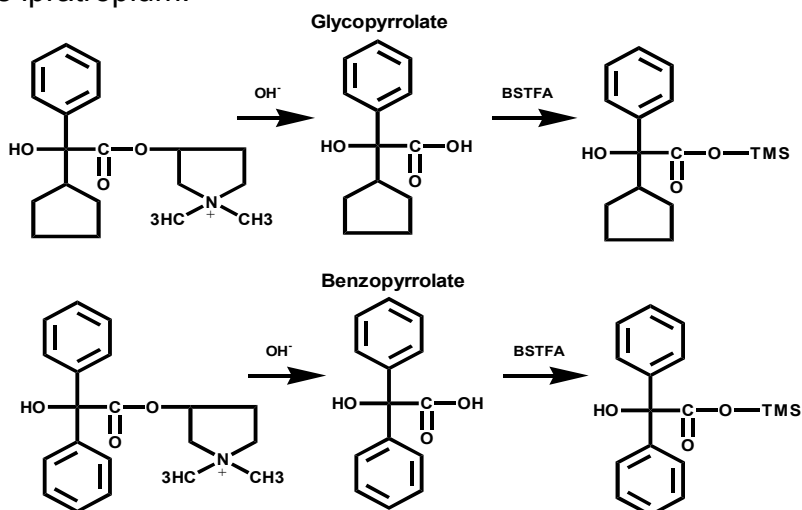


Figure 1. Analysis scheme for GC/MS identification of glycopyrrolate, and the possibility of unknown quaternary compounds generating α -hydroxy- α -phenyl-benzene acetic acid

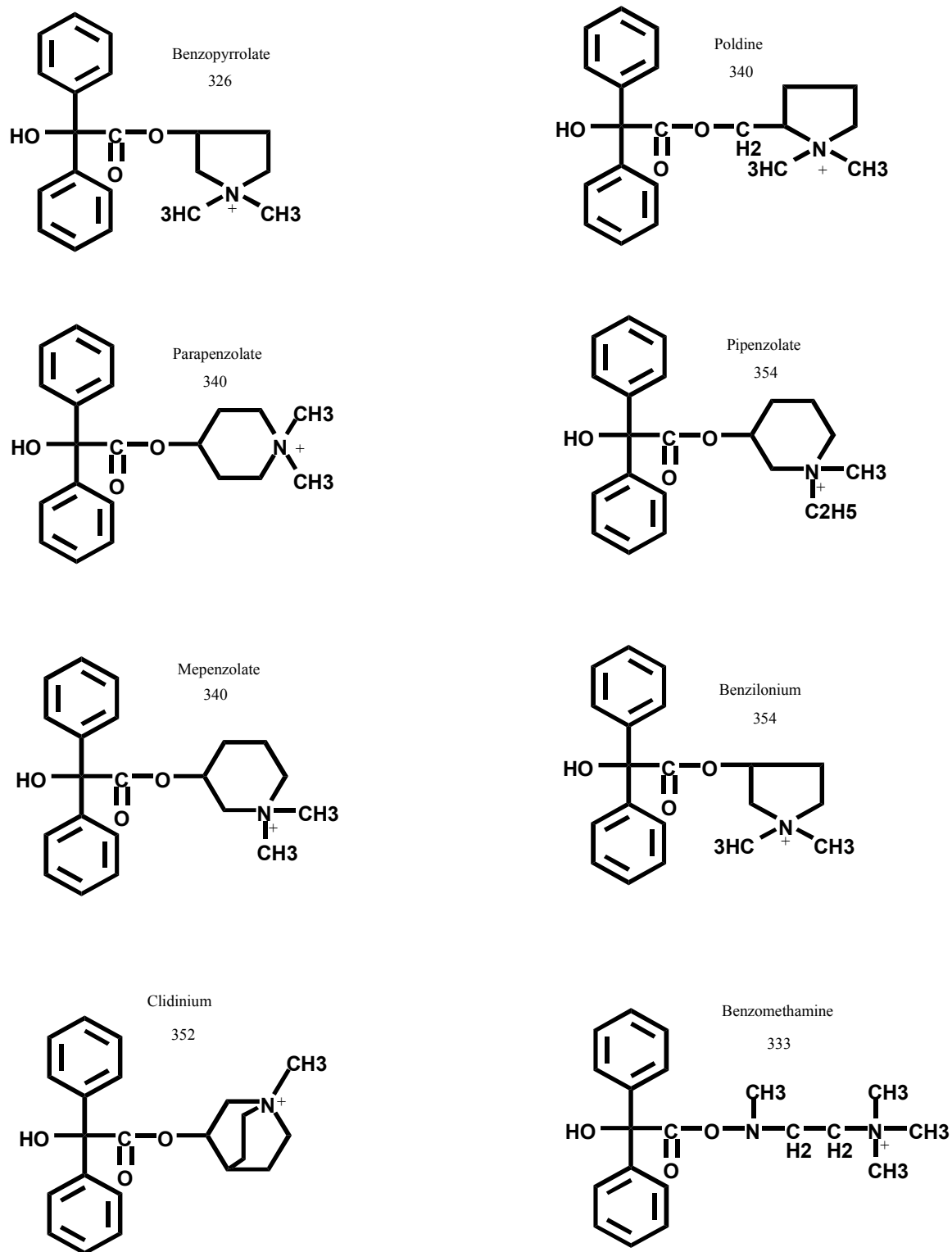


Figure 2. Possible compounds with the α -hydroxy- α -phenyl-benzene acetic acid substructure.

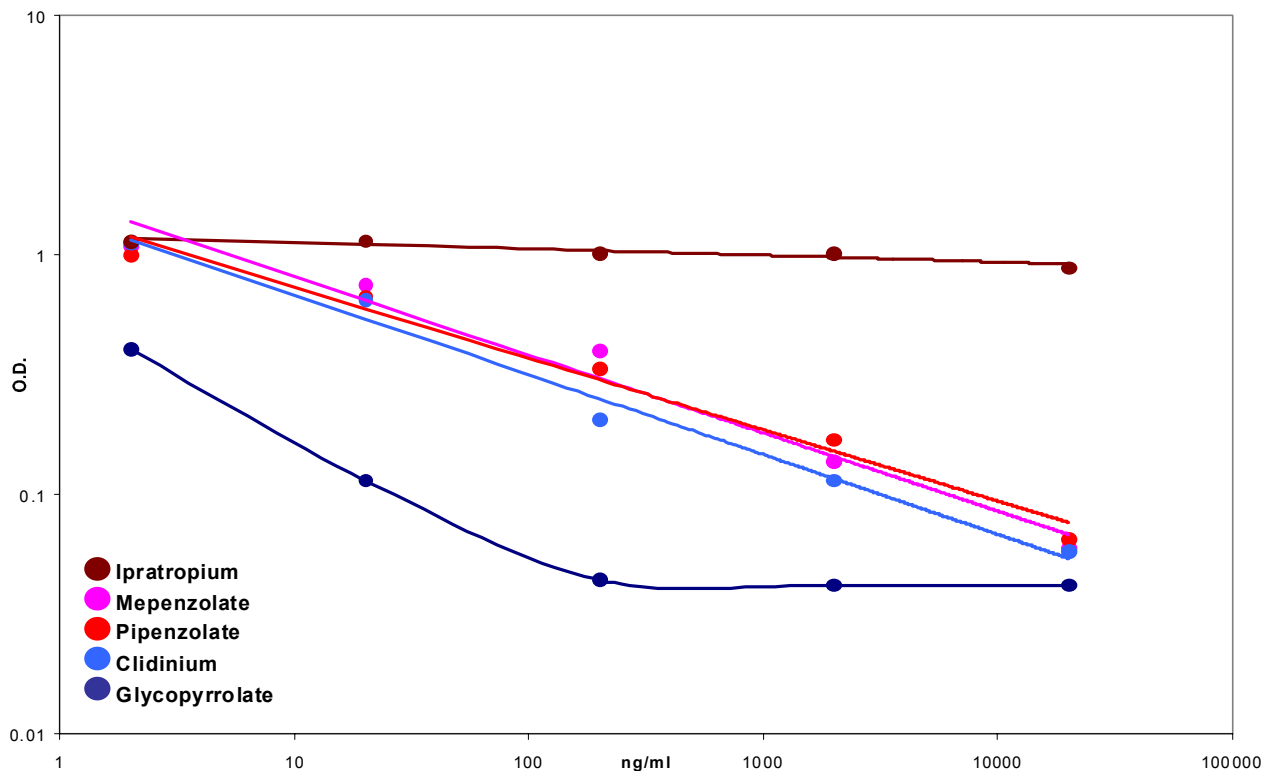


Figure 3. Response of Quaternary Compounds to Glycopyrrolate ELISA (Neogen)

From the above (figure 3.) it can be noted that Ipratropium does not cross-react on the Glycopyrrolate ELISA immunoassay kit (Neogen), and the hydrolysis product is not sufficiently unique (figure 4. below) to allow unequivocal presumptive identification. Faced with this challenge, a method involving liquid-liquid extraction, followed by LC/MS screening and LC/MS/MS full scan confirmation and quantification, was developed.

Ipratropium MW 332

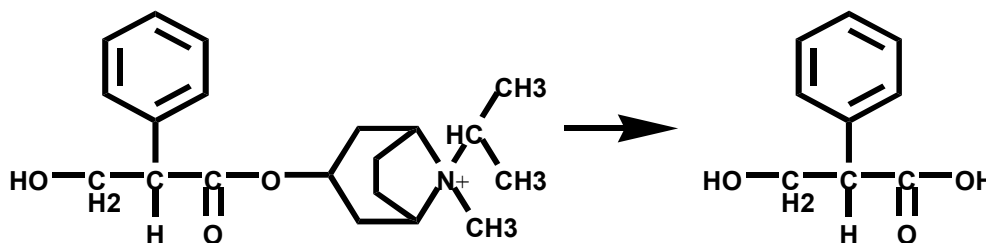


Figure 4. Hydrolysis of Ipratropium, (endo,syn)-(+/-)-3-(3-Hydroxy-1-oxo-2-phenylpropoxy)-8-methyl-8-(1-methylethyl)-8-azoniabicyclo[3.2.1]octane, leading to the production of α -hydroxymethyl benzene acetic acid.

Scope

The method described in this SOP utilizes heptane sulfonate ion-pair liquid-liquid extraction of equine urine into dichloromethane. Presumptive screening utilizes an LC-MS screen in positive electrospray ionization mode (ESI⁺), followed by MS/MS (ESI⁺) full scan quantification and confirmation of the suspect analyte. Suspect samples are screened, then pooled into groups representing an individual target analyte. Each group is then freshly prepared along with negative controls, blanks, and calibrators utilizing internal standardization. These groups are then re-analyzed for confirmation by full scan ESI⁺ spectrum identification and quantification. We have shown that this method is applicable to the identification, quantification, and confirmation of the following ten anticholinergic drugs:

Primary:	Glycopyrrolate	Secondary:	Methscopolamine
	Ipratropium		Methylhomatropine
	Mepenzolate		Anisotropine
	Pipenzolate		Neostigmine
	Clidinium		Isopropamide

Limitations

Ipratropium does not cross-react with the enzyme-linked immunosorbent assay (ELISA) test kit for glycopyrrolate (Neogen). For this reason, screening for this agent is achieved primarily by instrumental techniques. This method describes the instrumental screening for ipratropium by LC/MS. This instrumental screening method is also applicable to other anticholinergic compounds listed above.

Screening Extraction

Liquid-liquid ion-pair extraction is utilized to isolate the analytes for LC/MS screening and confirmation. A modification of a procedure of Sams² (1991) is used.

1. To 2 mL urine, add 3 mL 0.5 M ammonium acetate (pH 5.0)
2. Add 5 mL dichloromethane (DCM) and rotorack for 10 minutes
3. Centrifuge @ 2000 x g and discard DCM layer
4. Add 2 mL aqueous 0.1 M sodium heptane sulfonate to the aqueous sample supernatant from step 3
5. Add 5 mL DCM and rotorack for 10 minutes
6. Centrifuge @ 2000 x g rpm and aspirate aqueous (top) layer to waste
7. Transfer DCM layer to a clean-dry test tube (16x125 mm) and evaporate at 65°C (hot block)
8. Add 100 uL acetonitrile injection solvent to each test tube
9. Mix the contents of the tubes by vortexing to ensure the sides are rinsed, then centrifuge @ 2000 x g to concentrate all liquid to the bottom of the tube
10. Transfer injection solvent in step 9 to liquid chromatograph (LC) autosampler vials fitted with limited volume inserts. Cap vials and place in autosampler tray for analysis.

Liquid Chromatography Conditions

Instrument: Hewlett Packard 1100 LC
Column: Hewlett Packard LC-MS Zorbax SB-CN 5 micron 2.1x 50 mm
(part number 860975-905)
Mobile Phase: 80:20 acetonitrile: 50 mM aqueous ammonium acetate (pH 5)
Flow rate: 0.2 mL/min
Run Time: 3 minutes
Injection Volume: 30 µL

ESI⁺ MS Screening Conditions

Instrument: MicroMass Q-ToF
Mass Range: 200-450 daltons (amu)
Cone Voltage: 25
Scan Time: 1 sec
Inter-Scan Time: 0.1 sec
Acquisition Time: 0-35 minutes
Focus Voltage: 112
Capillary Voltage: 3000
Probe Heaters: 100 °C

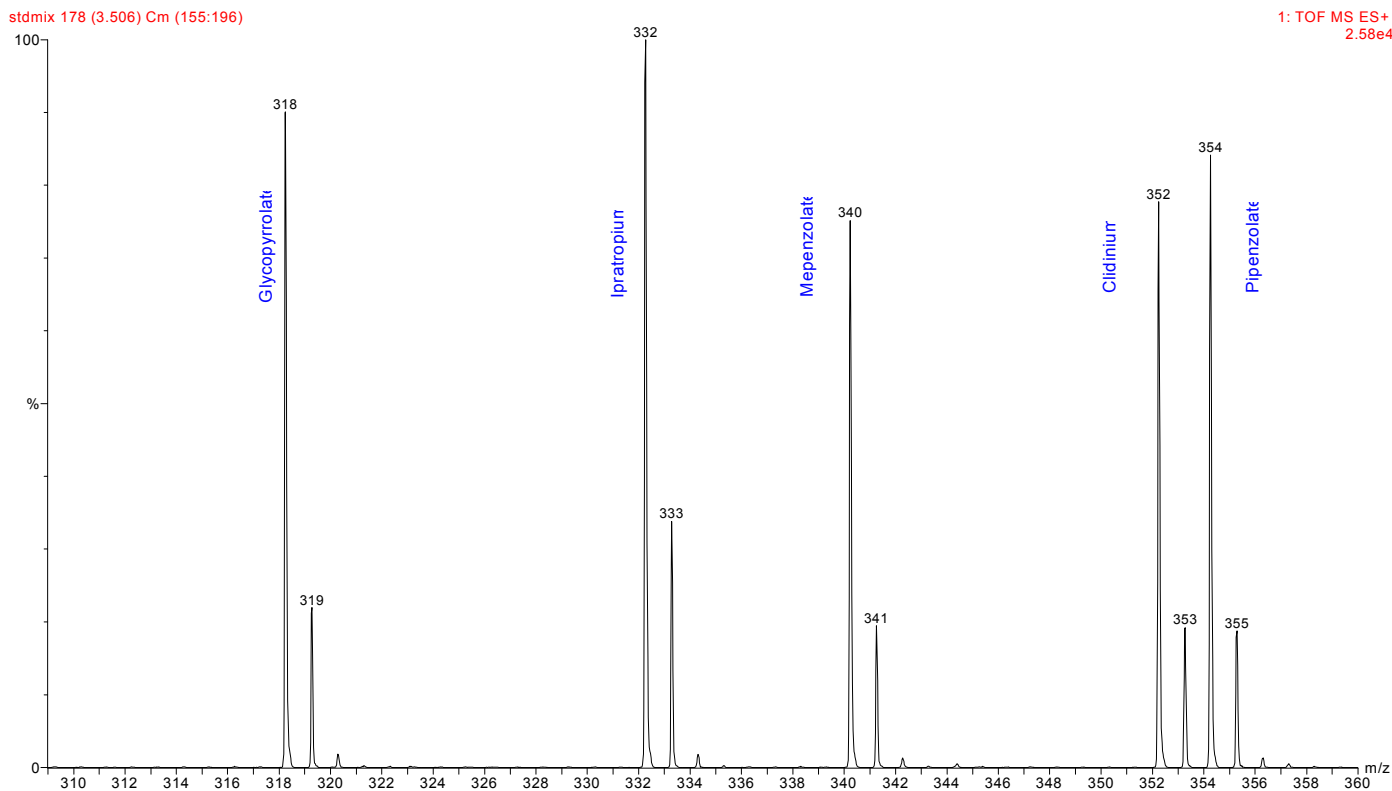


Figure 5. MS1 screen scan showing MS molecular ion clusters for primary analytes

MS/MS Verification of MS Screening of Suspect Samples

Upon completion and analysis of MS screening results, the same vials are analyzed (re-injected) by MS/MS to verify the identity of the preliminary finding. Any samples found to contain target analytes are logged, and grouped by target analyte.

ESI⁺ MS/MS Full Scan Conditions for Screening Suspect Samples

Samples exhibiting presumptive MS pseudo-molecular ions are re-injected and analyzed under MS/MS conditions for verification of the suspect identity. All settings remain the same as those under the MS conditions with the following exceptions:

Collision Gas: On
Collision Energy: 25
Parent Ions: Analyte Dependent (See below)
Internal Standard

ESI⁺ MS/MS Confirmation and Quantification

All presumptive findings are batch-grouped by target analyte. For each target analyte the following panel of analyses are prepared:

<u>Analyte</u>	<u>Parent Ion</u>	<u>Internal Standard</u>	<u>Parent Ion</u>
Ipratropium	332.2	Methyl Scopolamine	318.2
Glycopyrrolate	318.2	Methyl Homatropine	290.2
Mepenzolate	340.2	Methyl Scopolamine	318.2
Clidinium	352.2	Methyl Scopolamine	318.2
Pipenzolate	354.2	Methyl Scopolamine	318.2

The MS/MS method consists of two simultaneous parent ion experiments. The first function parent ion must be edited for the appropriate suspect ion, and the second function parent ion must be edited for the appropriate internal standard parent ion. For quantification, a minimum of 5 calibrator points are used.

Ipratropium & Glycopyrrolate

Negative Control

Sample

\

\

0 ng/mL Calibrator

1 ng/mL Calibrator

10 ng/ mL Calibrator

50 ng/ mL Calibrator

100 ng/ mL Calibrator

250 ng/ mL Calibrator

\ repeat a Negative Control for each additional sample.

Preparation of Samples, Controls, and Calibrators

MS/MS confirmation and quantification are performed on separate, freshly prepared samples and calibrators. 10, 1.0, and 0.1 µg/ml reference standard solutions are used for the calibrator and internal standard (IS) additions according to the following table:

Tube identifier	Sample type	µl Calibrator	µl IS (10 µg/mL)
Negative Control	Negative Control	NA	100
Sample	Unknown Sample	NA	100
Blank	Blank, 0 Calibrator	NA	100
Cal1	Calibrator 1 ng/mL	(20 of 0.1 µg/mL)	100
Cal10	Calibrator 10 ng/mL	(200 of 0.1 µg/mL)	100
Cal50	Calibrator 50 ng/mL	(100 of 1.0 µg/mL)	100
Cal100	Calibrator 100 ng/mL	(200 of 1.0 µg/mL)	100
Cal250	Calibrator 250 ng/mL	(500 of 1.0 µg/mL)	100

The panel of tubes is prepared according to the liquid-liquid ion-pair extraction procedure previously described. A negative control must be analyzed immediately before every unknown sample included in the panel.

MSMS Full Scan ESI⁺ Confirmation and Quantification

The LC and MSMS conditions used are those previously described with the appropriate modifications made for the specific target compound and its corresponding internal standard. The only additional consideration is the inclusion of the quantification method. To assure the validity of the automatic quantification report, it is required that each data file be manually inspected to ensure the validity of the automatic integration process at the conclusion of the analysis sequence. Manual reintegration is performed on any data file with unacceptable integration. Unacceptable integration is defined as peak splitting, extra peak or baseline inclusion, or failure of a peak being detected. Results with correlation less than 0.95 must be re-prepared and re-analyzed.

MSMS Full Scan ESI⁺ Acceptance Criteria

Full scan MS/MS spectra are unacceptable if any standard ion greater than 15% is absent from the suspect spectrum, or any ion not indicative of the reference spectrum greater than 15% is present in the suspect sample spectrum.

Method Characterization

Recovery of Method > 80%
 LOD of Method < 200 pg/mL
 LOC of Method < 300 pg/mL

Interferences

No interferences are known at this time. Stability of quaternary esters has been shown to be susceptible to degradation in urine at elevated temperatures over time. Quantification is performed to reference administration study data to avoid the reporting of incidental concentrations (> 24 hours post administration).

DETERMINATION OF RUGGEDNESS

Stability Studies: Effect of Temperature

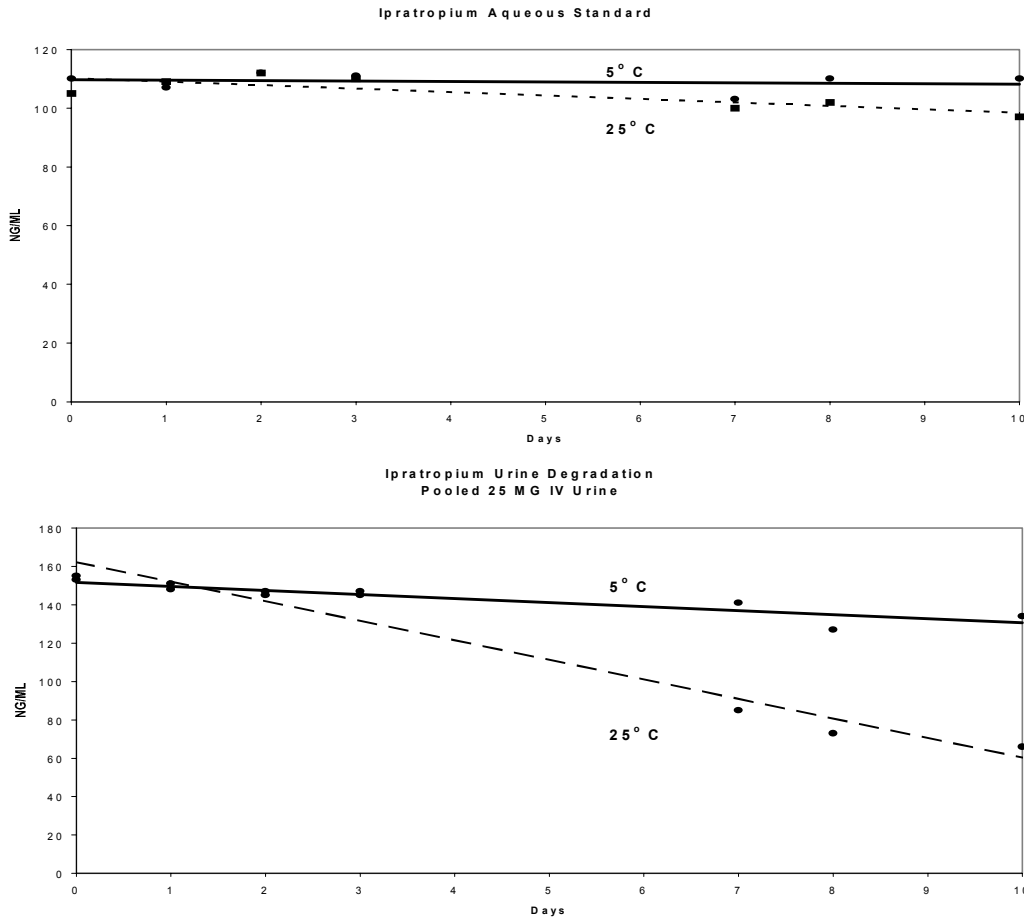


Figure 6. Ten day stability study of Ipratropium in analyte supplemented water and equine administration urine at 5°C and 25°C..

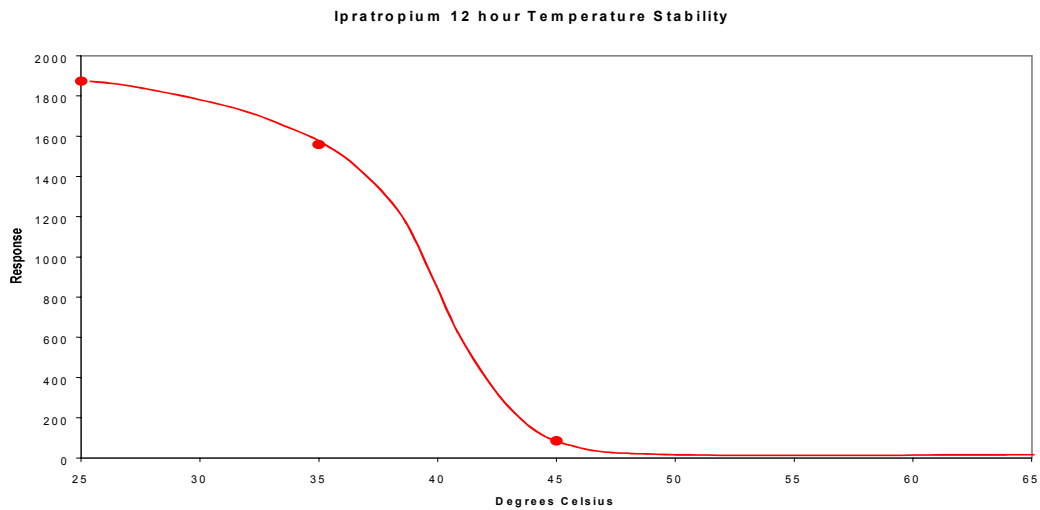


Figure 7. 12 hour stability study of Ipratropium equine administration urine_at increasing temperature.

Analyte Stability Following Freeze-Thaw cycles.

Ipratropium Freeze-Thaw Stability

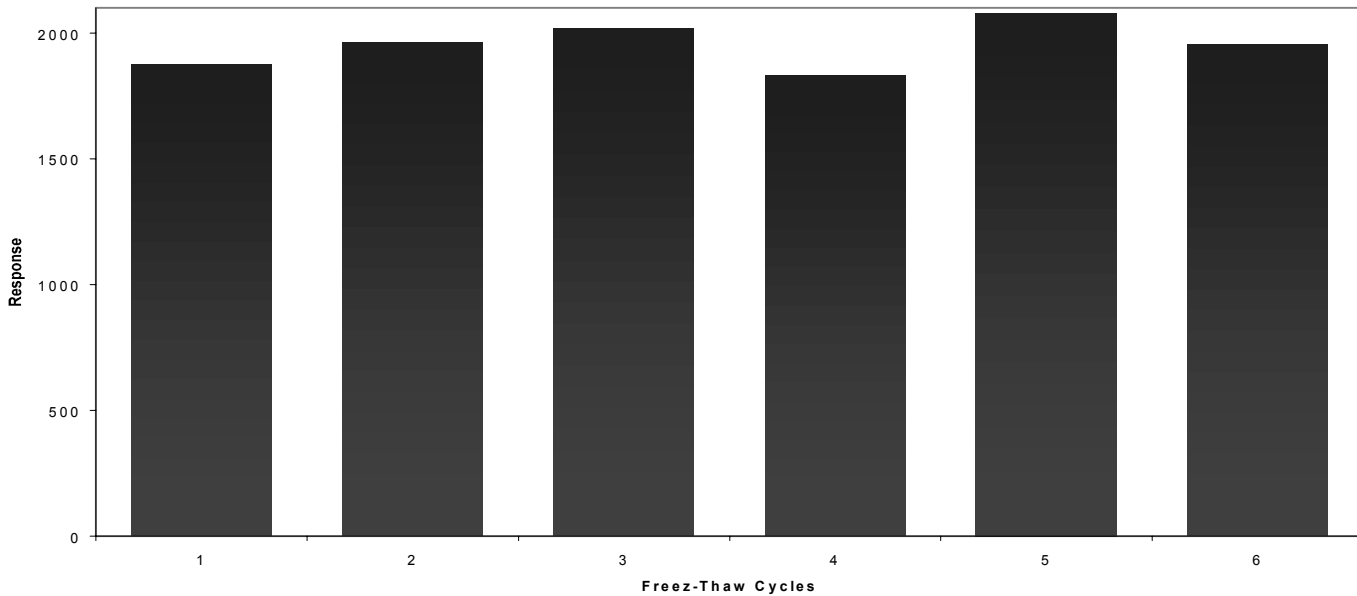


Figure 8. Stability of Ipratropium equine administration samples through five freeze-thaw cycles (CONTROL = #1)

EXTRACTION RECOVERY STUDIES

Analyte recovery as a function of ion-pair buffer

ul Ion Pair Buffer

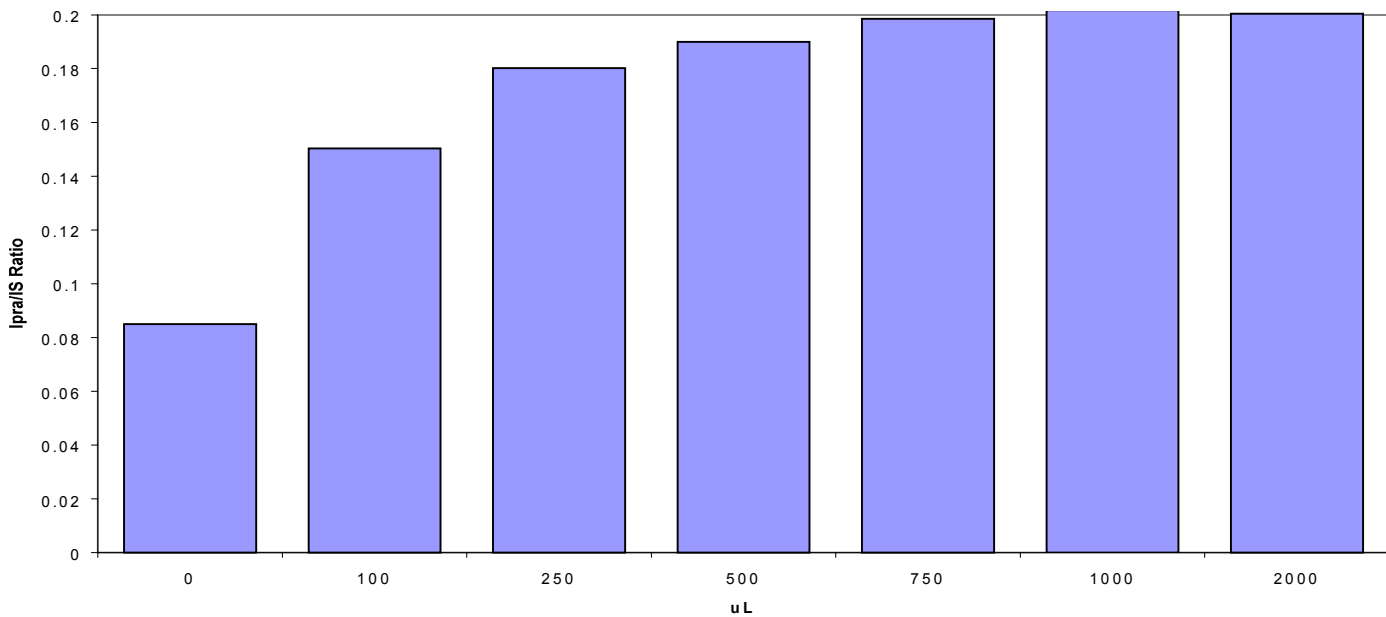


Figure 9. Ipratropium recovery efficiency as a function of molar volume of ion-pair reagent

Analyte recovery as a function of pH

Recovery of Ipratropium as a Function of pH

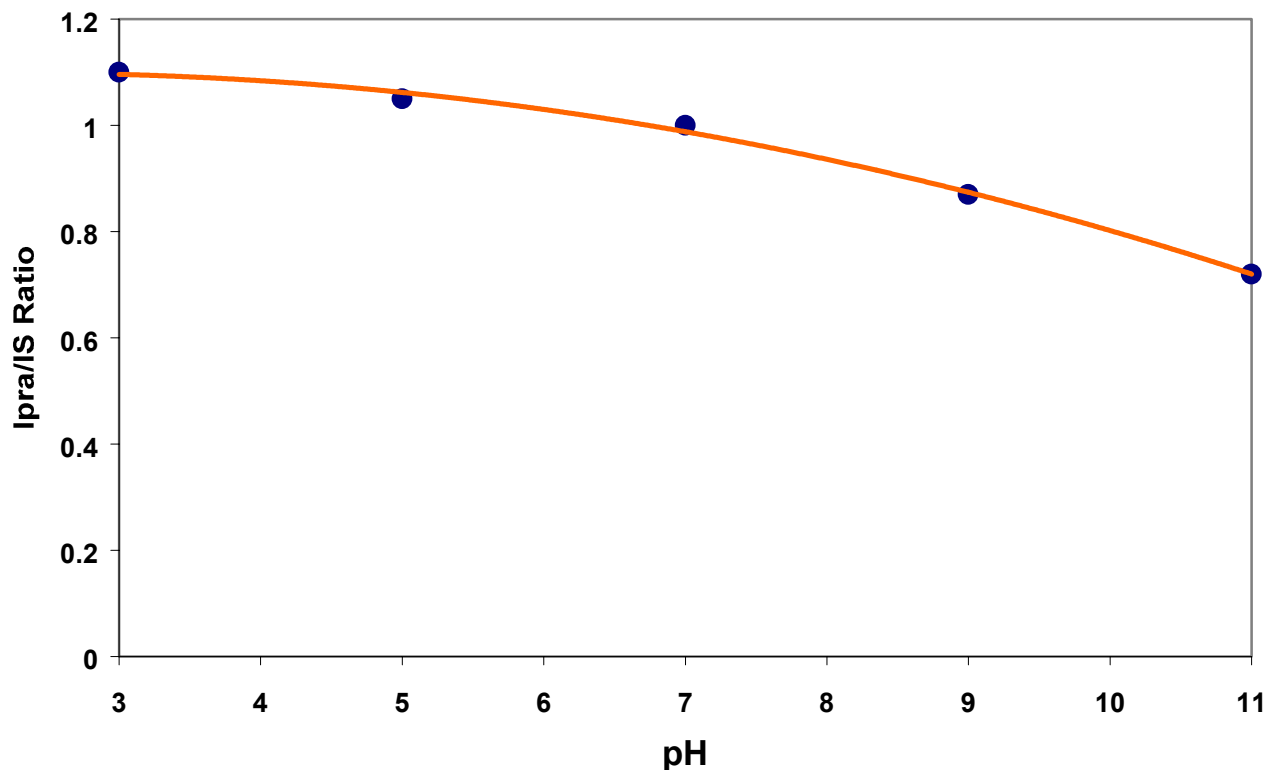


Figure 10. Recovery of Ipratropium from equine administration urine as a function of adjusted pH

Analyte recovery as a function of extraction solvent

Solvent Selectivity for Ion-Pair Extraction

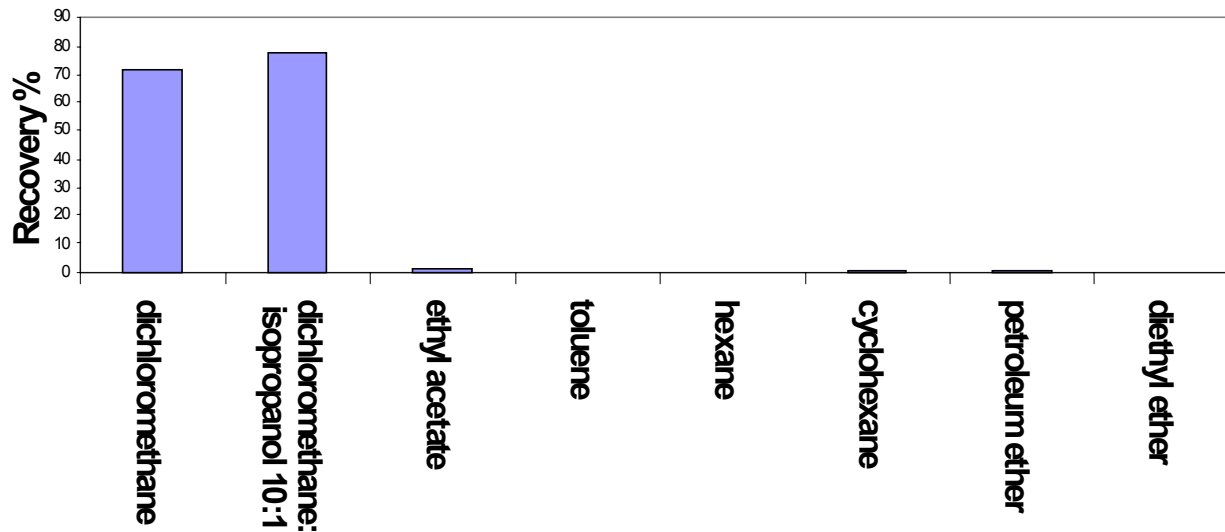


Figure 11. Recovery of Ipratropium from analyte supplemented water as a function of extraction solvent

IPRATROPIUM ADMINISTRATION CLEARANCE CURVES

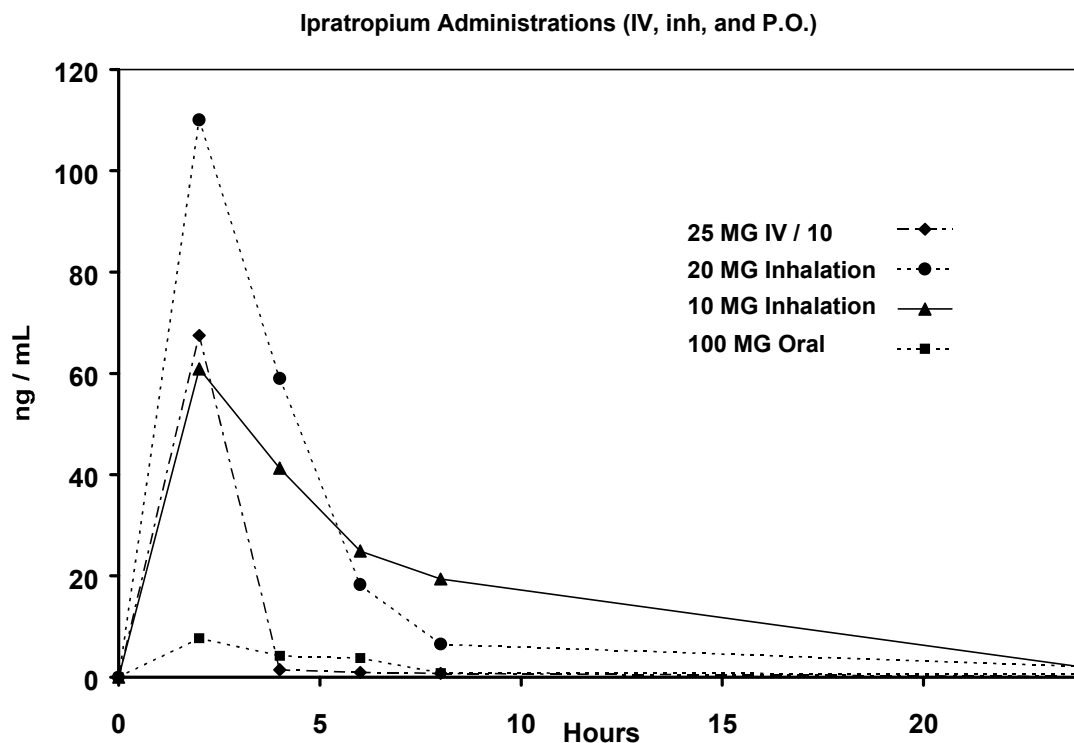


Figure 12. Elimination profiles of Ipratropium in equine urine following intravenous (IV), inhalation (Inh), and oral (PO) administrations of Ipratropium

DISCUSSION

Since Ipratropium is not detected by immunoassay screening for glycopyrrolate, an instrumental screening, quantification, and full scan confirmation was developed utilizing LC/MS/MS. Quantification is performed to provide perspective for dosage and time-frame of administration. Quantification can be adversely affected by storage and handling conditions, leading to lower quantification results, presumably due to degradation of the analyte in the matrix. Thus, proper storage and handling conditions must be provided during shipment and storage of the samples upon arrival in the laboratory. This method is reliable and applicable to the screening, quantification, and confirmation of a variety of quaternary anticholinergic drugs.

Materials and Reagents

Source and Catalog #

Sodium Heptane Sulfonate	Fisher O-3013
Water HPLC grade	Fisher W5-4
Acetonitrile HPLC Grade	Fisher A998-4
Dichloromethane HPLC Grade	Fisher D143-4
Glacial Acetic Acid	Fisher A38 ^c -212
Ammonium Hydroxide	EM AX1303-3
Ipratropium	Boehringer Ingelheim RM-0421
Glycopyrrolate	A.H Robbins 0031-7890-83
Clidinium	Sigma C-0414
Mepenzolate	Sigma M-5651
Methscopolamine	Sigma S-8502
MethylHomatropine	Sigma H-1503
Pipenzolate	Sigma P-6085
Isopropamide	Sigma I-7882
Anisotropine	Sigma A-5181
Neostigmine	Sigma N-2001

Preparation of Reagents

0.1 M aqueous Sodium Heptane Sulfonate – 2.2 gms in 100 ml HPLC water

0.5 M Ammonium Acetate (pH 5.0) – 29 mL Acetic acid per 900 ml HPLC water,
pH 5.0 with Ammonium Hydroxide, then q.s.to 1000 ml with HPLC water.

Preparation of Reference Stock Solutions

Ipratropium Reference Stock solutions Accurately weigh Ipratropium Bromide (~10 mg) and record weight. Multiply this number by 0.806 and add the resultant volume HPLC water (in milliliters). This will produce an aqueous 1 mg/mL working stock solution (Ipra A).

Add 100 µL of Ipra A to 9.9 mL acetonitrile to produce 10 µg/mL solution (Ipra B).

Add 1 mL of Ipra B to 9 mL acetonitrile to produce 1 µg /mL solution (Ipra C).

Add 1 mL of Ipra C to 9 ml acetonitrile to produce 1 µg /mL solution (Ipra D).

MethylHomatropine Reference Stock solutions Accurately weigh Homatropine MethylBromide (~10 mg) and record weight. Multiply this number by 0.784 and add the resultant volume HPLC water (in milliliters). This will produce an aqueous 1 mg/mL working stock solution (MHom A).

Add 100 µL of MHom A to 9.9 mL acetonitrile to produce 10 µg/mL solution (MHom B).

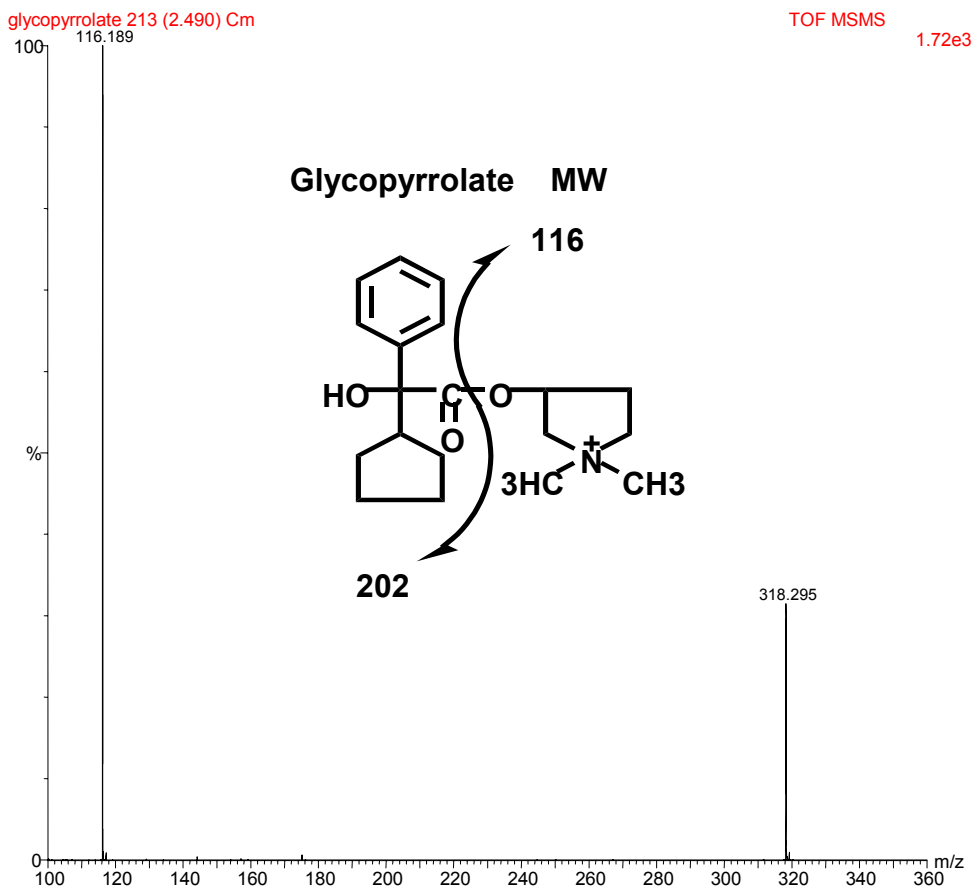
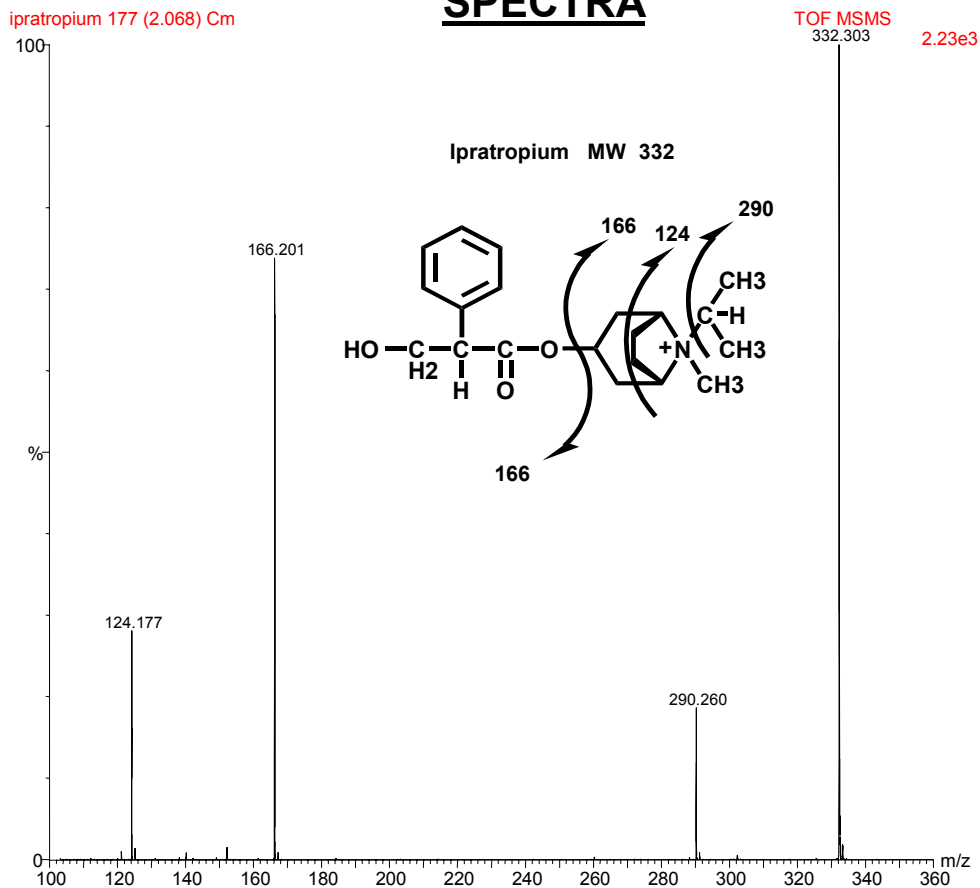
Add 1 mL of MHom B to 9 mL acetonitrile to produce 1 µg/mL solution (MHom C).

Add 1 mL of MHom C to 9 ml acetonitrile to produce 1 µg/mL solution (MHom D).

References

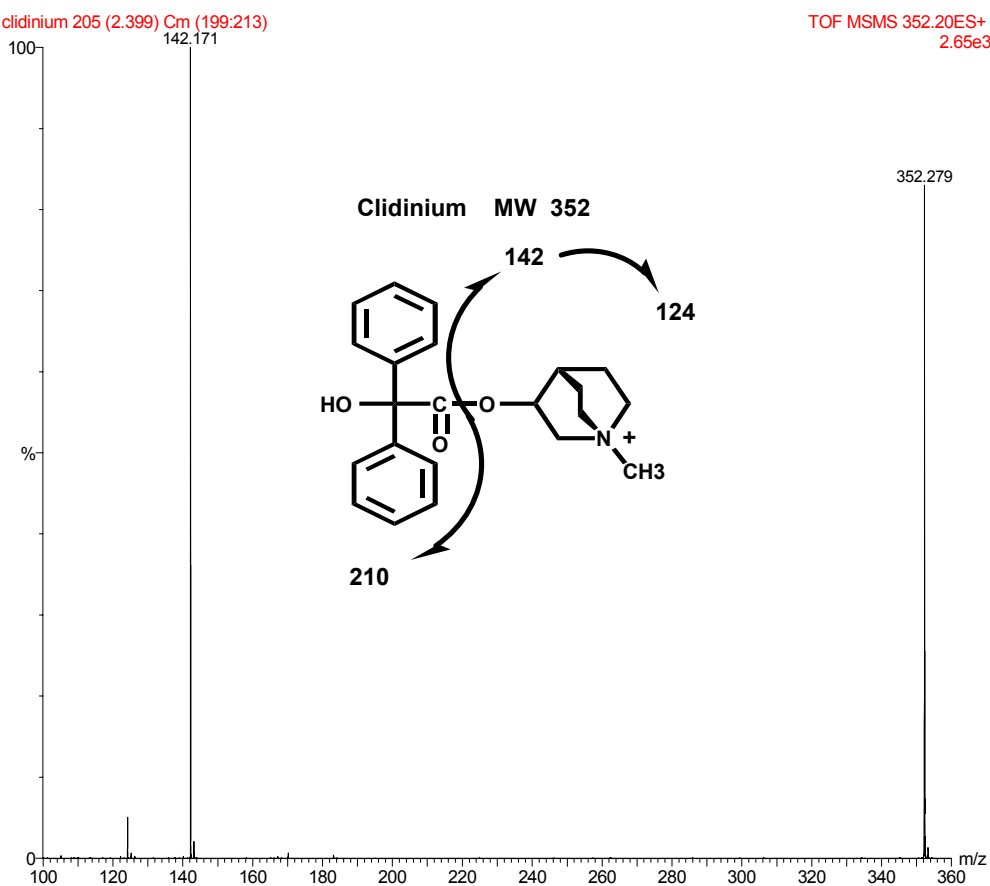
1. Mann Testing Laboratories Ltd., 1990, Glycopyrrolate Analysis
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3. Matassa, Beaumier, Leavitt, et al, J. Chrom B, 573 (1992) 43-48; *Solid-phase extraction techniques for the determination of glycopyrrolate from equine urine by liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry*
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5. Ryan, Mui, Downey, Proceedings of the 11th International Conference of Racing Analysts and Veterinarians, 1996, 448-493; *Detection and Confirmation of Clidinium Bromide in Equine Urine using LCMSMS and GCMS Techniques*
6. Rudy, et al, March 2000, 7th Testing Integrity Workshop @ UC Davis, Davis, California; *Identification and Confirmation of Quaternary Ammonium Compounds by LC/MS/MS.*
7. Wan, Tang, Leung, 13th International Conference of Racing Analysts and Veterinarians, August 2000; *Analysis of Quaternary Ammonium Drugs by CE/MS*

SPECTRA



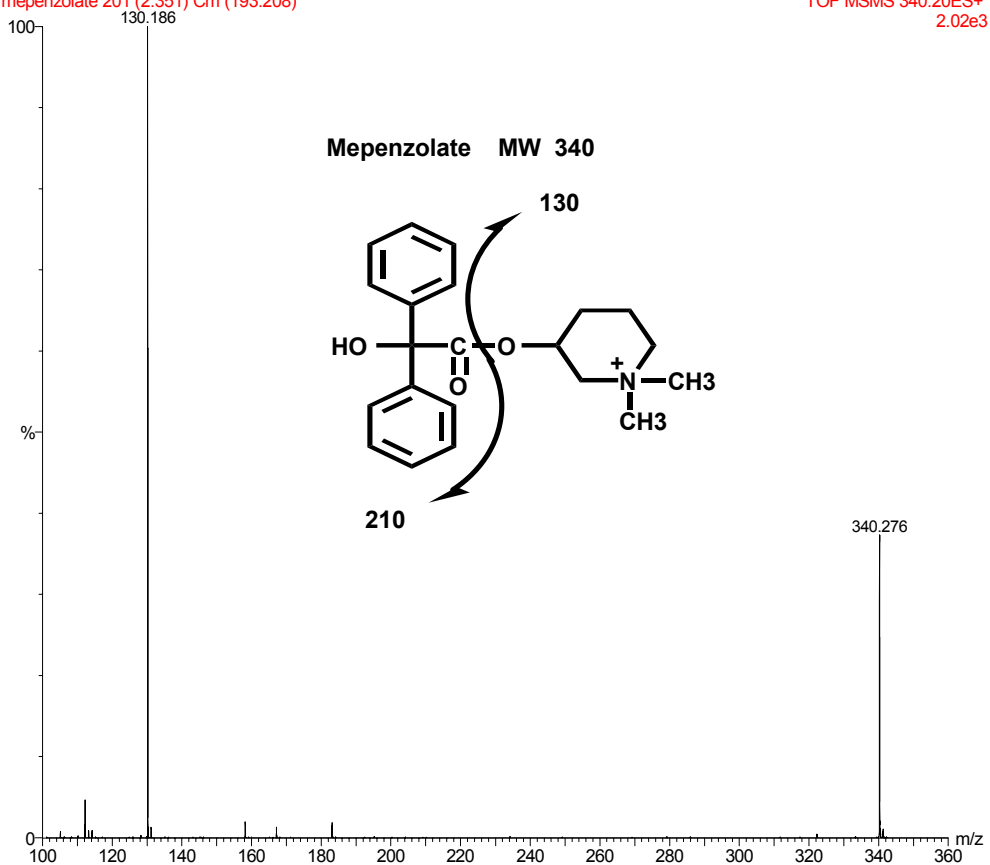
clidinium 205 (2.399) Cm (199:213)

TOF MSMS 352.20ES+
2.65e3



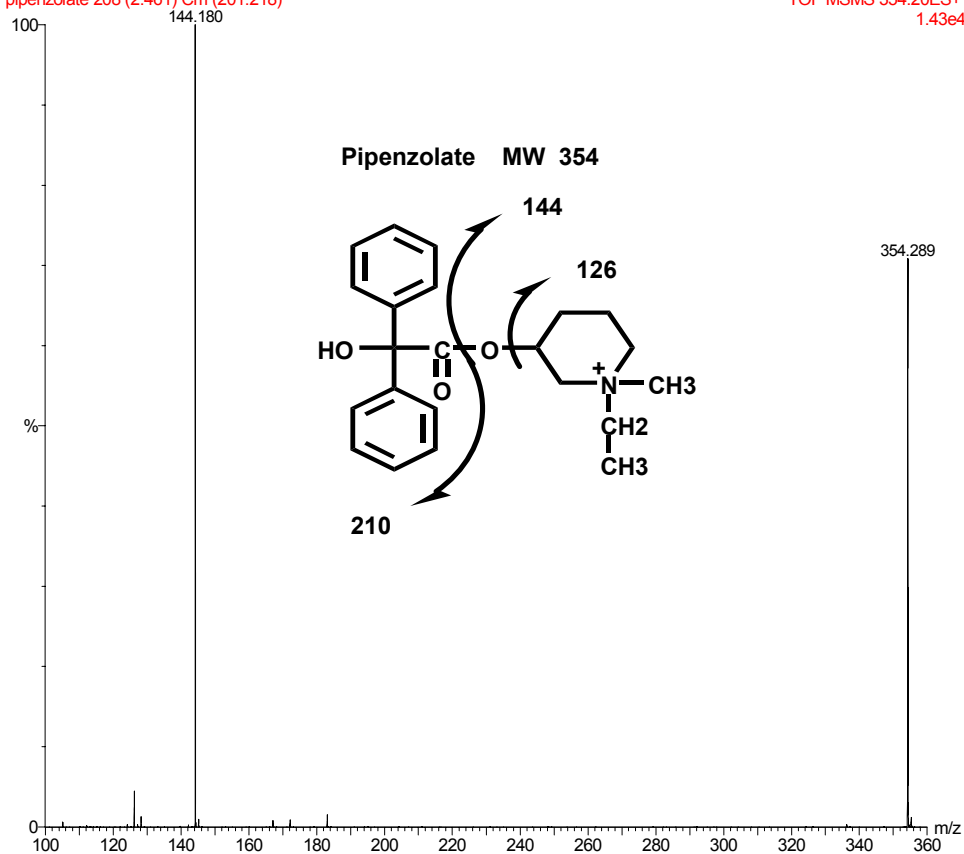
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TOF MSMS 340.20ES+
2.02e3



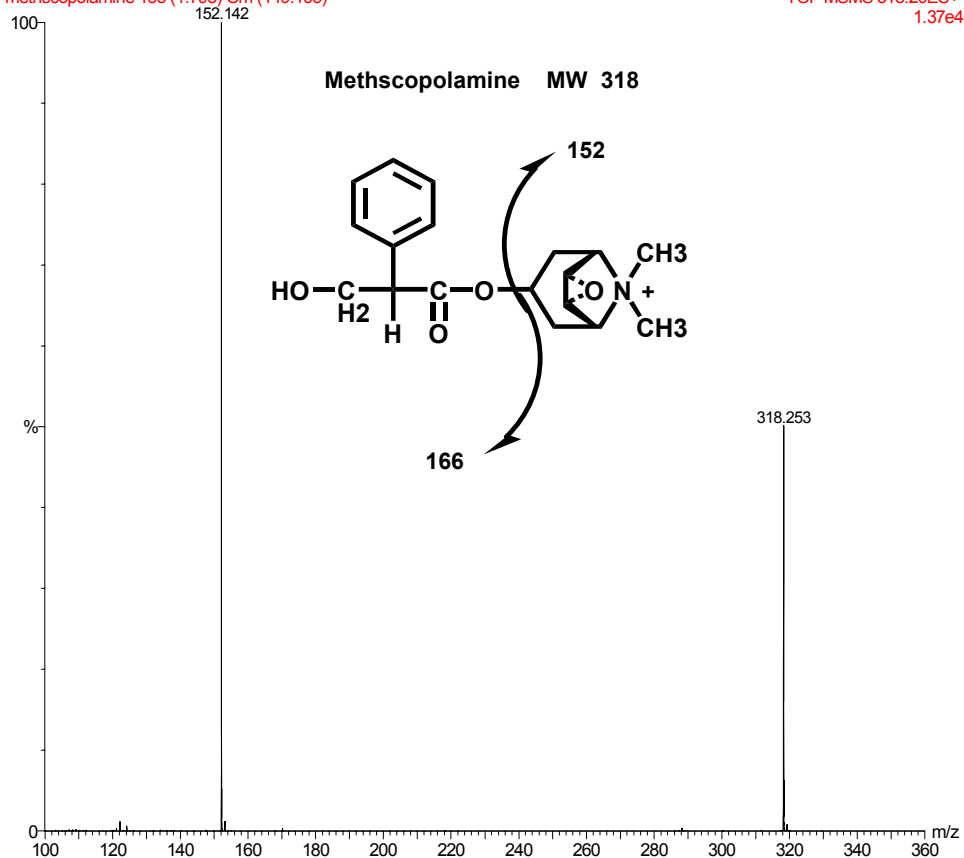
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1.43e4



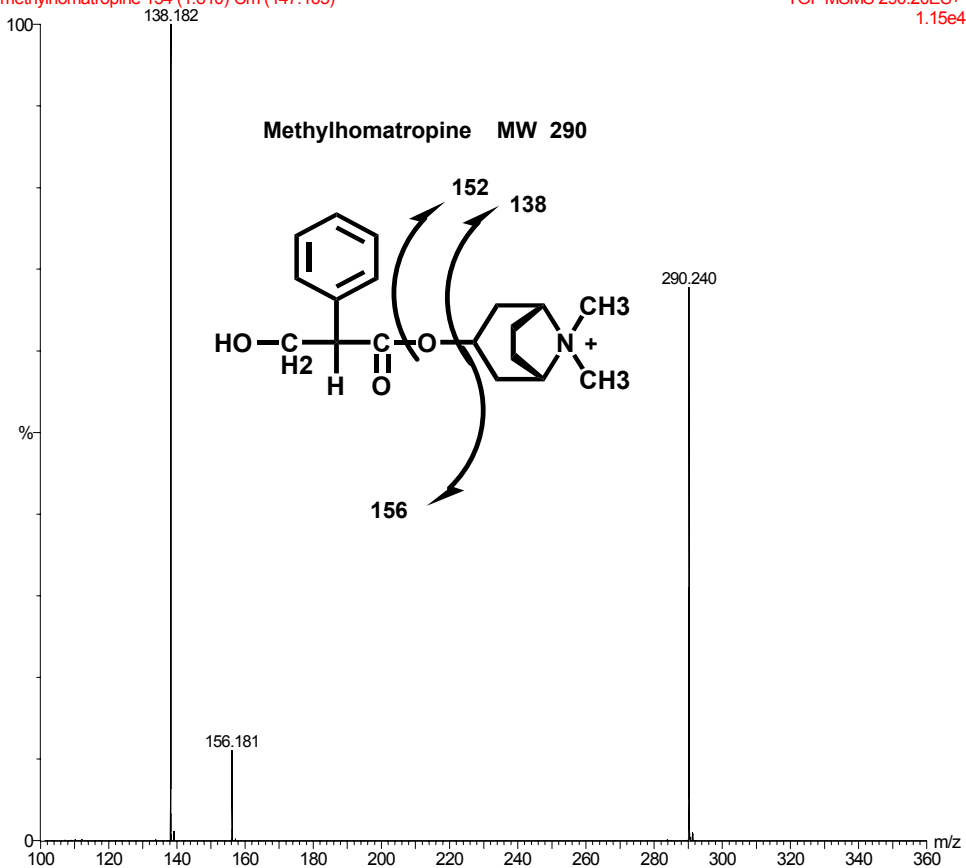
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TOF MSMS 318.20ES+
1.37e4



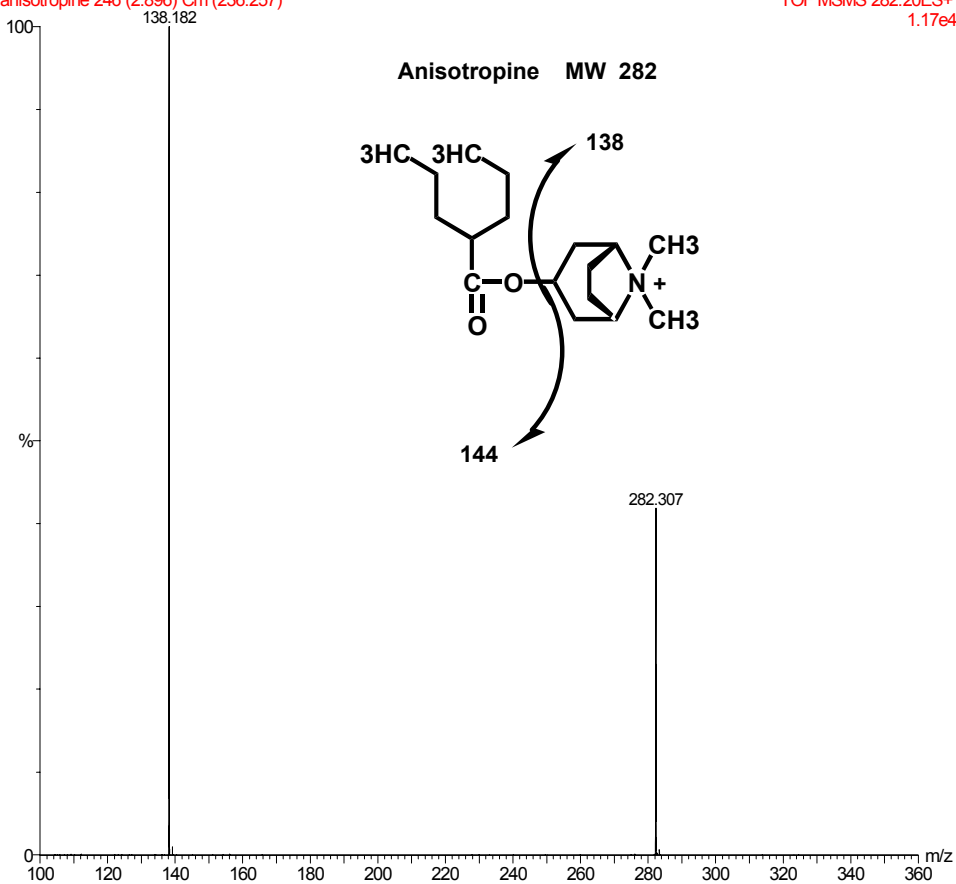
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TOF MSMS 290.20ES+
1.15e4



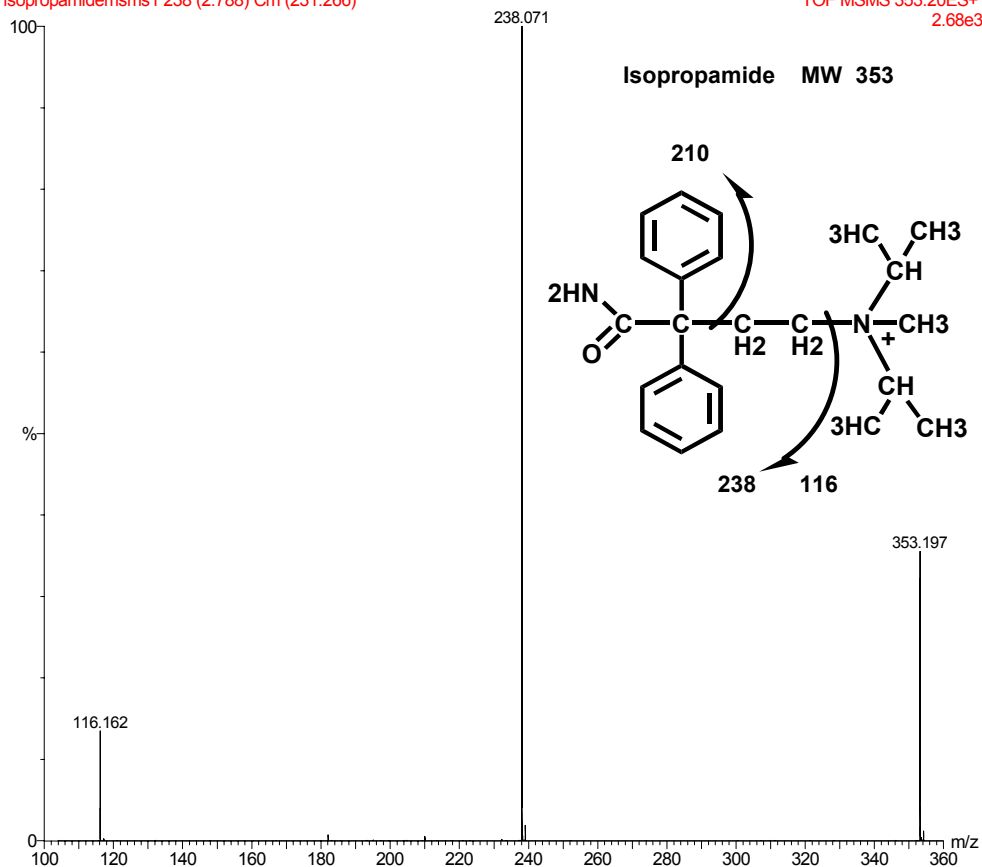
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TOF MSMS 282.20ES+
1.17e4



isopropamidemsms1 238 (2.788) Cm (231:266)

TOF MSMS 353.20ES+
2.68e3



neostigmine 162 (1.894) Cm (154:173)

TOF MSMS 223.20ES+
4.74e3

