
TIP Approved SOP

IDENTIFICATION AND CONFIRMATION OF PROPANTHELINE AND 16 QUATERNARY AMMONIUM DRUGS IN EQUINE PLASMA AND URINE BY ION TRAP LCMSMS

Developed by

PA EQUINE TOXICOLOGY & RESEARCH LABORATORY

220 EAST ROSEDALE AVENUE
WEST CHESTER UNIVERSITY
DEPARTMENT OF CHEMISTRY
WEST CHESTER, PA 19382
Phone: (610) 436-3501
Phone: (610) 436-3504

Director: Dr. Cornelius Uboh

E-mail: ubohcorn@vet.upenn.edu
cuboh@state.pa.us

Laboratory Manager: Jeffrey Rudy

E-mail: jeffrudy@ccis.net

Method Development: Jeffrey Rudy

Drug Administration:

**University of Pennsylvania School of Veterinary
Medicine**

New Bolton Center

834 West Street Road

Kennet Square, PA 19483

Phone: (610) 444-5800 ext 2265

Contact: Dr. Lawrence R. Soma

E-mail: soma@vet.penn.edu

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I. INTRODUCTION

Quaternary ammonium drugs are anticholinergic agents used to maximize pulmonary capacity under stressful conditions. Respiratory disease conditions are one of the most common conditions that affect racehorses. Treatment of respiratory diseases in racehorses is ranked among the largest number of reported clinical cases per year. In competitive sports, controversial mechanisms for increasing the oxygen carrying capacity of blood are gaining public attention. Consequently scientific attention to these agents is also dramatically increasing. These mechanisms include increasing red cell counts (blood packing, erythropoietin, darbepoetin), and increasing blood carrying capacity (hemoglobin polymers and perfluorinated hydrocarbons). A logical extension of these practices is to maximize the quantity of oxygen that the lungs can deliver to the bloodstream. We had previously reported a procedure for the screening and confirmation of eight quaternary anticholinergic drugs by Liquid-Chromatography Tandem Mass Spectrometry (LCMSMS) including Glycopyrrolate and Ipratropium. The successful regulation of these drugs in racehorses has led to the usage of other agents believed to be less detectable.

Previous methods presented at the 17th Montreux Symposium on LC-MS-2000 in Montreux, Switzerland and described in our earlier SOP for Quaternary Ammonium Drugs involved confirmation following screening by enzyme linked immunosorbent assay (ELISA). These methods included gas chromatography-mass spectrometry (GC-MS)¹ following hydrolysis and derivatization, and liquid chromatography – mass spectrometry (LC-MS)^{2,4}. These methods were limited to quaternary compounds included within a panel of ELISA cross reactivity. Other methods for more general instrumental screening of quaternary compounds not limited to ELISA target confirmation have included LC-MS⁴ and capillary electrophoresis-mass spectrometry (CE-MS)³. Gas chromatography was limited by the non-unique nature of the derivatized hydrolysis product to a very limited number of quaternary drugs. ELISA based LC-MS screening and confirmation was limited in scope by the ELISA target drug reactivity. More general instrumental screens included more drugs based on pharmacological or method chemistry similarities. However, most atmospheric pressure ionization (API) mass spectrometers sacrifice target drug sensitivity when increasing the number of target compounds. Solutions to this limitation include data dependent switching between MS and MSMS mode, and time segmentation of multiple scan events in MSMS mode.

In the present approach, we are presenting an instrumental method describing the screening and confirmation of 17 quaternary ammonium drugs with a 6-minute analysis times and limits of detection and confirmation less than 100 pg/mL using a Thermo Electron Deca XP Plus ion trap mass spectrometer.

II. SCOPE

This standard operating procedure (SOP) describes identification and confirmation procedures for 17 quaternary anticholinergic drugs utilizing a Thermo Electron Deca XP Plus ion trap liquid chromatograph-mass spectrometer (LCMS/MS) operated in positive ion electrospray ionization mode with subsequent MS and MS² analysis. Extraction procedures for equine plasma and urine are presented. Since no threshold exists for these compounds, the reporting limit is the limit of confirmation. However, quantitative considerations are included for system and quality control, as well as for value-added quantitative estimates to further characterize the reported results.

III. PRIMARY DRUG STANDARD REFERENCE MATERIALS

#	Drug Name	Source	Cat #
1	Ipratropium	Boehringer Ingelheim	RM-0421
2	Glycopyrrolate	A.H Robbins	0031-7890-83
3	Clidinium	Sigma	C-0414
4	Mepenzolate	Sigma	M-5651
5	Methscopolamine	Sigma	S-8502
6	MethylHomatropine	Sigma	H-1503
7	Pipenzolate	Sigma	P-6085
8	Isopropamide	Sigma	I-7882
9	Anisotropine	Sigma	A-5181
10	Neostigmine	Sigma	N-2001
11	Propantheline	Sigma	P-8891
12	Bretylium	Sigma	B-8406
13	Edrophonium	Sigma	E3256
14	Tubocurarine	Sigma	T2379
15	Pancuronium	Sigma	P-1918
16	Pyridostigmine	Sigma	P-9797
17	Oxyphenonium	Sigma	O5501-1G

Obtain these materials from the pharmacy and record accession on the pharmacy log sheet.

IV. PREPARATION OF PRIMARY REFERENCE STOCK SOLUTIONS

Weigh between 8 and 15 mg of the quaternary salt. Dilute to volume using HPLC grade (or better) methanol (Volume **Y.yy** mL = **X.xx mg** x **Z.ZZ** where **Z.ZZ is the Salt/Free ratio where applicable**).

1 mg/mL MeOH reference stock solutions were prepared as follows:

#	Drug Name	Free Quaternary ratio	Free MW	Salt MW	LABEL	mg/mL
1	Ipratropium	0.8062	332.465	412.368	IPRATSTOCK121803	1
2	Glycopyrrolate *	0.9142	318.438	348.341	GPYRSTOCK121803	0.2
3	Clidinium	0.8152	352.455	432.358	CLIDSTOCK121803	1
4	Mepenzolate	0.8099	340.444	420.347	MEPSTOCK121803	1
5	Methscopolamine	0.7994	318.394	398.292	SCOPSTOCK121803	1
6	Methylhomatropine	0.7842	290.384	370.287	MHOMASTOCK121803	1
7	Pipenzolate	0.8161	354.471	434.374	PIPSTOCK121803	1
8	Isopropamide	0.6817	327.492	480.434	ISOPROPSTOCK121803	1
9	Anisotropine	0.7795	282.448	362.352	ANISSTOCK121803	1
10	Neostigmine	0.7365	223.296	303.199	NEOSTOCK121803	1
11	Propantheline	0.8218	368.498	448.401	062904STOCKPROPAN	1
12	Bretylum	0.5868	243.167	414.363	062904STOCKBRETYL	1
13	Edrophonium	0.8242	166.244	201.697	062904STOCKEDROP	1
14	Tubocurarine	0.8960	610.753	681.658	080304STOCKTUBO	1
15	Pancuronium	0.7819	572.876	732.683	080304STOCKPANCUR	1
16	Pyridostigmine	0.6940	181.215	261.119	080304STOCKPROPAN	1
17	Oxyphenonium	0.8135	348.508	428.411	0810STOCKPROPAN	1

Cap and mix until compound is completely dissolved in methanol.

- * Glycopyrrolate was prepared by diluting commercial Robinul® injectable solution into methanol

Complete Balance Use Log and QA Primary Reference Standard Log for this process. Label the primary reference stock solutions by some form of the following format: DRUGSTOCKmmddyy.

V. PREPARATION OF FLOW INJECTION ANALYSIS (FIA) and LIQUID CHROMATOGRAPHY (LC) COLUMN TEST WORKING STOCK SOLUTIONS

Materials Needed: Primary reference stock solutions and 50:50:1 MeCN:Water:Formic acid

10 ug/mL FIA solutions and 100 ng/mL LC Test solutions were prepared in 50:50:1 (Water:MeCN:1% Formic Acid) as follows:

Using Propantheline as an example, all FIA and LC test solutions are prepared as follows:

Drugs	µL Used	mL 50:50:1	Label Used	Label	Concentration
Propantheline	100	9.9	062904FIAPropan	062904FIAPropan	10 µg/mL

Drugs	µL Used	mL 50:50:1	Label Used	Label	Concentration
Propantheline	100	9.9	062904LCPropan	062904LCPropan	100 ng/mL

An LC column test solution containing all 17 quaternary ammonium compounds was prepared as follows:

Drugs	µL Used	mL 50:50:1	Label Used	Label	Concentration
1-17	100	8.3	062904FIAdrug	062904LC17QUATS	100 ng/mL

Via. PREPARATION OF SCREENING POSITIVE CONTROLS

Urine screening mixed positive controls are prepared at 5 and 25 ng/mL using the combined LC Test mixture and negative control urine as follows:

Drug	µL Used	mL urine	Label Used	Label	Target Concentration
1-17	250	0.75	062904LC17QUATS	PC25	25 ng/mL
1-17	50	0.9	062904LC17QUATS	PC5	5 ng/mL

Vib. PREPARATION OF CALIBRATION STOCK SOLUTIONS

Using Propantheline as an example, secondary spiking stock solutions are prepared as follows:

Drug	µL Used	mL 50:50:1	Label Used	Label	Target Concentration
Propantheline	1000	9	062904FIAPropan	062904Propan1000	1000 ng/mL
Propantheline	1000	9	062904Propan1000	062904Propan100	100 ng/mL
Propantheline	1000	9	062904Propan100	062904Propan10	10 ng/mL

Vic. PREPARATION OF PLASMA AND URINE CALIBRATION STOCK SOLUTIONS

Confirmation calibrators for respective quaternary drugs detected by screening are prepared using the appropriate stock solutions described above (see section Vib) and appropriate negative control matrix (plasma or urine) previously determined to be negative for these compounds by this method.

Target Conc. ng/mL	uL added	Using	Used for: P=plasma U=urine
0.1	10	10 ng/mL	P
0.25	25	10 ng/mL	P
0.5	50	10 ng/mL	P
0.75	75	10 ng/mL	P
1	10	100 ng/mL	P,U
5	50	100 ng/mL	P,U
10	100	100 ng/mL	P,U
25	25	1000 ng/mL	U
50	50	1000 ng/mL	U
75	75	1000 ng/mL	U
100	100	1000 ng/mL	U

VII. SAMPLE REQUIREMENTS FOR ANALYSIS

- A. Mobile phase blank
 - 1. Designate MB1.....MBn
- B. Negative (control) sample (with and without internal standard)
 - 1. Designate plasma NC1 (no internal standard) and NC-IS.
 - 2. Prepare negative (Control) samples using negative (Control) plasma.
- C. Positive control samples
 - 1. Designate plasma or urine QC (or PC)1.....QC (or PC)n.
 - 2. Prepare positive control samples as described in Section VIII.
- D. Calibrators (confirmation only)
 - 1. Prepare a set of calibrators for analysis of plasma or urine samples.
 - 2. Calibrator concentrations as designated.
 - 3. Prepare plasma calibrators using negative (control) plasma from standard working solutions as described in Section VI.
- E. Test samples
 - 1. Designated to use the date on which the sample is analyzed and
*.raw data file designated to use sequence row number.

VIII. Sample Extraction by Liquid-Liquid Ion Pair Extraction

Safety Requirements: Lab coat, fume hood, eye protection

Materials needed: 0.1 M Sodium Heptane Sulfonate, 0.5 M Ammonium Acetate (pH 5.0), dichloromethane, 16x125 mm screw cap culture tubes and caps, 16x100 mm culture tubes, negative control urine, combined 100 ng/mL LC Test Mixture solution

17 Drug Screening Sample Extraction (no internal standard):

- 1. Transfer 1 mL urine, into labeled 16x125 mm screw cap culture tubes
- 2. Add 0.3 mL 0.1 M Sodium Heptane Sulfonate, 3 mL 0.5 M Ammonium Acetate (pH 5.0)
- 3. Add 5 mL dichloromethane
- 4. Include 1 negative control and 2 positive controls (5 & 25 ng/mL)
- 5. Cap and mix thoroughly (10 minutes roto-rack)
- 6. Centrifuge at 2,500 ~ 3,000 rpm for 5 minutes.
- 7. Aspirate to waste the top aqueous layer
- 8. Carefully transfer bottom dichloromethane layer to clean, labeled 16x100 mm culture tubes
- 9. Dry the extracts in a fume hood at approximately 65 °C under a stream of air.
- 10. Reconstitute the residues with 100 µL of 50:50:1 (MeCN:Water:Formic Acid).
Transfer the above solution into auto-sampler vials fitted with 200 µL limited volume inserts. All the samples are now ready for LC/MS/MS analysis.

Single Drug Confirmation Sample Extraction – Example: Propantheline (using Pipenzolate as internal standard, if Pipenzolate is suspected, then use Mepenolate):

Plasma:

1. Transfer 1 mL suspect sample plasma (in triplicate), and negative control plasma, into labeled 16x125 mm screw cap culture tubes [2 negative controls (NC & NC+IS) ,2 positive controls (0.5 & 5 ng/mL)in duplicate, and calibrators]
2. Add 100 uL internal standard to all tubes except NC
3. Add 0.3 mL 0.1 M Sodium Heptane Sulfonate, 3 mL 0.5 M Ammonium Acetate (pH 5.0)
4. Add 5 mL dichloromethane
5. Cap and mix thoroughly (10 minutes roto-rack)
6. Centrifuge at 2,500 ~ 3,000 rpm for 5 minutes.
7. Aspirate to waste the top aqueous layer
8. Carefully transfer bottom dichloromethane layer to clean, labeled 16x100 mm culture tubes
9. Dry the extracts in a fume hood at approximately 65 °C under a stream of air.
10. Remove test tubes from the drying block, place in a rack, and allow cooling to room temperature.
11. Reconstitute the residues with 100 µL of 50:50:1 (MeCN:Water:Formic Acid)
12. Transfer the above solution into auto-sampler vials fitted with 200 µL limited volume inserts. All the samples are now ready for LC/MS/MS analysis.

Urine:

1. Transfer 1 mL suspect urine sample (in triplicate) and negative control urine, into labeled 16x125 mm screw cap culture tubes [2 negative control (NC & NC+IS) and 2 positive controls (5 & 50 ng/mL) in duplicate, and calibrators]
2. Add 100 uL internal standard to all tubes except NC
3. Add 0.3 mL 0.1 M Sodium Heptane Sulfonate, 3 mL 0.5 M Ammonium Acetate (pH 5.0)
4. Add 5 mL dichloromethane
5. Cap and mix thoroughly (10 minutes roto-rack)
6. Centrifuge at 2,500 ~ 3,000 rpm for 5 minutes.
7. Aspirate to waste the top aqueous layer
8. Carefully transfer bottom dichloromethane layer to clean, labeled 16x100 mm culture tubes
9. Dry the extracts in a fume hood at approximately 65 °C under a stream of air.
10. Remove test tubes from the drying block, place in a rack, and allow cooling to room temperature.
11. Reconstitute the residues with 100 µL of 50:50:1 (MeCN:Water:Formic Acid)
12. Transfer the above solution into auto-sampler vials fitted with 200 µL limited volume inserts. All the samples are now ready for LC/MS/MS analysis.

IX. SEQUENCE ORDER FOR ANALYSIS

A. The sequence order for screening and confirmation is the same, except for unknown samples. Screening samples are sequentially ordered by track and sample, with blanks bracketing the individual track sets. Confirmation is independent, repeat preparation of all QC and calibrators, with target samples prepared in triplicate, and triplicates bracketed by blanks.

1. Blank
2. Column Test
3. Blank
4. Negative Control
5. Negative Control +Internal Standard (Blue entries not included in general screen)
6. QC1
7. QC2
8. QC3 (optional)
9. Blank
10. C1
11. C2
12. C3

13. C4
14. C5
15. C6
16. C7

17. Blank 18. Track A, Samples 1 thru N 19. Blank

20. Repeat 17 thru 19 as needed

21. Blank
22. QC1
23. QC2
24. QC3 (optional)
25. Blank
26. C1
27. C2
28. C3
29. C4
30. C5
31. C6
32. C7
33. Blank

Blank Track A, Sample X ₁ Track A, Sample X ₂ Track A, Sample X ₃ Blank
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Repeat as needed for the number of samples for confirmation

Screening analysis uses no waste injection, to allow for repeat analysis in case of power failure, sequence error, retention drift, or other unforeseen need for reanalysis. Confirmation uses partial loop injection due to lower sample throughput and better repeatability statistics at these concentrations.

X. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION AND CONFIRMATION OF QUATERNARY AMMONIUM DRUGS

A. Instrumentation

1. Thermo-Finnigan Deca XP Plus ion trap mass spectrometer with Xcalibur V1.3 for system control and data acquisition and processing
2. Thermo-Finnigan Surveyor quaternary HPLC pump, auto-sampler, column compartment and on-line degasser. No waste injection mode is used for the screening procedure and partial loop injection mode is used for the confirmation procedures.

B. HPLC conditions

1. Ace C18 Analytical Column, 3 x 50 mm, 5 micron particle size (Part # ACE – 121-0503, Mac-Mod Analytical, 127 Commons Court, PO Box 2600, Chadds Ford, PA 19317)
2. LC Guard Column
 - a) Type: Ace 3 C18 (Part No. ACE-111-0103GD, Mac-Mod Analytical, 127 Commons Court, PO Box 2600, Chadds Ford, PA 19317).
 - b) Dimension: 2.1 x 12.5 mm
 - c) Particle size: 5 micron
 - d) Temperature: ambient
3. Pre-Column Filter Column-Saver (Part # MMCCS210 – Mac-Mod Analytical, 127 Commons Court, PO Box 2600, Chadds Ford, PA 19317)
4. Mobile Phase
 - Mobile phase A: 2.3 mM Formic Acid (pH 5.0)
 - Mobile phase C: Acetonitrile 0.1% Formic Acid

	min	%A	%C	μL/min
0	0	40	60	20
1	0.2	40	60	20
2	0.3	40	60	200
3	0.5	40	60	200
4	3.5	100	0	200
5	4	100	0	200
6	4.01	40	60	400
7	4.82	40	60	400
8	4.83	40	60	20
9	5.5	40	60	20

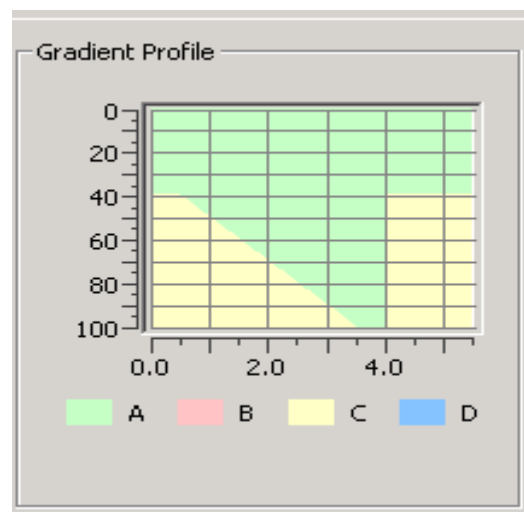
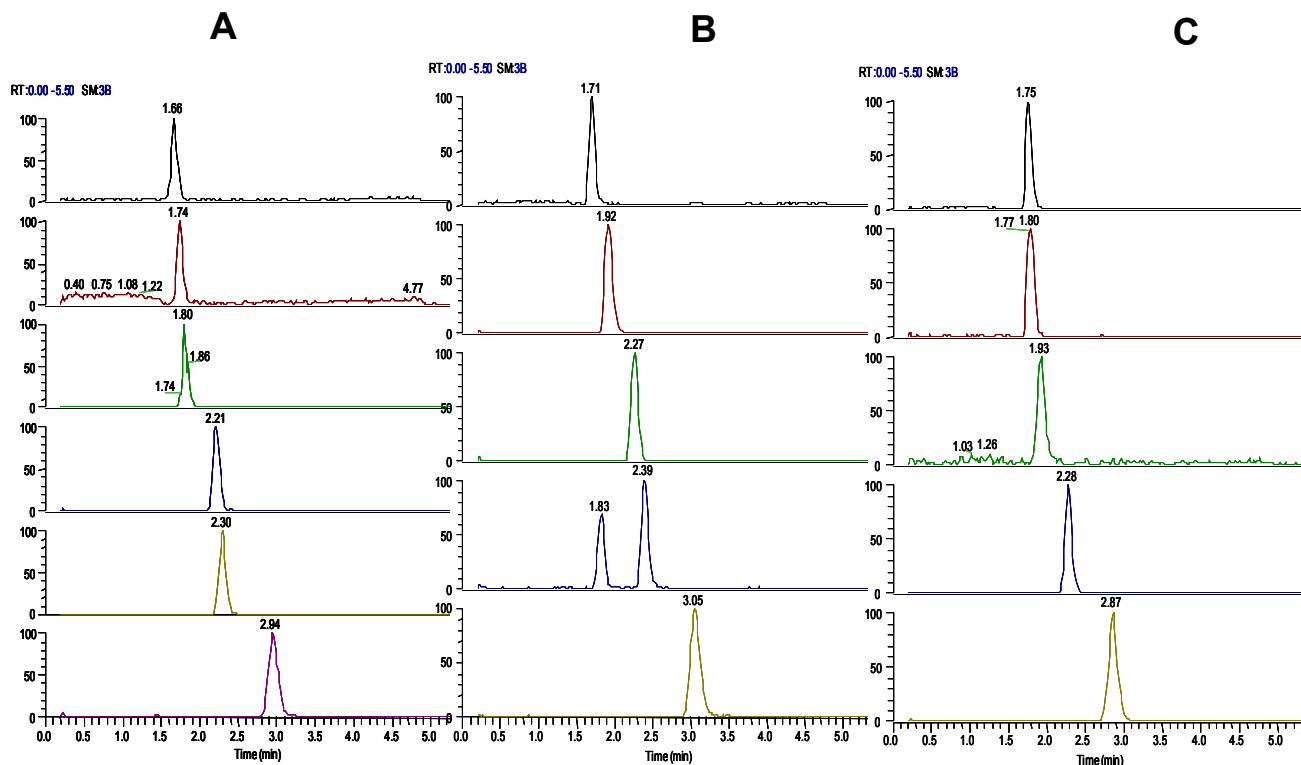


Figure 1. LC Gradient Table



Tubocurarine +2*
 Edrophonium
 Pancuronium +2*
 Mepenzolate
 Pipenzolate
 Oxyphenonium

Pyridostigmine
 Ipratropium
 Clidinium
 Glycopyrronium & MethScopolamine
 Propantheline

Neostigmine
 Methylhomatropine
 Bretylium
 Isopropamide
 Anisotropine

Figure 2. Retention order for MSMS screens for Groups A, B, and C

C. Discussion

The 17 drugs can be screened in urine using the ion trap operating in MS data dependant mode and the liquid chromatographic conditions described above. However, it was noticed that the lower limit of detection was compromised on several precursor ion channels due to high baseline, failure to trigger the dependent MSMS scans, or both. Data inspection of large sample sets was also a formidable task. Sensitivity below 100 pg/mL was achieved using separate MSMS scan methods run sequentially (above). This simplifies data inspection using Excalibur Qual Browser as well as providing a more reliable integration report based on product ions using Excalibur Quan Browser. Administration data for urine clearance of Glycopyrronium, Ipratropium, and Propantheline (see Appendix) suggest that 100 pg/mL (urine) is an order of magnitude lower than would be necessary to detect race day administration of these drugs in urine. Therefore, the MS data dependent conditions will also be described.

D. Mass Spectrometer Conditions

MS Data Dependent Screen

Segment 1 Information

Duration (min): 5.50
 Number of Scan Events: 2
 Tune Method: Prom091203

Scan Event Details:

1: + c norm (60.0-570.0)
 2: + c norm Dep MS/MS Most intense ion from (6).

Data Dependent Settings:

Parent Mass List: 166.00 181.00 223.00 242.00 282.00
 286.00 290.00 305.00 318.00 332.00
 340.00 348.00 352.00 353.00 354.00 368.00

Reject Mass List: 279.00 324.00 391.00
 Default Charge State: 2
 Default Isolation Width: 1.3
 Normalized Collision Energy: 35.0
 Activation Q: 0.250
 Activation Time: 30.000
 Min. Signal Required: 100000
 Min. MSn Signal Required: 5000

MSMS Screen and Confirmation Table

DRUG	PRECURSOR	COLLISION %	~ RT	SCREEN GROUP	SCAN RANGE
Tubocurarine +2*	305	32	1.51	A	80-600
Edrophonium	166	29	1.63	A	50-180
Pancuronium +2*	286	22	1.71	A	75-490
Mepenzolate	340	33	2.04	A	90-352
Pipenzolate	354	33	2.11	A	95-370
Oxyphenonium	348	35	2.67	A	85-360
Pyridostigmine	181	32	1.56	B	50-250
Ipratropium	332	33	1.79	B	90-342
Clidinium	352	33	2.08	B	95-362
Glycopyrronium & MethScopolamine	318	31	2.19	B	85-330
Propantheline	368	29	2.76	B	100-400
Neostigmine	223	38	1.63	C	60-236
Methylhomatropine	290	31	1.66	C	75-306
Bretylium	242	29	1.8	C	65-260
Isopropamide	353	25	2.08	C	95-362
Anisotropine	282	31	2.55	C	75-300

Figure 3. The conditions in the table above form the basis for the MSMS screen conditions as well as for confirmation and determination of screening suspects

MSMS Screen Group A

Segment 1 Information

Duration (min): 5.50
Number of Scan Events: 6
Tune Method: Prom091203

Scan Event Details:

- 1: + c norm ·(305.1)->o(80.0-600.0)
MS/MS: Amp. 32.0% Q 0.250 Time 30.000 IsoW 1.3
- 2: + c norm ·(166.1)->o(50.0-180.0)
MS/MS: Amp. 29.0% Q 0.250 Time 30.000 IsoW 1.3
- 3: + c norm ·(286.1)->o(75.0-490.0)
MS/MS: Amp. 22.0% Q 0.250 Time 30.000 IsoW 1.3
- 4: + c norm ·(340.2)->o(90.0-352.0)
MS/MS: Amp. 33.0% Q 0.250 Time 30.000 IsoW 1.3
- 5: + c norm ·(354.2)->o(95.0-370.0)
MS/MS: Amp. 33.0% Q 0.250 Time 30.000 IsoW 1.3
- 6: + c norm ·(348.2)->o(85.0-360.0)
MS/MS: Amp. 35.0% Q 0.250 Time 30.000 IsoW 1.3

MSMS Screen Group B

Segment 1 Information

Duration (min): 5.50
Number of Scan Events: 5
Tune Method: Prom091203

Scan Event Details:

- 1: + c norm ·(181.1)->o(50.0-250.0)
MS/MS: Amp. 32.0% Q 0.250 Time 30.000 IsoW 1.3
- 2: + c norm ·(332.2)->o(90.0-342.0)
MS/MS: Amp. 33.0% Q 0.250 Time 30.000 IsoW 1.3
- 3: + c norm ·(352.2)->o(95.0-362.0)
MS/MS: Amp. 33.0% Q 0.250 Time 30.000 IsoW 1.3
- 4: + c norm ·(318.2)->o(85.0-330.0)
MS/MS: Amp. 31.0% Q 0.250 Time 30.000 IsoW 1.3
- 5: + c norm ·(368.2)->o(100.0-400.0)
MS/MS: Amp. 29.0% Q 0.250 Time 30.000 IsoW 1.3

MSMS Screen Group C

Segment 1 Information

Duration (min): 5.50
Number of Scan Events: 5
Tune Method: Prom091203

Scan Event Details:

- 1: + c norm ·(223.1)->o(60.0-236.0)
MS/MS: Amp. 38.0% Q 0.250 Time 30.000 IsoW 1.3
- 2: + c norm ·(290.1)->o(75.0-306.0)
MS/MS: Amp. 31.0% Q 0.250 Time 30.000 IsoW 1.3
- 3: + c norm ·(242.1)->o(65.0-260.0)
MS/MS: Amp. 29.0% Q 0.250 Time 30.000 IsoW 1.3
- 4: + c norm ·(353.2)->o(95.0-362.0)
MS/MS: Amp. 25.0% Q 0.250 Time 30.000 IsoW 1.3
- 5: + c norm ·(282.2)->o(75.0-300.0)
MS/MS: Amp. 31.0% Q 0.250 Time 30.000 IsoW 1.3

MSMS Confirmation Example: Propantheline with internal standard

Segment 1 Information

Duration (min): 5.50

Number of Scan Events: 2

Tune Method: Prom091203

Scan Event Details:

1: + c norm ·(368.3)->o(100.0-375.0)

MS/MS: Amp. 29.0% Q 0.250 Time 30.000 IsoW 1.3

2: + c norm ·(354.2)->o(95.0-370.0)

MS/MS: Amp. 33.0% Q 0.250 Time 30.000 IsoW 1.3

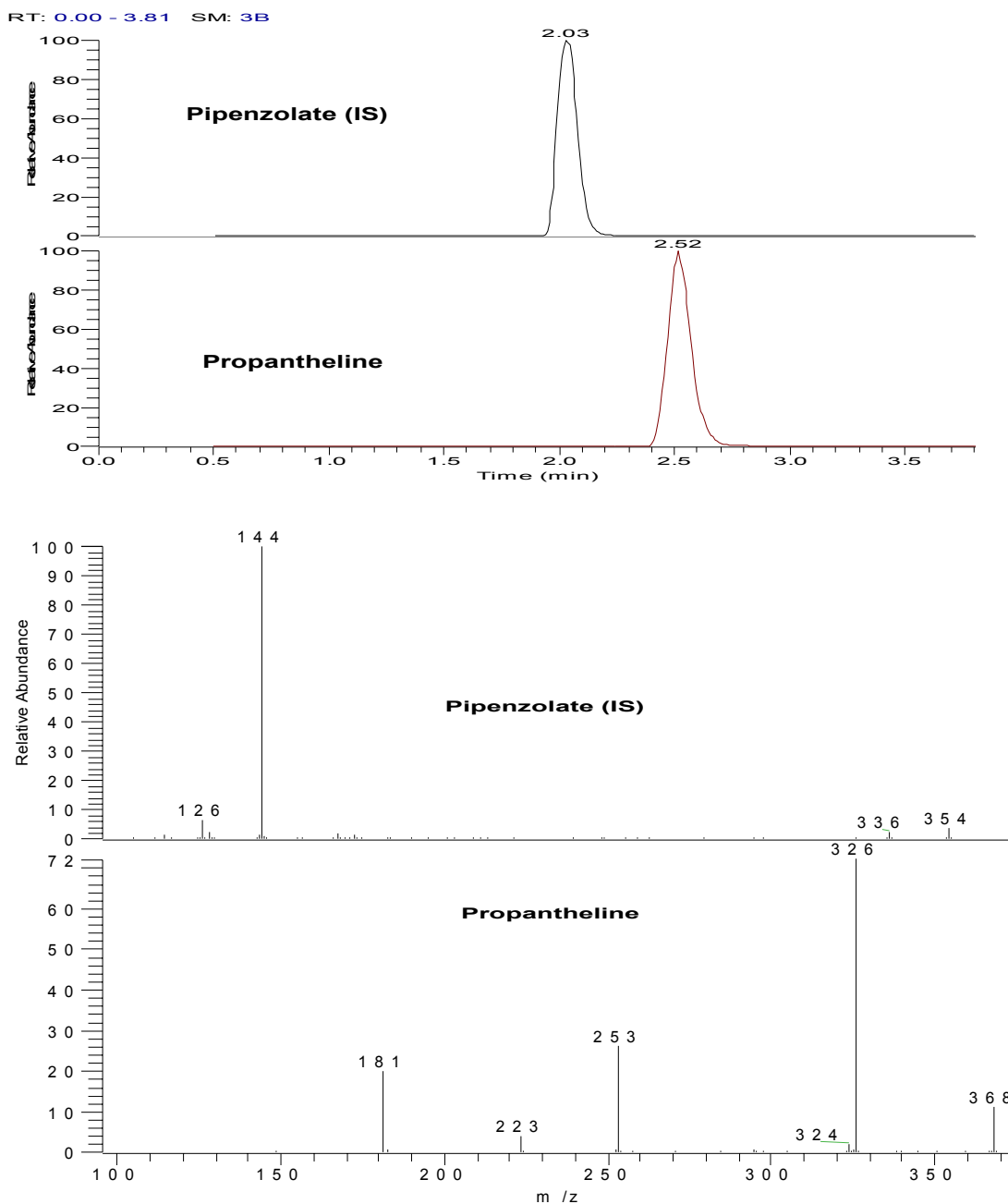


Figure 4. LC Retention and MS2 Spectra for Pipenzolate (internal standard) and Propantheline

XIa. METHOD STATISTICS – PROPANTHELINE LINEARITY

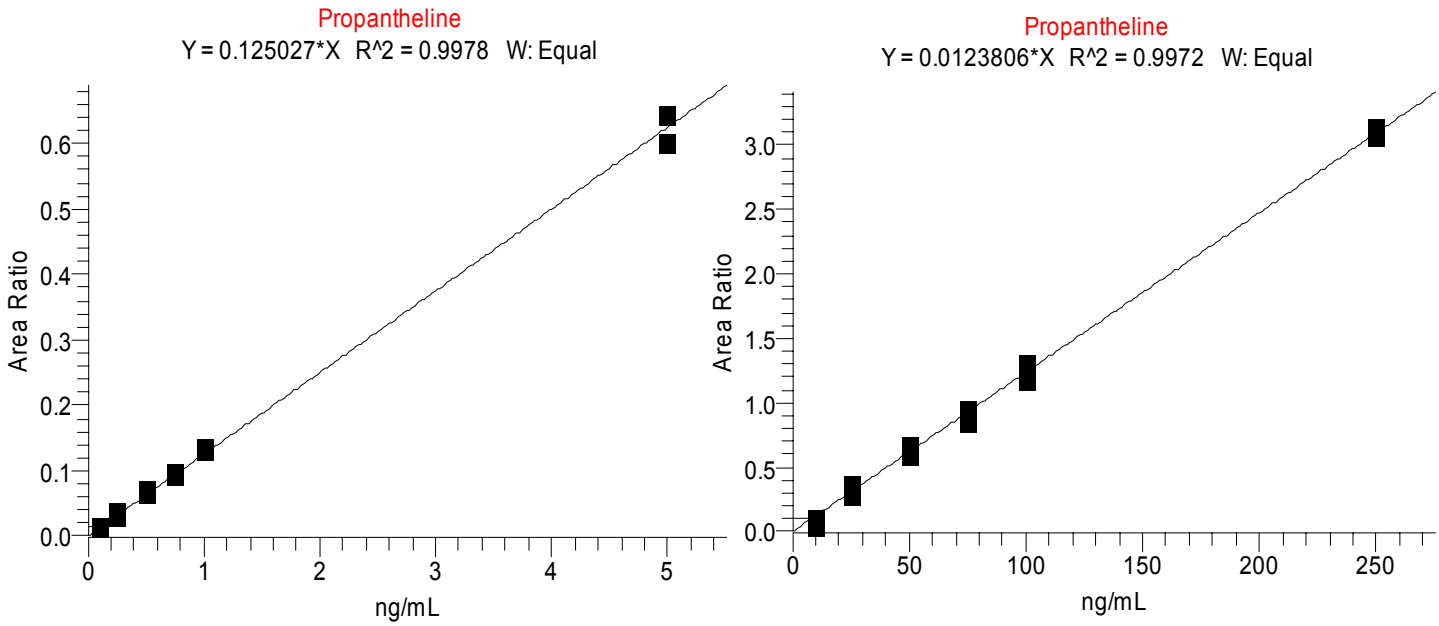


Figure 5. Linearity of pre-run and post-run plasma calibrators (left) and urine calibrators (right)

Propantheline: Urine Clearance – 50 MG IV

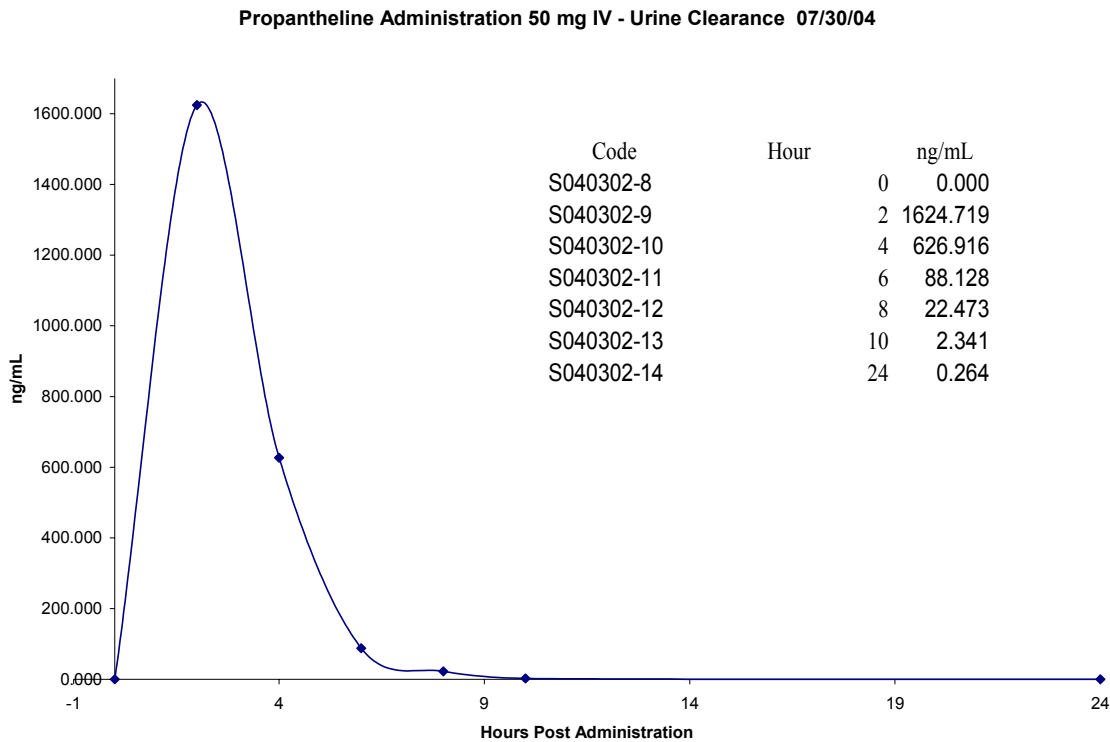


Figure 6. Propantheline: Urine Clearance Curve. 24-hour urine sample is confirmable by MS2 spectral matching.

Propantheline: Plasma Clearance – 50 MG IV

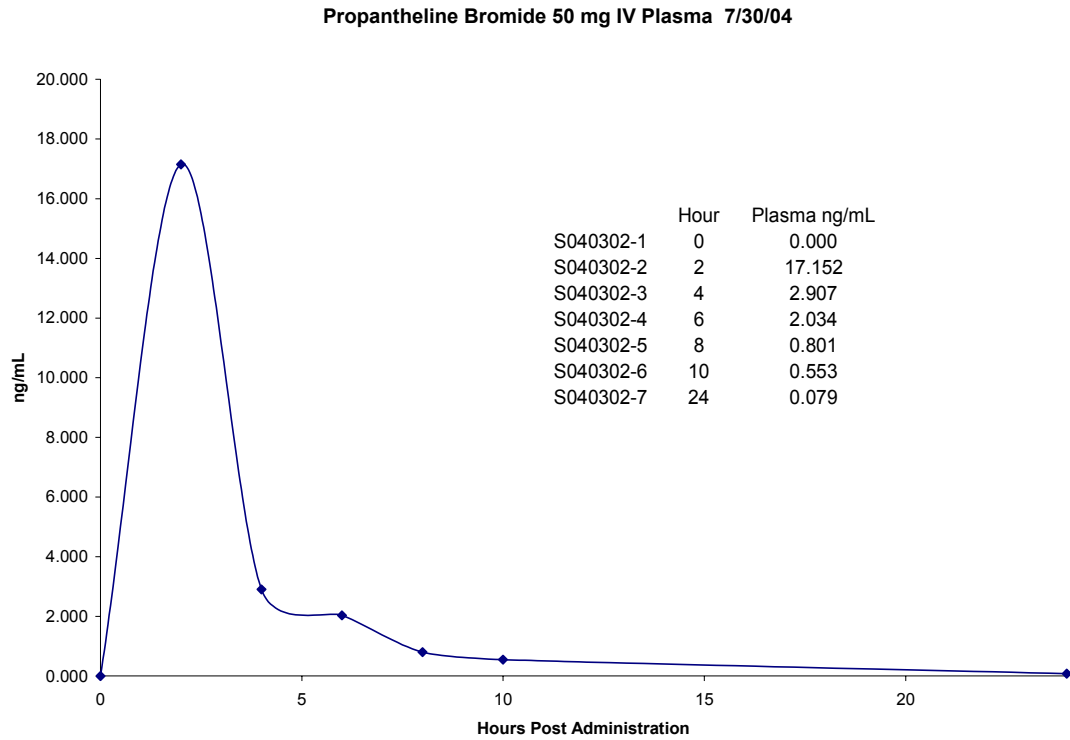


Figure 7. Propantheline: Plasma Clearance curve. 24-hour plasma sample is confirmable by MS2 spectral matching.

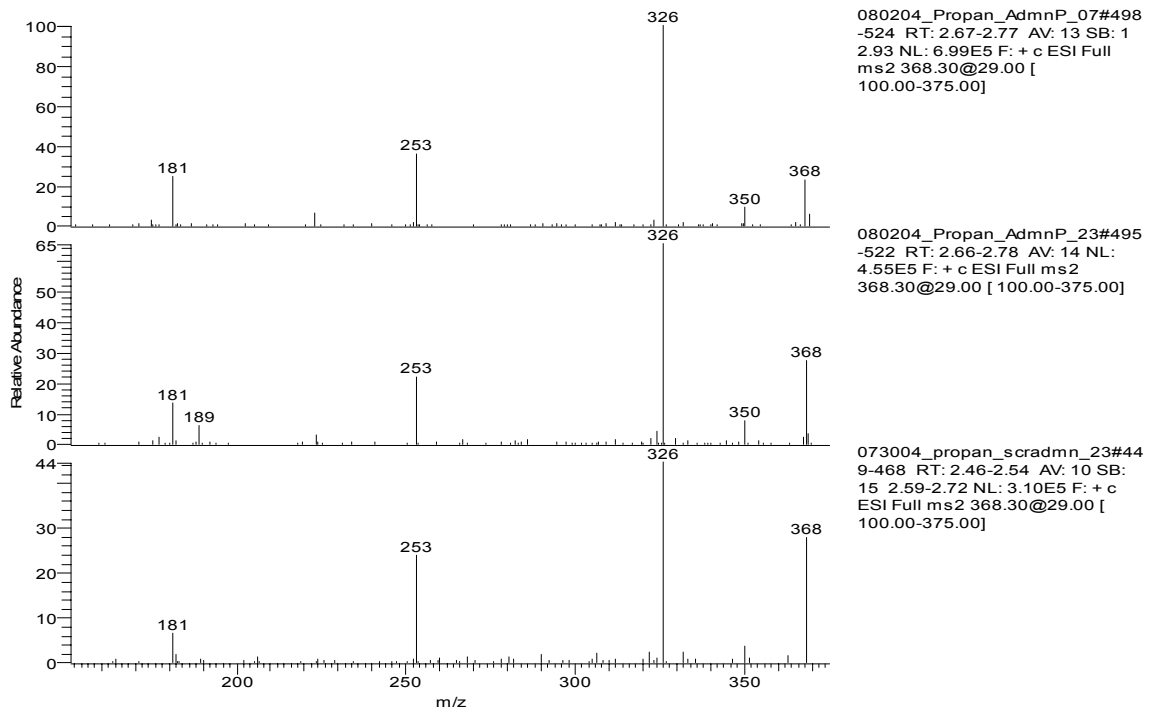


Figure 8. MS2 Propantheline spectra of 100 pg/mL calibrator, 24-hour plasma (79 pg/ml), and 24-hour urine (264 pg/mL).

Propantheline: Urine Precision and Accuracy

Propantheline Urine Pre-run and Post-run Calibrators

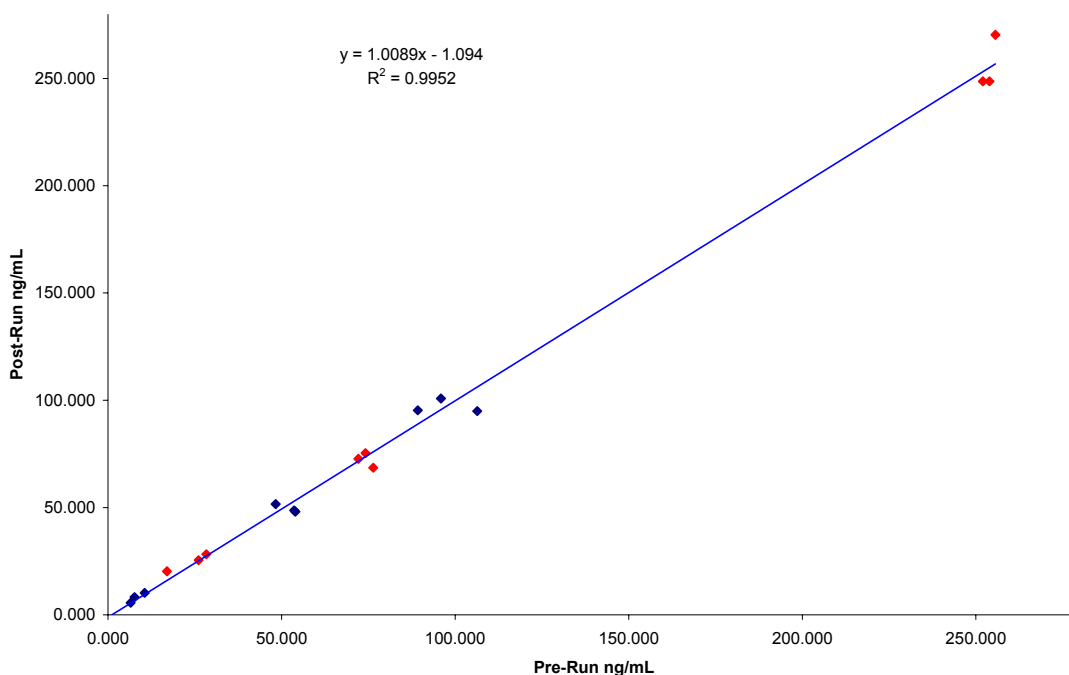


Figure 9. Youden plot of 3 independent analyses of pre-run and post-run urine calibrators bracketing official plasma confirmation samples, indicating combined within-run and between-run dispersion of results. Within-run and between-run accuracy and precision of Propantheline Urine Calibrators below.

Within Run Accuracy and Precision (Urine)

	AR%	CV%
10	80-103	3.0-12
25	84-113	0.3-12
50	99-103	4.6-8.1
75	97-100	0.5-7.6
100	93-101	3.6-7.8
250	100-105	0.9-3.9

Between Run Accuracy and Precision (Urine)

	AR%	CV%
10	81.387	24.376
25	96.929	18.926
50	101.412	5.341
75	97.613	3.790
100	98.770	4.446
250	101.939	3.167

MSMS SENSITIVITY (for Propantheline)

Instrument Lower Limit of Detection (LOD_i) less than 10 pg injected

Method Lower Limit of Detection (LOD_m) less than 0.1 ng/mL (< 10 pg injected)

Lower Limit of Quantitation (LLOQ) less than 0.1 ng/mL (@ 25% CV)

Lower Limit of Confirmation (LOC) less than 0.1 ng/mL

XIb. METHOD STATISTICS – CRITERION

This SOP is intended strictly for screening and confirmation purposes, but quantitative considerations are included to characterize method performance, deterioration of system responsiveness, and as value added estimates for positive test results. The positive threshold for this SOP is the limit of confirmation.

Criteria for Identification of Propantheline from Equine Plasma or Urine

Identification of Propantheline

The diagnostic ions for Propantheline are 181, 253, 326, and 368 m/z and the retention time for the suspect sample, calibrator, and QC control must agree to +/- 0.15 minutes.

Confirmation of Propantheline

All diagnostic ions for Furosemide are present in the full scan MSMS spectrum of the suspect sample, +/- 25% relative, of that ion compared with Propantheline calibrator and control spectra, with no interfering ions > than 20%. Spectra may be averaged and/or subtracted.

XII. SEQUENCE OF POSITIVE SAMPLE DATA PACKET

- A. SAMPLE TRANSFER SHEET (WS#32)
- B. SAMPLE USAGE SHEET (FORM #7)
- C. SAMPLE LIST
- D. LC METHOD
- E. MS METHOD
- F. EXTRACTED ION CHROMATOGRAM COMPARISON
- G. SPECTRA COMPARISON
- H. CONFIDENCE REPORT
- I. QUANTIFICATION REPORT
- J. QUANTIFICATION CALIBRATION CURVE
- K. COLUMN TEST CHROMATOGRAM AND SPECTRA

Other Required Documentation

In addition to the positive data packet, the following documentation is required:

Sequence Sample list prints are maintained in the Deca XP three ring binder.

Instrument usage logbook completion (and maintenance log if needed), indicating date and project.

Data packets for samples determined to be negative will contain the following elements:

- a. Sample Transfer Sheet (WS # 32)
- b. Sample Usage Sheet (Form #7)
- c. Confidence Determination Report
- d. Quantification Report

XV. REAGENTS

- A. Methanol, Optima grade (Cat. No. A 454-4, Fisher Scientific.)
- B. Acetonitrile, Optima grade (Cat. No. A 996-4, Fisher Scientific.)
- C. Water, Optima grade (Cat. No. W7-4, Fisher Scientific.)
- D. Formic Acid, SupraPur (Cat. No. 11670-1, EM Science)
- E. Ammonium hydroxide 28% (Cat. No. AX1303-3, EM Science)
- F. Water, HPLC grade (Cat. No. 4218-03, J.T. Baker)
- G. Dichloromethane, HPLC Grade (Cat. No. D143-4, Fisher Scientific)
- H. Sodium Heptane Sulfonate (Cat. No. O-3013, Fisher Scientific)
- I. Glacial Acetic Acid (Cat. No. A38^c-212, Fisher Scientific)

XVI. FORMULAE

Safety Requirements: Lab coat, fume hood, gloves, safety glasses. CAUTION: Strong alkali solutions generate heat upon mixing.

- A. 2.3 mM Formic Acid: (Using D. and C. above), add 400 μ L Formic Acid (D.) to 1000 mL of Water (C.). Mix thoroughly. Adjust pH to 5.0 using ammonium hydroxide (E.). Check pH. Cap, label, and record pH.
- B. 1:1 Ammonium Hydroxide: Water: (Using E. and F. above), add 500 mL Ammonium Hydroxide (E.) to 500 mL HPLC grade water (F.). Mix thoroughly, Cap, label.
- C. 50:50:1: (Using B, C, and D above), add 10 mL Formic Acid (D.) to 500 mL Water (C.) and Acetonitrile (B.).
- D. 0.1 % Formic Acid: Add 1 mL formic acid (D.) to 1000 mL water (F.)
- E. 0.1 M aqueous Sodium Heptane Sulfonate: Add 2.2 gm (H.) to 100 mL HPLC water (F.)
- F. 0.5 M Ammonium Acetate (pH 5.0): Add 29 mL Acetic Acid (I.) to 900 mL HPLC water (F.). Adjust pH to 5.0 with ammonium hydroxide (Formula D.), then bring to final volume with HPLC water (F.)

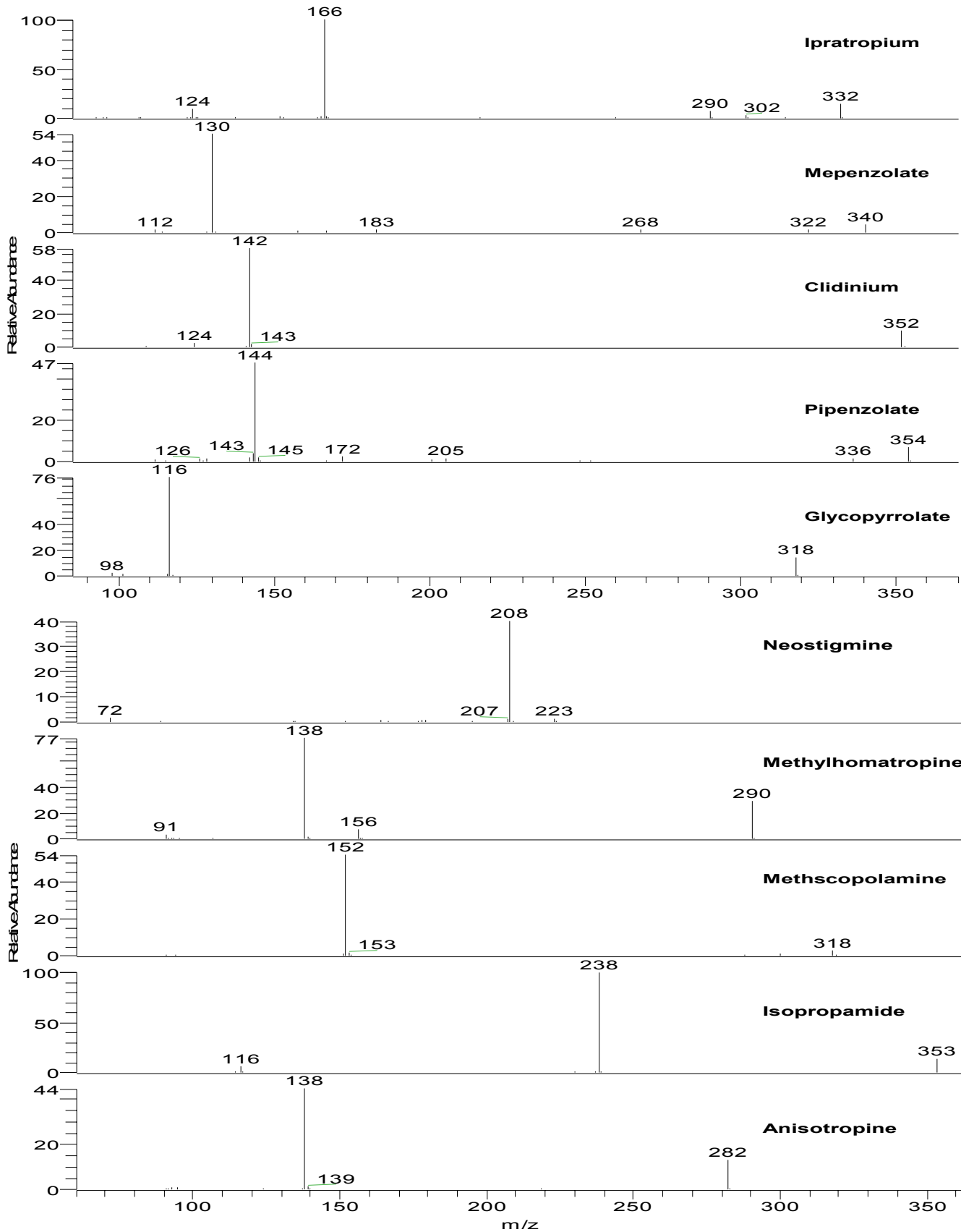
XVII. MATERIALS

- A. 16 × 100 mm test tubes.
- B. 16 × 125 mm screw-top test tubes.
- C. 16 × 150 mm screw-top test tubes
- D. Polypropylene Caps
- E. Test tube rack
- F. Pipettes and tips.
- G. Rotorack
- H. Centrifuge
- I. Vortex mixer (Scientific Industries, Inc.)
- J. Branson Ultrasonic Water Bath, 8510 (Fisher Scientific or equivalent)
- K. pH meter (IQ Scientific Instruments)
- L. Sample Concentrator (Dri-Block DB-3, Techne)
- M. IEC HN-SII Centrifuge (International Equipment Company)
- N. Rotorack (Speci-Mix, Thermolyne)
- O. 2 mL autosampler vials
- P. 200 uL Insert (Target PP Polyspring, National Scientific Company)

XIX. References and Additional Information:

1. R. Sams, ARCI Informational Set #4 (April 1991), **Glycopyrrolate Ion-Pair Extraction**
2. Jeffrey Rudy (Method Author): TIP SOP; **Ipratropium: Screening, Quantification and Confirmation by LC/MS/MS, November 2000.**
3. Wan, Tang, Leung, 13th International Conference of Racing Analysts and Veterinarians, August 2000; **Analysis of Quaternary Ammonium Drugs by CE/MS**
4. Wan, Yiu, Ho, Chromatographia Supp 59, 2004, 545-550, **Detection of Quaternary Ammonium Drugs in Equine Urine by LC-MS**

SPECTRA OF QUATERNARY AMMONIUM DRUGS



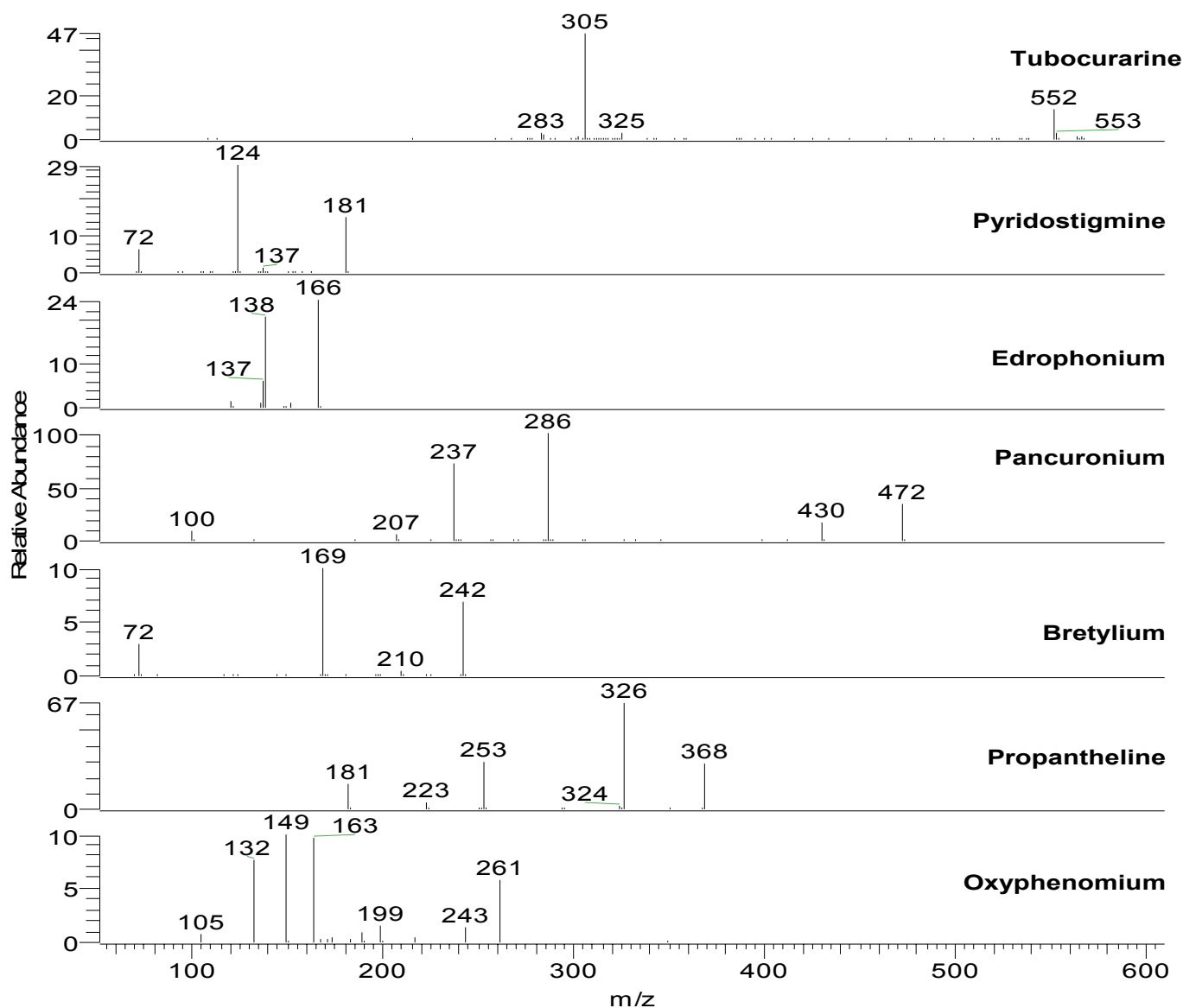


Figure 10. MSMS Spectra of 17 Quaternary Ammonium Drugs. Note that Tubocurarine and Pancuronium utilize the $m/+2z$ half mass as the precursor ion, and that loss of one charge yields singly charged product ions greater than the doubly charged precursor ion (see Table # 5, page 5.).

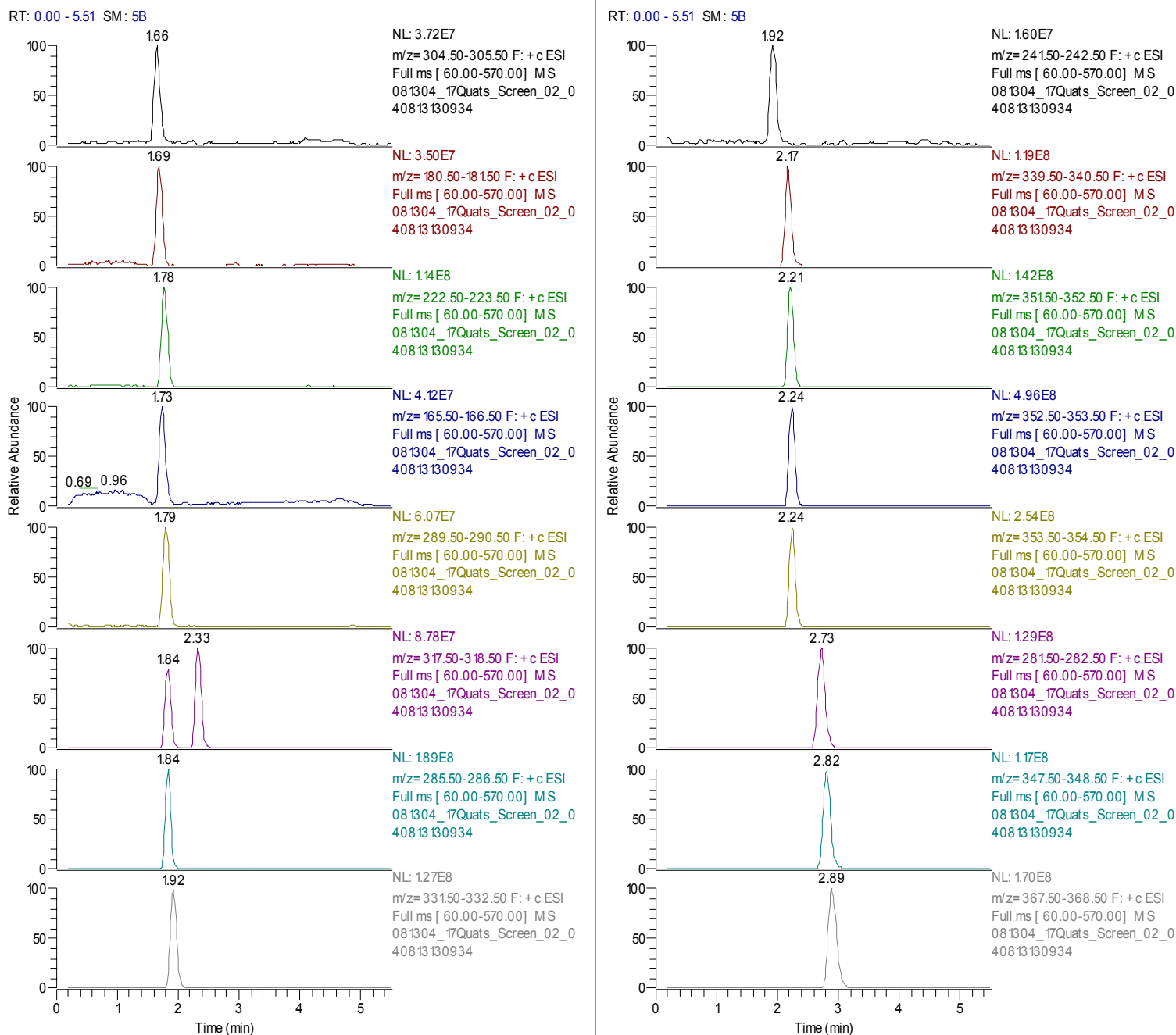


Figure 11. MS chromatogram of 17 Quaternary Ammonium Drugs column test mix. Note that Tubocurarine and Pancuronium utilize the $m/z+2z$ half mass as the detection ion. This is the Qual Browser layout (2 vertical cells) for evaluating MS data dependent screen runs.