

SCREENING AND CONFIRMATION OF 2-1(HYDROXYETHYL) PROMAZINE SULFOXIDE BY ION TRAP LC/MS/MS

I. INTRODUCTION

Phenothiazine-derived tranquilizers have been widely used in veterinary medicine since the 1950's. As a class, the phenothiazine derivatives induce tranquilization and block conditioned responses; but unlike other tranquilizers, the depressant action on the central nervous system is mostly sub cortical. These drugs are used in horses to relieve apprehension prior to general anesthesia and as chemical restraint for ease of shipping and handling. Low dose pre-race administration is rumored to occur in order to prevent nervous expenditures of energy prior to racing. Since there is little effect on coordinated motor responses, energy is conserved for actual competition, without nervous exhaustion prior to racing^{5,6}.

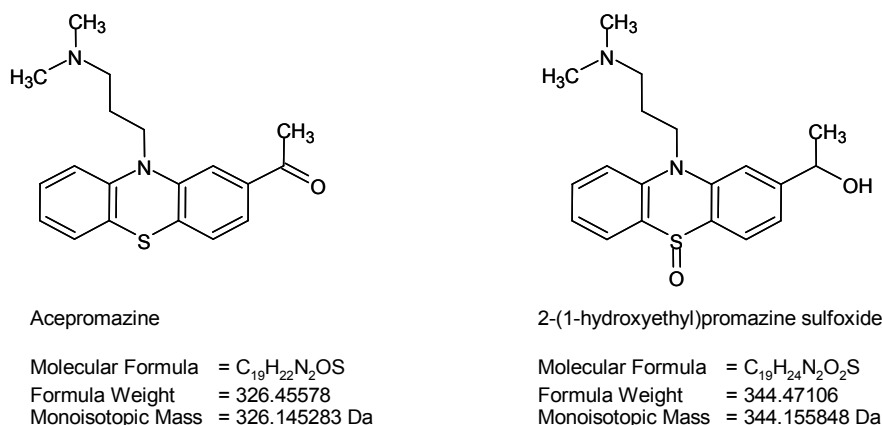


Figure 1. Chemical structures of Acepromazine and its major equine metabolite, 2-1(hydroxyethyl) promazine sulfoxide.

II. SCOPE

This standard operating procedure (SOP) describes identification and confirmation procedures for the presence of 2-1(hydroxyethyl) promazine sulfoxide (**HEPS**) in plasma and/or urine using the Deca XP Plus Ion Trap LC/MS/MS (Thermo-Finnigan). Additional information will be provided for Acepromazine (**ACE**), and for all metabolites detectable by this method. The relative abundances of these analytes may be affected by drug formulation, route of administration, individual animal differences, age and condition of samples, and/or method chemistry. Enzyme Linked Immuno-Sorbent Assay (ELISA) presumptive findings using the Neogen Promazine ELISA may not contain Acepromazine or its metabolites. This test has a wide range of cross reactivity, and interpretation of results obtained by promazine ELISA test kit should be correlated to other ELISA test kits (i.e. Tricyclics). Additionally, concentrations predicted by ELISA testing may be high relative to any single analyte detected in urine or plasma.

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IIB. BACKGROUND

The Racing Commissioners International (RCI) Uniform Classification Guidelines for Foreign Substances designate Acepromazine as a class three drug². The Thoroughbred Owners and Breeders Association (TOBA) Graded Stakes Medication Guidelines lists the minimum urinary concentrations of the presumed major respective metabolite to be 20 ng/mL³. The National Thoroughbred Racing Association (NTRA) Task Force Guidelines indicate 3 jurisdictions use a 25 ng/mL urine metabolite threshold, 1 jurisdiction uses a 10 ng/mL threshold, 1 jurisdiction uses a 2 ng/mL threshold and 22 jurisdictions use no threshold urinary limit^{2,4}.

ELISA screening and LCMS(MS) confirmation and quantification sensitivities permit detection and reporting of urinary concentrations, which could exceed a 72-hour window prior to racing^{2,3,4}. Potentially trivial urinary findings can be further defined by monitoring plasma and urine concentrations of all detectable analytes. Additionally, plasma screening by this standard operating procedure would detect possible attempts to circumvent post-race urine testing by administration of Acepromazine just prior (within 2 hours) to racing.

III. PRIMARY DRUG AND INTERNAL STANDARD REFERENCE MATERIALS

Source of analytical reference and internal standard materials:

Compound	Source	Cat #	Reference Label
2-(1-hydroxyethyl)promazine sulfoxide	NEOGEN Corporation	# 110230	R-21OHETHYLPROMS-1
Acepromazine	Sigma	# A-6908	S-Ace-1
d3-Codeine	Alltech	# 1913	R-CodD3-1

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

IV. PREPARATION OF PRIMARY REFERENCE STOCK SOLUTIONS

- 2-(1-hydroxyethyl) promazine sulfoxide is commercially available as 200 ug/mL in methanol by NeoGen Corporation, Catalog Number 110230. (phone: 1-800-447-8201).
- Acepromazine is supplied as the maleate salt. Weigh between 5 and 10 mg (X.xx mg) into a glass bottle and label. Dilute to volume using HPLC grade (or better) methanol (Volume Y.yy mL = X.xx mg/Z.ZZ where Z.ZZ is the Salt/Free ratio if applicable). Cap and mix until compound is completely dissolved in methanol.
- d3-Codeine is supplied as 100 ug/mL in methanol by Alltech, (1-800-437-3784), Catalog Number 1913.

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

Materials Needed: Primary Stock Solution A & B (IV. A, B above), 50:50:1 (MeCN:Water:Formic Acid)
 10 ug/mL FIA solutions were prepared as follows:

Drug	Using	Volume	mL 50:50:1	Label
2-(1-Hydroxyethyl)Promazine Sulfoxide	R-21OHETHPROS-1	200 uL	3.8	21OHEPSFIA121703
Acepromazine	S-ACE-1	100 uL	9.9	ACEFIA121703

From these, 100 ng/mL LC solutions were prepared as follows:

Drug	Using	Volume	mL 0.1% FA	Label
2-(1-Hydroxyethyl)Promazine Sulfoxide	21OHEPSFIA121703	100 uL	9.9	21OHEPSLC121703
Acepromazine	ACEFIA121703	100 uL	9.9	ACELC121703

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Preparation of d3-Codeine Internal Standard Working Stock Dilution

Materials Needed: Primary Stock C

Add 100 uL R-CodD3-1 to 9.9 mL 0.1% Formic Acid to yield 1000 ng/mL and Label with **MMDDYYd3COD1000**.

Via. PREPARATION OF CALIBRATION STOCK SOLUTIONS

Working Calibrator Solutions are prepared according to the following table:

Using the ACE and HEPS FIA solutions, the following calibrators were prepared as described here:

CAL#	Concentration ng/mL	Concentration Used For:	mL of Cal (Both ACE and HEPS)	Using Cal	mL 50:50:1	Label	Matrix Used for: (U or P)
2	5,000	500	1 (2 tot)	10ug/mL	0	040604ACE_HEPSCAL5K	U
3	2,500	250	0.5 (1 tot)	10ug/mL	1	040604ACE_HEPSCAL2.5K	U
4	1,000	100	0.8	2	3.2	040604ACE_HEPSCAL1K	U
5	500	50	0.4	2	3.6	040604ACE_HEPSCAL500	U
6	250	25	0.2	2	3.8	040604ACE_HEPSCAL250	U
7	100	10	0.4	4	3.6	040604ACE_HEPSCAL100	P, U
8	50	5	0.2	4	3.8	040604ACE_HEPSCAL50	P, U
9	10	1	0.4	7	3.6	040604ACE_HEPSCAL10	P, U
10	7.5	0.75	0.3	7	3.7	040604ACE_HEPSCAL7.5	P
11	5.0	0.5	0.2	7	3.8	040604ACE_HEPSCAL5	P
12	2.5	0.25	0.1	7	3.9	040604ACE_HEPSCAL2.5	P
13	1.0	0.1	0.4	9	3.6	040604ACE_HEPSCAL1	P

100 uL of CAL# 1-9(above) are added to 0.9 mL Negative Control Urine. 100 uL of these spiked urines are ready for analysis with 100 uL aliquots of unknown urine sample. D3 Codeine is used as internal standard (see below)

100 uL of CAL# 7-13(above) are added to 0.9 mL Negative Control plasma. D3 Codeine is used as internal standard (see below)
 Using 031703d3Cod10, 100 ng/mL solution was prepared

CAL#	Concentration ng/mL	Concentration Used For:	mL of Stock	Using Cal#	mL 0.1% FA	Label
14	100	10	1	031703d3Cod1000	9	100903d3Cod100

Vib. PREPARATION OF URINE AND PLASMA CALIBRATORS

Urine analysis utilizes 100 uL of sample.

A 100 uL aliquot of CAL# 1 thru 9 is mixed with 0.9 mL urine in respectively labeled tubes. A 100 uL aliquot of these urine calibrators is then transferred into appropriately labeled 16 x 125 screw top culture tubes as calibrators and controls. Prepare duplicate 10 and 250 ng/mL QC controls for urine analysis.

Plasma analysis utilizes 1 mL of sample.

A 100 uL aliquot of CAL# 7 thru 13 is mixed with 0.9 mL of plasma in respectively labeled tubes. Prepare duplicate 0.5 and 5 ng/mL QC controls for plasma analysis.

100 uL of CAL# 14 (Internal Standard) is added to each tube except NC1 (negative control).

VII. SAMPLE REQUIREMENTS FOR ANALYSIS

A. Mobile phase blank

1. Designate Solvent Blank₁...Solvent Blank_n.

B. Negative (control) sample

1. Designate plasma or urine NC or plasma or urine blank.
2. Prepare negative (Control) sample from negative (Control) plasma or urine.

C. Positive control samples

1. Designate plasma or urine QC (or PC)₁.....QC (or PC)_n.
2. Prepare positive control samples as described Section VIb.

D. Calibrators

1. Prepare plasma urine calibrators using negative (control) plasma or urine from standard working solutions as described in Section VIb.

E. Test samples

1. Designated to use the date of which the sample is analyzed and
*.raw data file designated to use sequence row number.

VIIIa. Plasma: Liquid-Liquid Extraction

Safety Requirements: Lab coat, fume hood, eye protection

1. Add 1 mL plasma (controls, calibrators, and samples) into labeled 16 x 125 mm screw cap culture tubes.
2. Add 1 mL 1:1 ammonium hydroxide: water.
3. Add 100 uL spiking dilutions to respective calibrator and QC tubes.
4. Add 100 uL internal standard (**d3-Codeine**) to all tubes except NC1 (negative control - no internal standard).
5. Gently vortex all tubes.
6. Add 5 mL of 5:1 dichloromethane: chlorobutane into each tube, cap all screw-top tubes tightly and rotorack for 5 minutes.
7. Centrifuge at 2,500 ~ 3,000 rpm (839 ~ 1,409 g) for 5 minutes.
8. Decant the (top) organic layer into a labeled fresh test tube for each sample.
9. Bring the extracts in test tubes to dryness in a fume hood at approximately 40 °C under a steady stream of nitrogen.
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 0.1% formic acid (aqueous) for plasma analysis.
12. Transfer the above solution into a 200 µL insert and load in the auto sampler vials. All the samples are now ready for LC/MS/MS analysis.

VIIIb. Urine: Liquid-Liquid Extraction

Safety Requirements: Lab coat, fume hood, eye protection

1. Add 0.9 mL negative control urine into labeled 16 x 125 mm screw cap culture tubes for calibrators and controls. (Enzyme Hydrolysis is not required for HEPS in urine, although hydroxyl metabolites will be better detected.).
2. Add 100 μ L spiking dilutions to respective calibrator and QC tubes and gently mix.
3. Transfer 100 μ L of the calibrators and controls into respectively labeled 16 x 125 mm screw cap culture tubes
4. Transfer 100 μ L unknown urine to appropriately labeled tubes.
5. Add 4 mL pH 10 saturated borate buffer to all tubes.
6. Add 100 μ L internal standard (**d3-Codeine**) to all tubes except NC1 (negative control no internal standard).
7. Gently vortex all tubes.
8. Add 5 mL of 5:1 dichloromethane: chlorobutane into each tube, cap all screw-top tubes tightly and rotorack for 5 minutes.
9. Centrifuge at 2,500 ~ 3,000 rpm (839 ~ 1,409 g) for 5 minutes.
10. Decant the (top) organic layer into a labeled fresh 16 x 100 culture tube for each sample.
11. Bring the extracts in test tubes to dryness in a fume hood at approximately 65 °C under a steady stream of nitrogen.
12. Remove test tubes from the drying block, place in a rack, and allow cooling to room temperature.
13. Reconstitute the residues with 200 μ L of 0.1% formic acid (aqueous).
14. Transfer the above solution into a 200 μ L insert and load in the auto sampler vials. All the samples are now ready for LC/MS/MS analysis.

Urine: Enzyme Hydrolysis Procedure (optional)

1. To 0.1 mL urine (samples, calibrators, and controls), add 1 mL 1 M sodium acetate (pH 5), 1 mL β -glucuronidase (*patella vulgata* - 5000 units/mL).
2. Cap and incubate at 65°C for 3 hours.
3. Cool tube and adjust to pH 9.0 with ammonium hydroxide: water (1:1).
4. Extract with 5 mL dichloromethane: isopropanol (10:1).
5. Centrifuge and aspirate aqueous layer to waste.
6. Extract organic layer remaining from step 5 with 3 mL 0.1 N sulfuric acid.
7. Centrifuge and aspirate organic layer to waste.
8. Adjust aqueous layer remaining from step 7 to pH 9.0 with ammonium hydroxide: water (1:1).
9. Extract with 5 mL dichloromethane: isopropanol (10:1).
10. Centrifuge and aspirate aqueous layer to waste.
11. Proceed from step 5 above (urine liquid-liquid extraction).

VIIIc. Sample Extraction Validation

2-1(hydroxyethyl)promazine sulfoxide and Acepromazine pH efficiency (DCM)

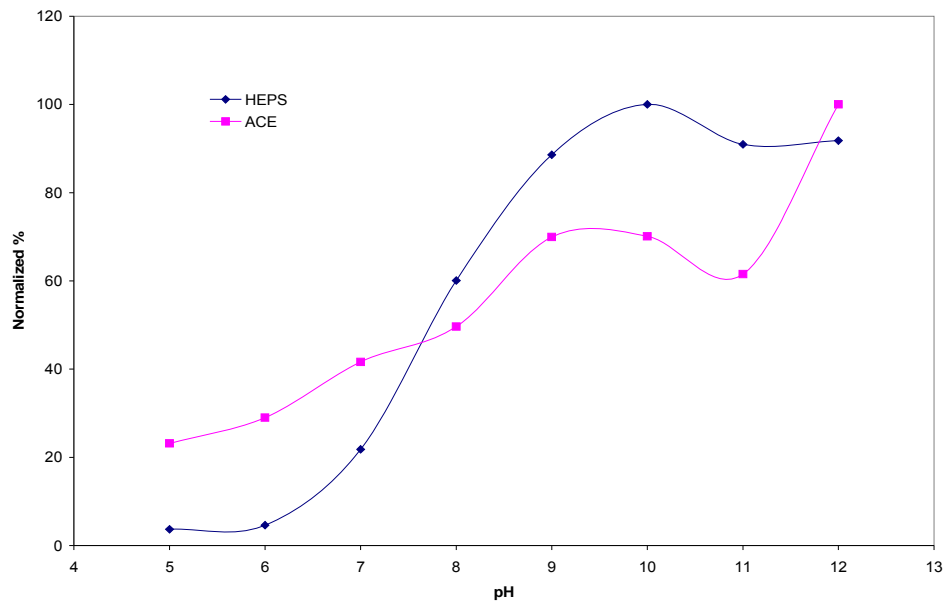


Figure 2. Optimum Extraction pH for Acepromazine (ACE) and 2-1(hydroxyethyl) promazine sulfoxide (HEPS)

2-1HEPS & Ace Solvent Comparison at pH 10

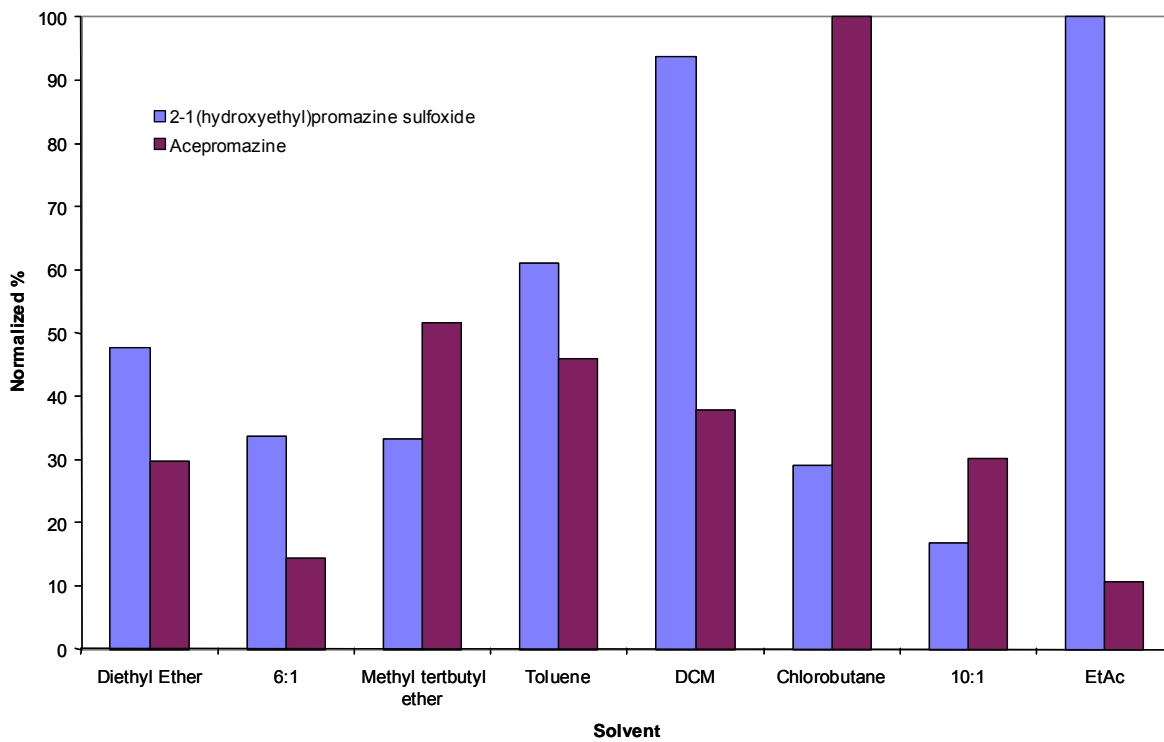


Figure 3. Extraction solvent comparison for ACE-HEPS. Note: No single solvent was optimum for both compounds.

Solvent Efficiency Fine Tune Mix (at pH 12 ammonium hydroxide)

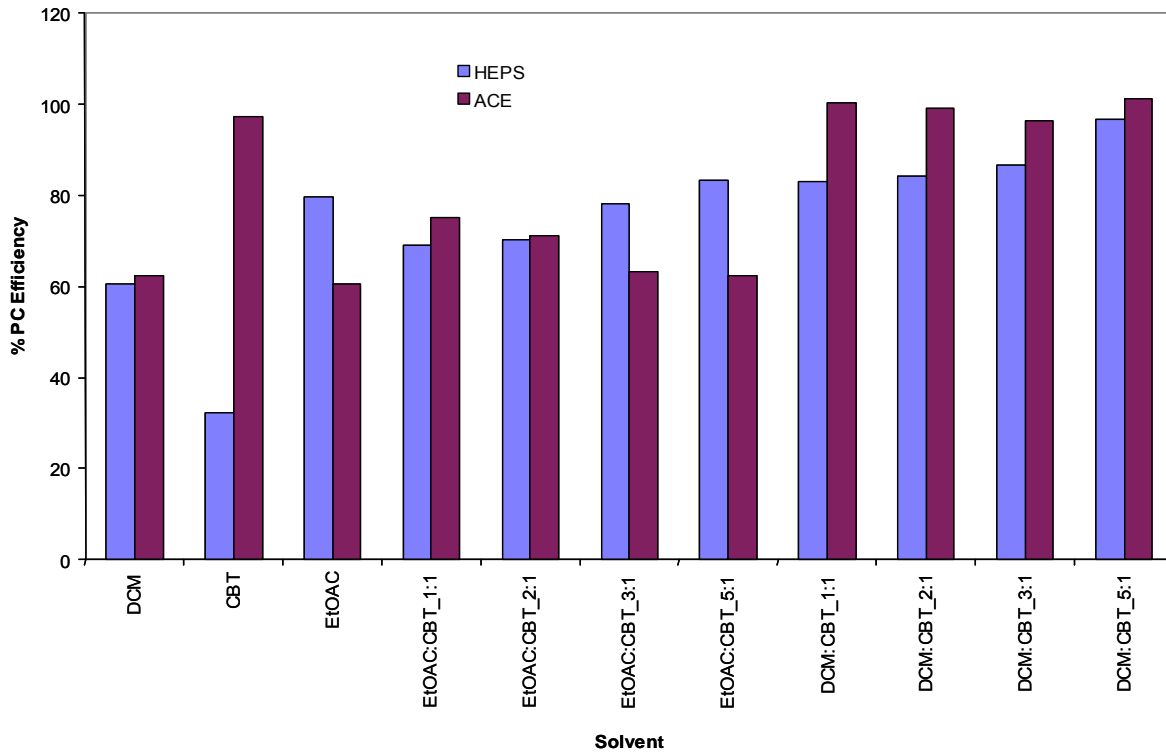


Figure 4. pH 12 1:1 ammonium hydroxide extraction solvent comparison and fine tuning. 5:1 DCM:CBT was selected as the extraction solvent for both plasma and urine.

HEPS Method Extraction Efficiency

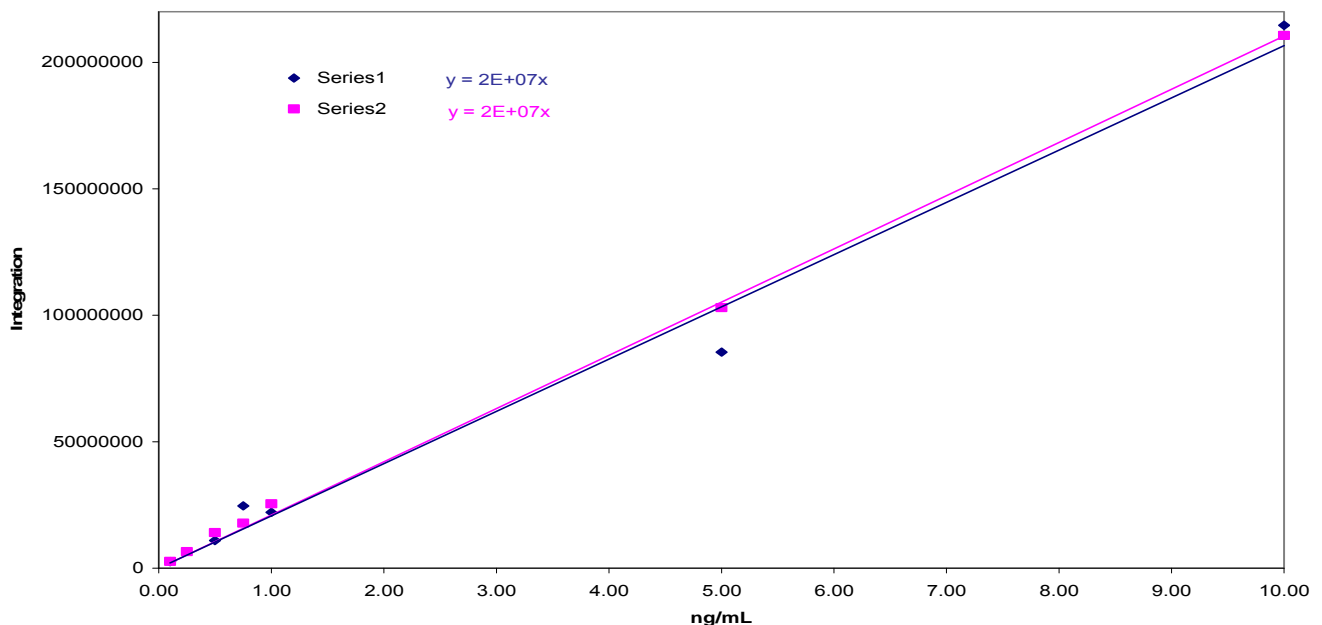


Figure 4b. Final extraction efficiency (recovery) of HEPS (~ 100 %)

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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
6. QC1
7. QC2
8. QC3
9. Blank
10. C1
11. C2
12. C3
13. C4
14. C5
15. C6
16. C7

- | |
|---|
| <ol style="list-style-type: none">17. Blank18. Track A, Samples 1 thru N19. Blank |
|---|

20. Repeat 17 thru 19 as needed

21. Blank
22. QC1
23. QC2
24. QC3
25. Blank
26. C1
27. C2
28. C3
29. C4
30. C5
31. C6
32. C7
33. Blank
34. Blank-Standby Method

<p>Blank Track A, Sample X₁ Track A, Sample X₂ Track A, Sample X₃ Blank</p>
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<p>Repeat as needed for the number of samples for confirmation</p>
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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.

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X. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION AND ESTIMATION OF 2-(hydroxyethyl) promazine sulfoxide

A. Instrumentation

1. Thermo-Electron Deca XP Plus ion trap mass spectrometer with Xcalibur V1.3 for system control and data acquisition and processing.
2. Thermo-Electron Surveyor quaternary HPLC pump, autosampler, column compartment and on-line degasser. Autosampler uses no waste injection in screen mode and partial loop injection mode in confirmation mode.

B. HPLC conditions

1. Ace C18 Analytical Column, 3.0 x 50 mm, 5 micron particle size (Part # ACE –
 - a) 111-0503, 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)

LC Conditions

	min	%A	%C	µL/min
0	0	80	20	20
1	0.2	80	20	20
2	0.3	80	20	200
3	0.5	80	20	200
4	3	5	95	200
5	4.5	5	95	200
6	4.51	80	20	400
7	5.9	80	20	400
8	5.91	80	20	20
9	6.2	80	20	20

A. 2.3 mM pH 5 Formic Acid

C. Acetonitrile 0.1% Formic Acid

Column MacMod Ace 3µ C-18 50x3 mm PN 111-0503

C. Mass Spectrometer Conditions

1. Positive Ion Electrospray Mode
2. Deca XP Plus Acquisition Parameters (W=Wideband Activation)

Segment 1		MSMS	% Collision	Isolation Width	Q	Time ms	Scan Range	Monitor Ions
HEPS	Scan Event 1	345.2	34	1.3	0.25	30	150-350	300,314
d-3 Codeine	Scan Event 2	303.2	33	1.3	0.25	30	150-310	285,243,215
Segment 2								
ACE	Scan Event 1	327.2	31	1.3	0.25	30	150-335	282,254,240

Other Detected Metabolites

	MSMS	% Collision	Isolation Width	Q	Time ms	Scan Range	Monitor Ions
ACE Sulfoxide	343.2	39W	1.3	0.25	30	150-350	298
Hydroxy ACE	343.2	39W	1.3	0.25	30	150-350	270
Hydroxy ACE Sulfoxide	359.2	45W	1.3	0.25	30	150-365	328
Hydroxy HEPS	361.2	31W	1.3	0.25	30	150-370	330

XIa. METHOD STATISTICS - STABILITY

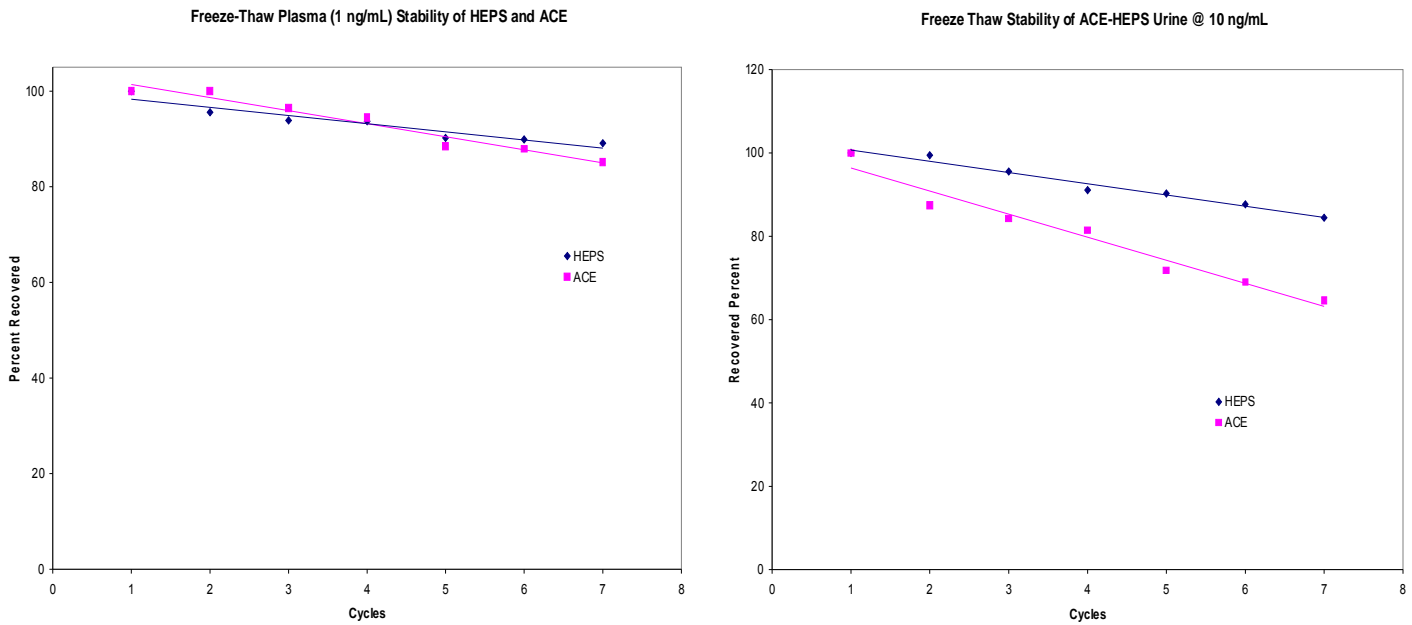


Figure 5. Freeze Thaw stability (1 ng/mL in plasma - left) and (10 ng/mL urine - right) for ACE and HEPS through six freeze-thaw cycles.

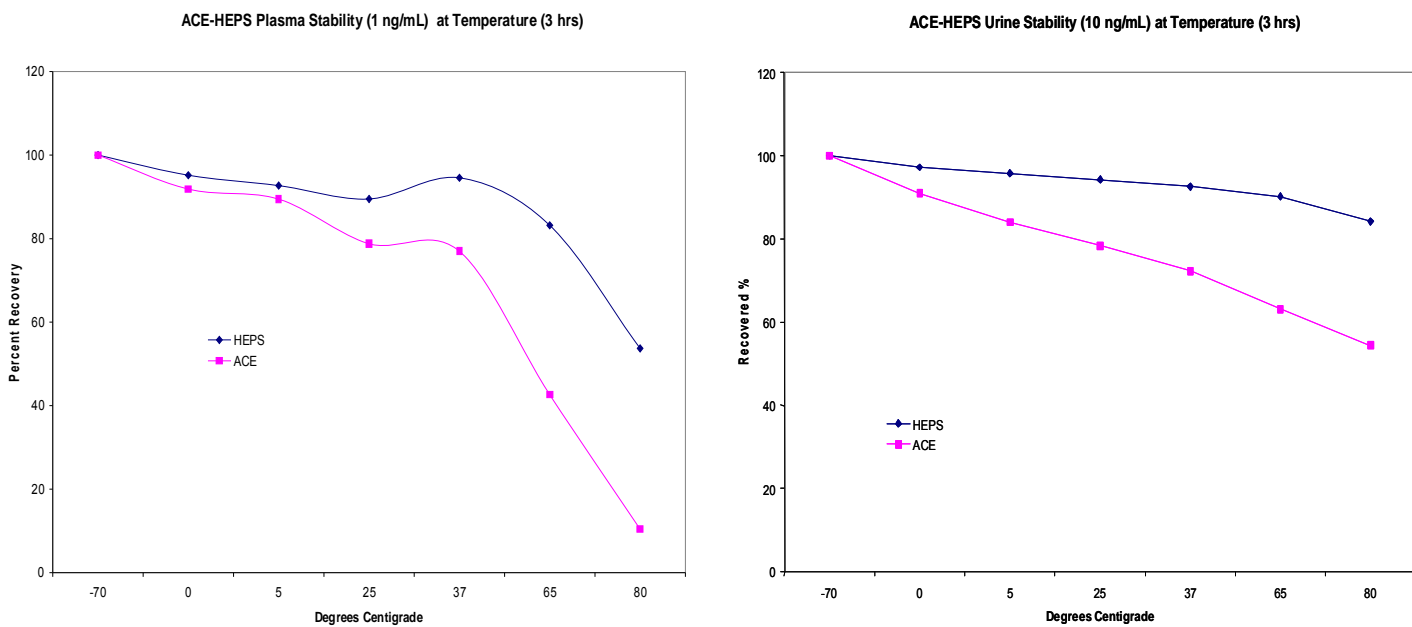
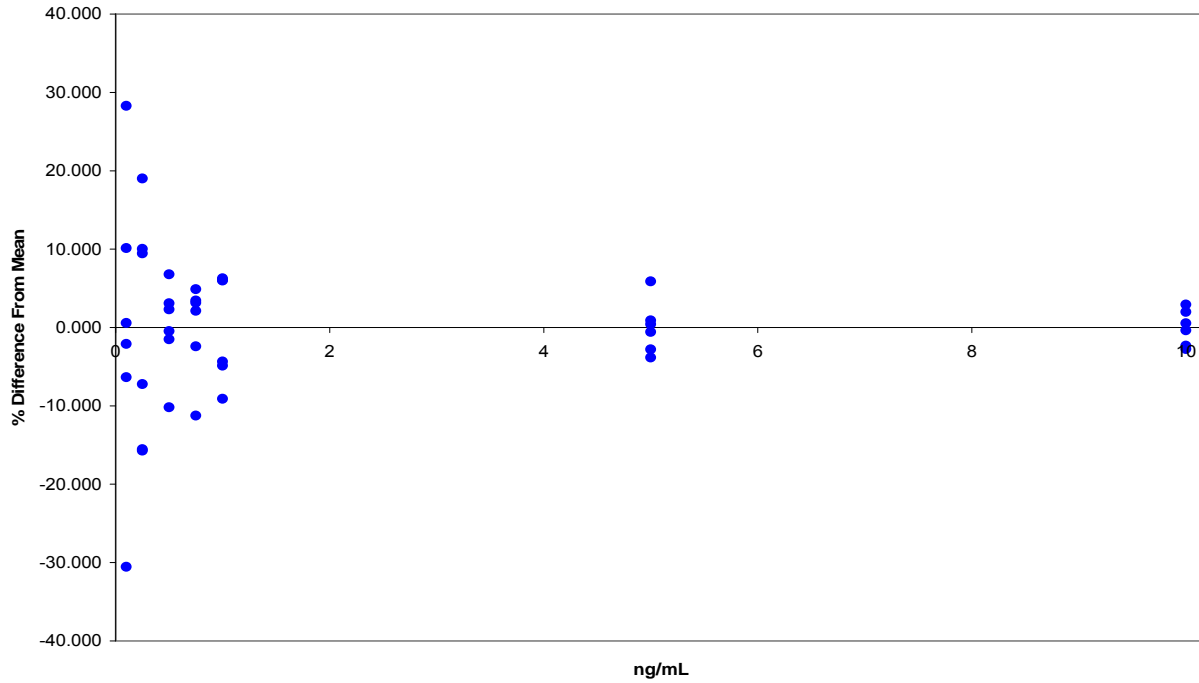


Figure 6. Temperature stability (1 ng/mL in plasma - left) and (10 ng/mL urine - right) for ACE and HEPS. The ranges through 45°C simulate conditions potentially encountered during shipping, handling, and storage of the unknown samples. The higher temperature challenges model forced thawing and hydrolysis procedures. ACE appears to be subject to degradation, with the more extreme decline in plasma partially due to plasma protein denaturation.

XIb. METHOD STATISTICS – PRECISION AND ACCURACY

Hydroxyethyl Promazine Sulfoxide Within Run Precision as % Difference From Mean



Hydroxyethyl Promazine Sulfoxide Between Run Precision as % Difference From Mean

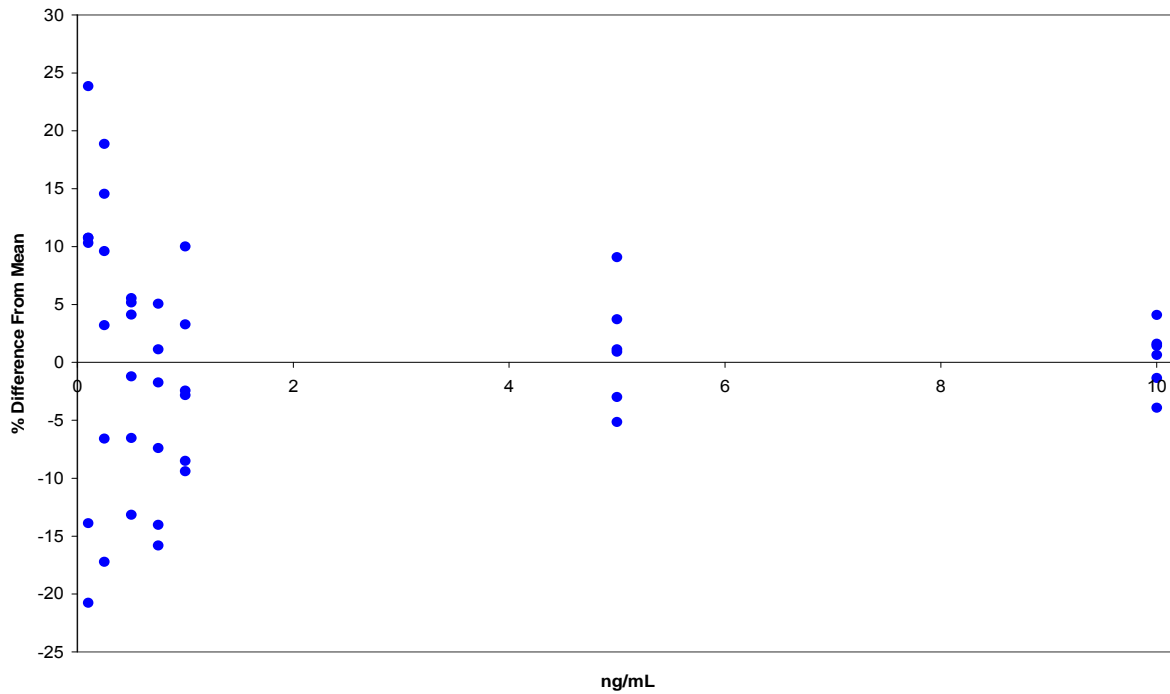


Figure 7. HEPS plasma within run (top) and between run (bottom) precision ($\sigma=3$).

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Within Run Precision and Accuracy
n=6

ng/mL	CV	Accuracy
0.10	25.13	91.45
0.25	17.65	100.62
0.50	13.84	102.20
0.75	6.41	98.19
1.00	8.46	113.79
5.00	7.12	98.61
10.00	5.05	102.39

Between Run Precision and Accuracy
n=7

ng/mL	CV	Acc
0.10	22.53	88.79
0.25	17.68	102.13
0.50	11.23	100.51
0.75	13.05	108.98
1.00	7.86	107.85
5.00	5.89	97.13
10.00	4.28	99.92

Figure 10. Within Run (left) and Between Run Precision and Accuracy

Pre-Run Versus Post Run Calibrators

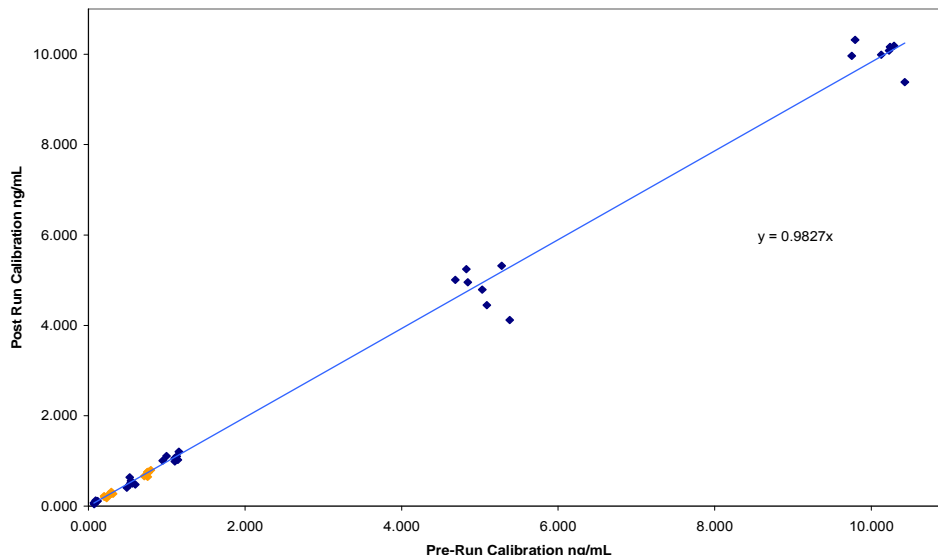


Figure 8. Youden Plot of Plasma calibrator pairs from analyses of greater than 40 plasma injections (excluding calibrators, blanks and controls). Plot indicates a representation of combined inter-assay and intra-assay variation (range represents $\sim 3\sigma$).

PETRL QUANTITATION PROCEDURE
 2-(1-hydroxyethyl)promazine sulfoxide plasma 0.5 ng/mL Control
 95% Interval

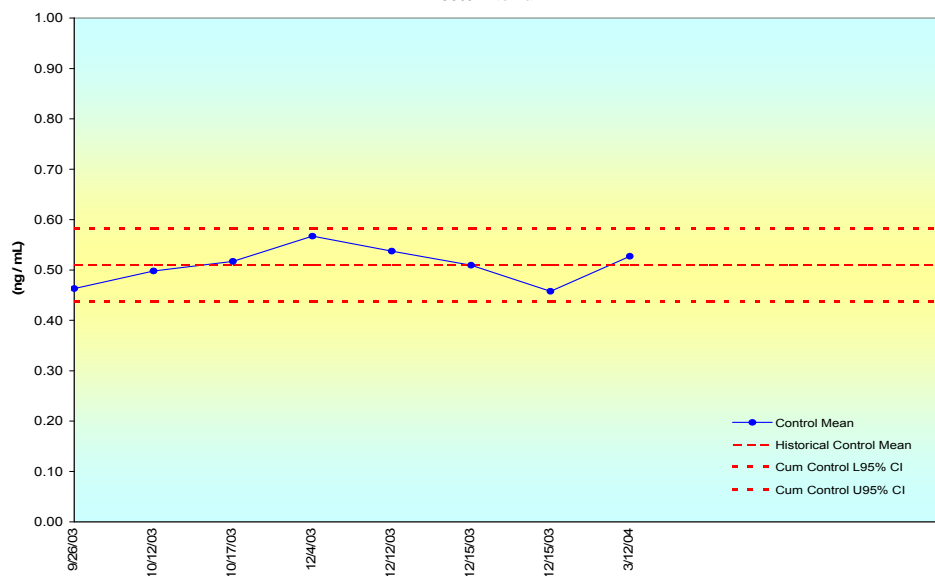


Figure 9. Control Plot of 0.5 ng/mL plasma control data (n=4; mean of 2 calibrators, 2 controls) from analyses of greater than 30 plasma injections (excluding calibrators, blanks and controls). Dispersion is reduced due to averaging of replicates. Plot indicates a representation of Type A method uncertainty (95% $\sim 2\sigma$) at this concentration.

XIc. METHOD STATISTICS – LIMITS

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 of the principle analyte hydroxyethyl promazine sulfoxide (HEPS) in plasma. This SOP may be used for confirmation of any urine analyte reported, but quantitative estimates are limited to Acepromazine (ACE) and HEPS due to the availability of standard reference material.

- A. Limit of detection in plasma of HEPS and ACE is less than 50 pg/mL, and less than 100 pg/mL in urine. Limit of detection is defined as the ability of automatic integration to report estimated concentrations that represent a chromatographic signal to noise level greater than 5:1 for the respective quantifying ions (or sum of quantifying ions).
- B. Limit of quantitation of HEPS and ACE is 100 pg/mL in plasma, and 250 pg/mL in urine. Limit of quantitation is defined as +/- 25% residual for the respective calibrator. The low end calibrators primarily serve as an indicator of system integrity and response.
- C. Limit of confirmation of HEPS and ACE in plasma is ~100 pg/mL, and ~300 pg/mL in urine. Limit of confirmation is defined as all qualifying and diagnostic ions present +/- 30% relative abundance, with no interfering ions > than 20%.

XId. Criteria for Identification of HEPS from Equine Urine and Plasma

Identification of HEPS and ACE

The diagnostic ions for HEPS are 314, 300, 256, and 238 m/z and the retention time of HEPS for the suspect sample, calibrator, and QC control must agree to +/- 0.15 minutes.

The diagnostic ions for ACE are 282, 254, and 240 m/z and the retention time of ACE for the suspect sample, calibrator, and QC control must agree to +/- 0.15 minutes.

Confirmation of HEPS and ACE

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

Primary quantification of HEPS will be based on plasma. Urine quantification data is included for informational (or requested) purposes only. ACE is only detectable within 4 hours post administration in plasma, and is quantifiable and confirmable within 2 hours post administration (25 mg Acepromazine, IV). The

presence of confirmable plasma ACE indicates administration within 2 hours of sample collection.

XII. SEQUENCE OF POSITIVE SAMPLE DATA PACKET

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

Other Required Documentation

In addition to the positive data packet, the following documentations are also required:

Sequence Sample list print-outs 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. Dichloromethane, HPLC grade (Cat. No. 9315-03 , J.T. Baker)
- E. Formic Acid, SupraPur (Cat. No. 11670-1, EM Science)
- F. Ammonium hydroxide 28% (Cat. No. AX1303-3, EM Science)
- G. Water, HPLC grade (Cat. No. 4218-03, J.T. Baker)
- H. Chlorobutane, HPLC grade (Cat. No. CX0914-1 , EM Science)

XVI. FORMULAE

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

- A. 0.1% Formic Acid: (Using E. and C. above), add 1000 uL Formic Acid (E.) to 1000 L of Water (C.). Mix thoroughly. Cap, label.
- B. 2.3 mM Formic Acid: (Using E. and C. above), add 400 uL Formic Acid (E.) to 1000 mL of Water (C.). Mix thoroughly. Check pH. Adjust to pH 5.0 with 1:1 Ammonium Hydroxide: Water. Cap, label, and record pH.
- C. 1:1 Ammonium Hydroxide: Water: (Using F. and G. above), add 500 mL Ammonium Hydroxide (F.) to 500 mL HPLC grade water (G.). Mix thoroughly, Cap, label.
- D. 50:50:1: (Using B, C, and E above), add 10 mL Formic Acid (E.) to 500 mL Water (C.) and Acetonitrile (B.).
- E. Saturated Sodium Borate, pH 10: (Using H., G., and F. above), add sufficient Sodium Borate (G.) to 1 L HPLC grade water (H.), till no more dissolves. Heat with stirring. Continue to add (G.) until no more dissolves. Cool mixture. Adjust to pH 10 with 50% Sodium Hydroxide (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 and caps
- P. 200 uL Insert

XVIII. Clearance Profiles of ACE and HEPS

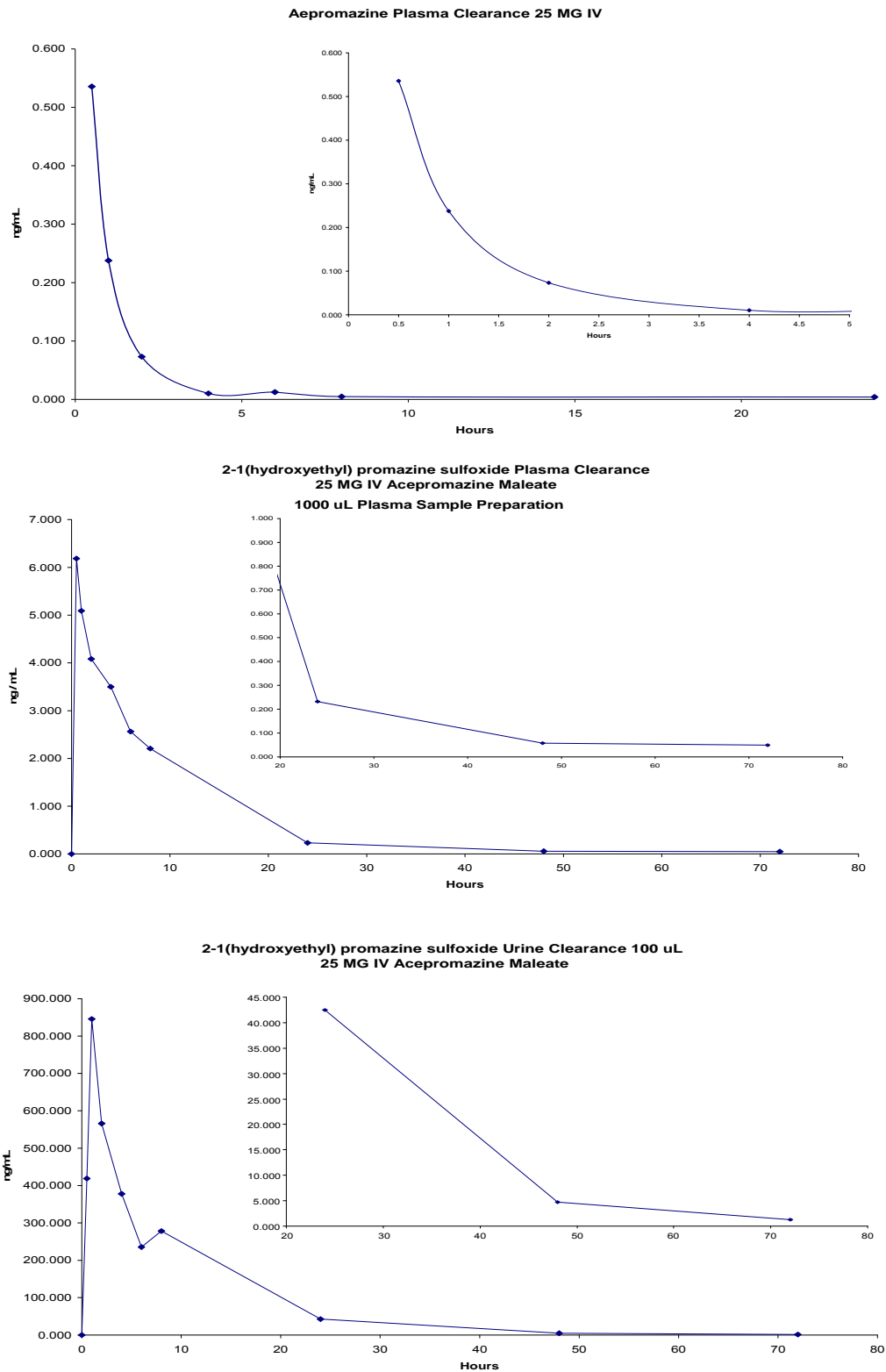
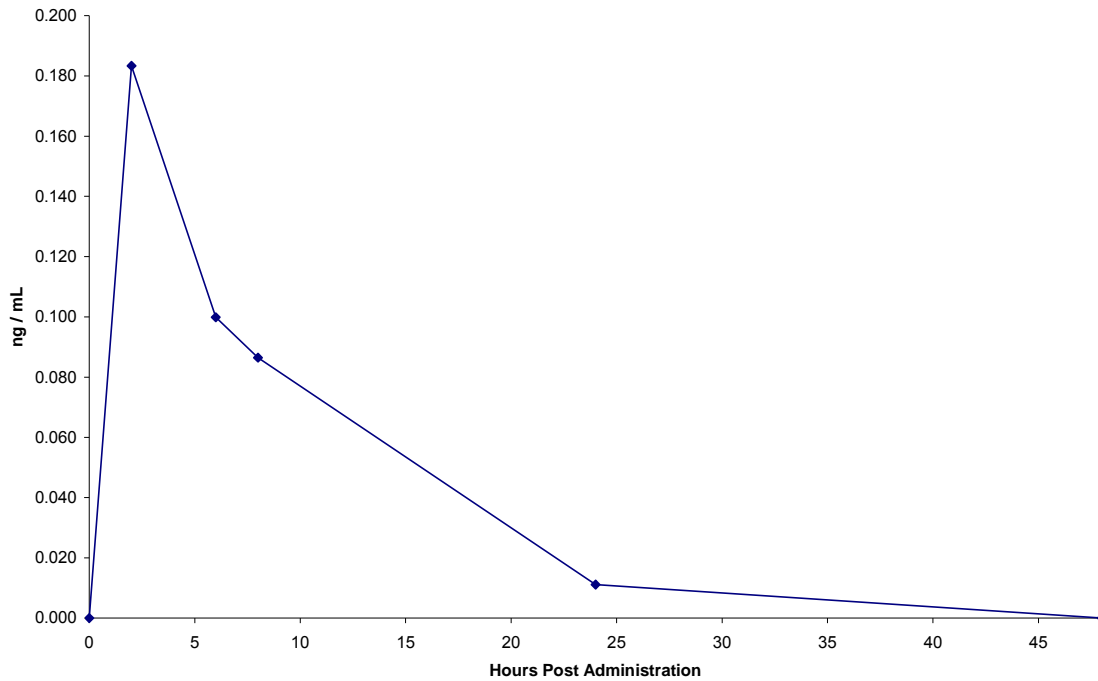


Figure 10. Plasma Clearance of ACE (top panel), Plasma Clearance of HEPS (middle panel), and urine clearance of HEPS (bottom panel) following 25 MG IV

5 MG IV Acepromazine 2-1 HEPS Plasma Clearance



5 MG IV Acepromazine 2-1 HEPS Urine Clearance

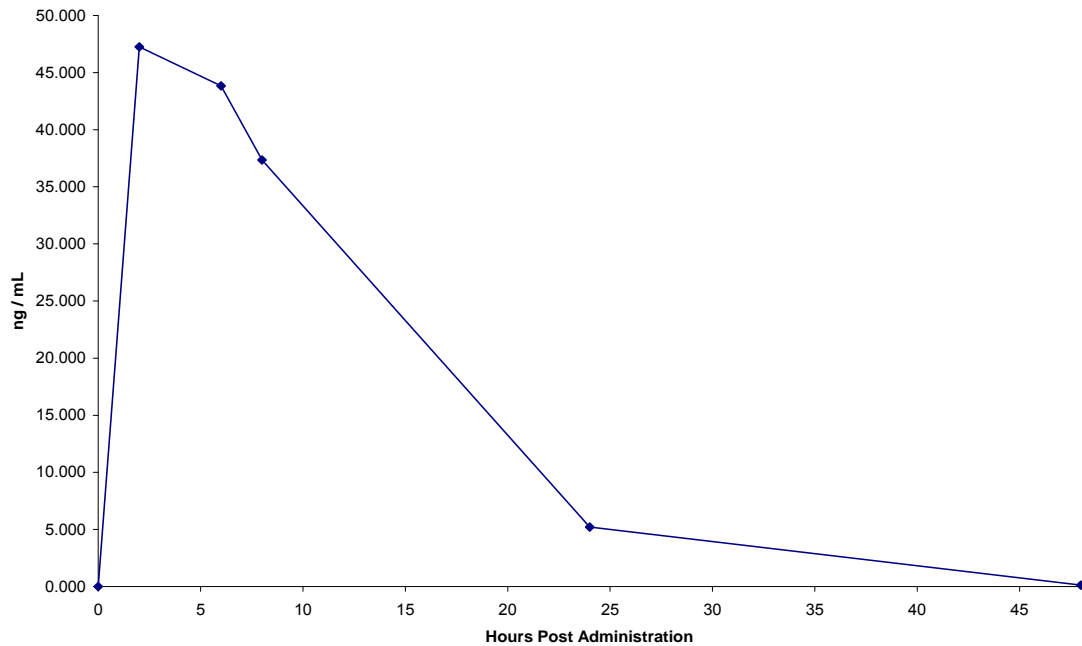


Figure 11. Plasma Clearance of ACE (top panel), Plasma Clearance of HEPS (middle panel), and urine clearance of HEPS (bottom panel) following 5 MG IV

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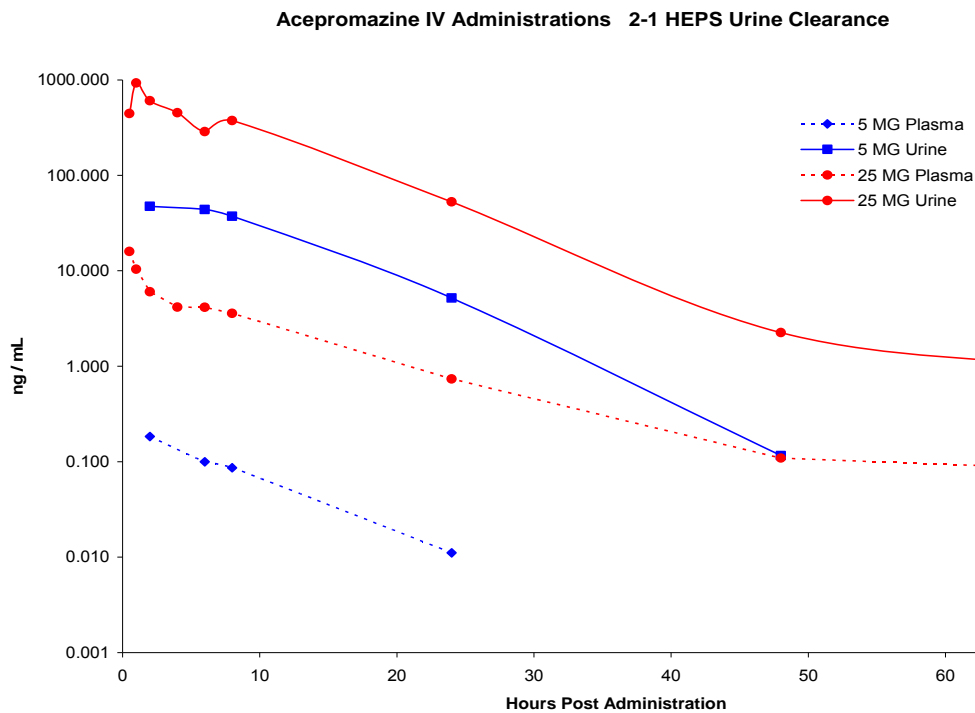


Figure 12. Combined log plot of plasma and urine clearance of HEPS from 5 MG (blue) and 25 MG (red) IV administration.

Urine Acepromazine Analytes Retention Times and Spectra

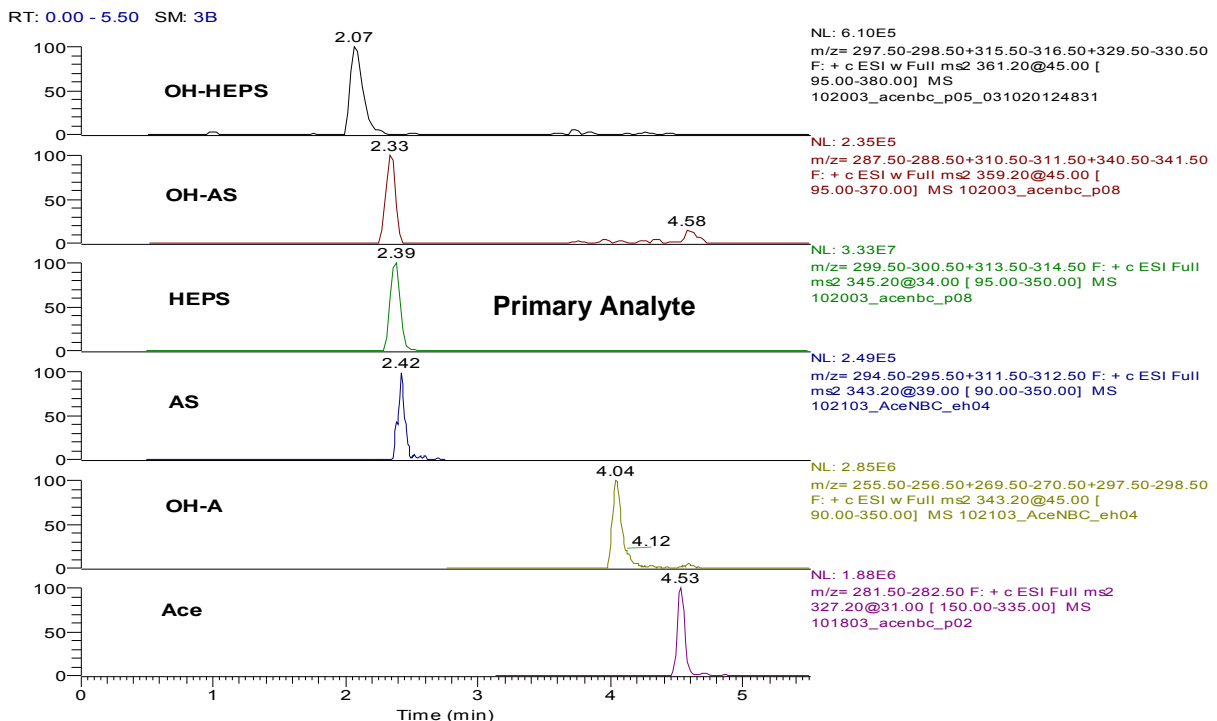
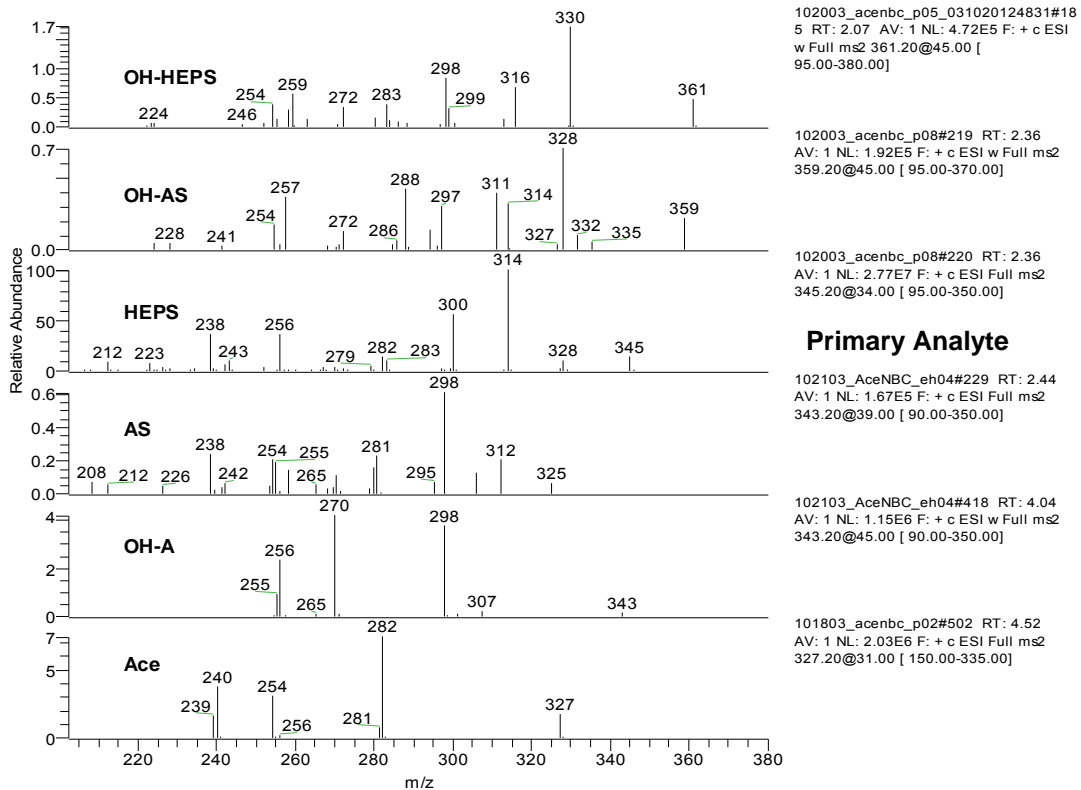


Figure 13. Detected urine metabolites of Acepromazine elution profile (above) and corresponding MSMS spectra (below)

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Plasma Acepromazine Analytes Retention Times

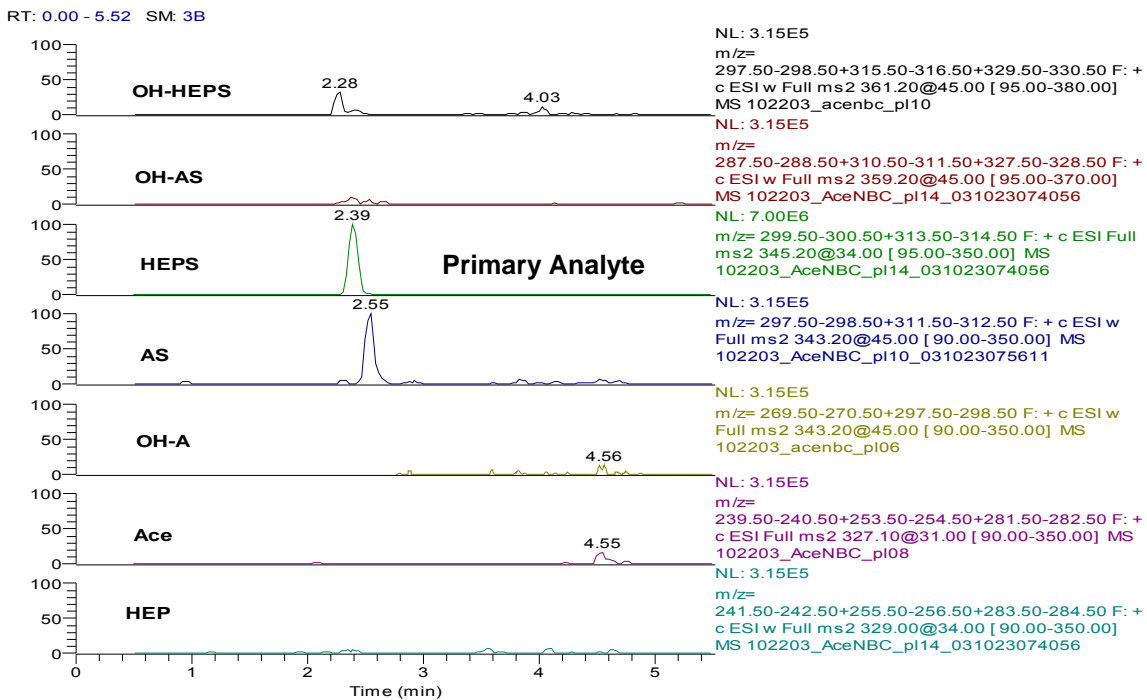
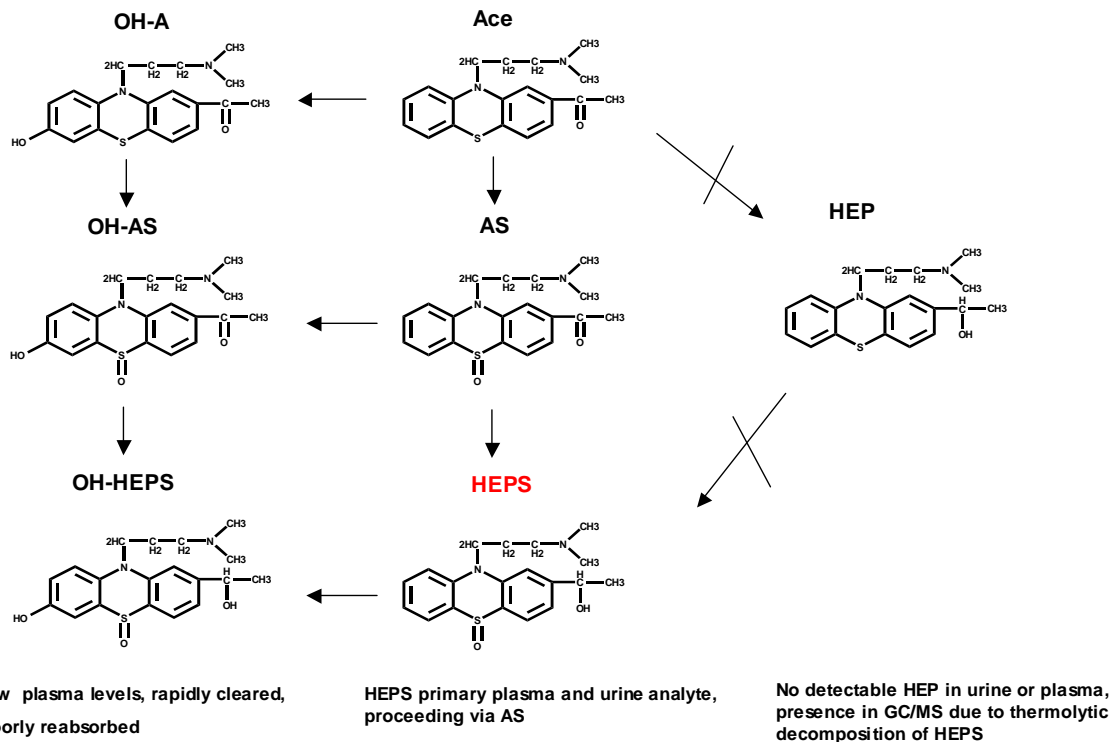


Figure 14. Extracted ion chromatograms of Acepromazine plasma metabolites following base extraction conditions previously described (NOTE: Acepromazine is detected only at low pg/mL plasma levels for no more than 4 hours). Proposed metabolic pathway (below) based on detected analytes. (NOTE: HEP detected in GC/MS analysis appears to be a thermal artifact of HEPS.)

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Acepromazine Oxidation Products

RT: 0.45 - 5.50 SM: 5B

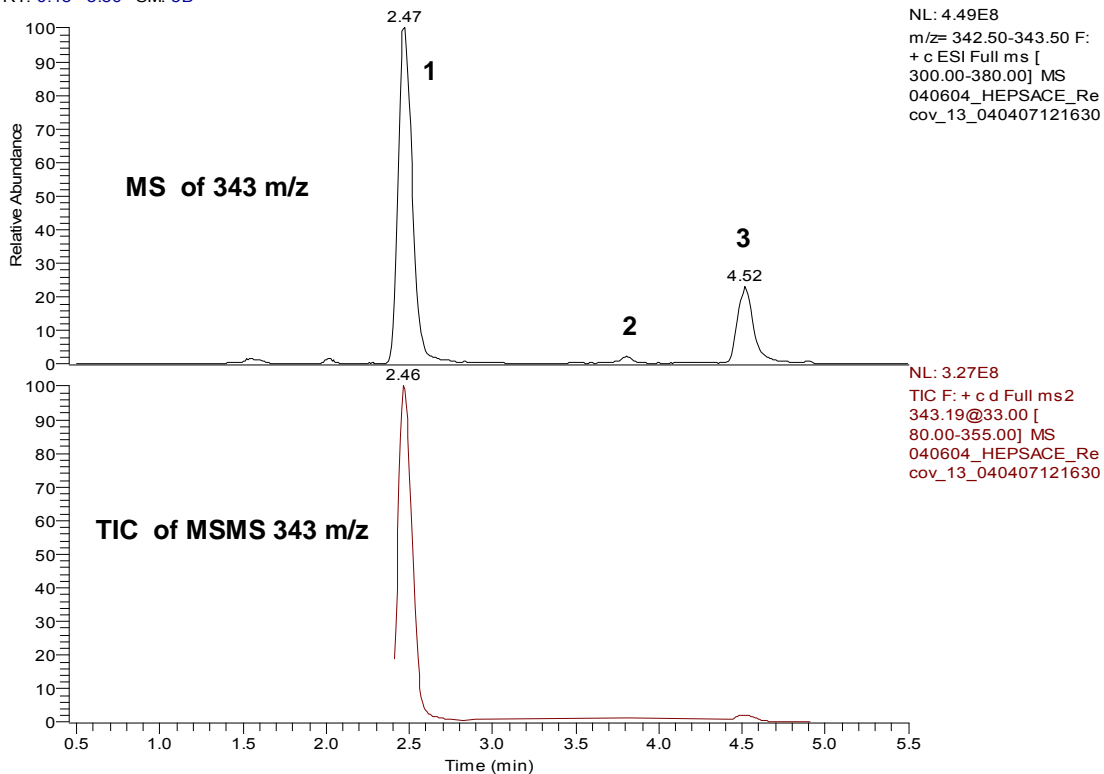


Figure 15. Peaks detected by MS Data Dependant analysis of degraded Acepromazine control stock solution

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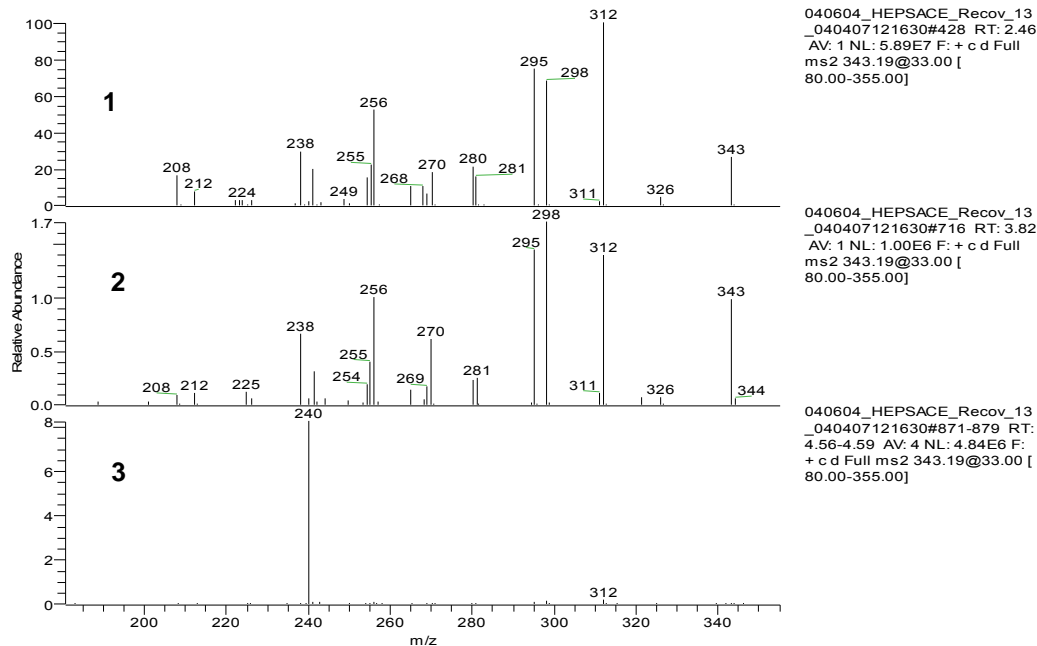


Figure 16. Spectra obtained by MS Data Dependent analysis of degraded Acepromazine control stock solution

Acepromazine instability indicated by studies cited previously appears to be primarily oxidative in nature, with S-oxide, N-oxide, and N-hydroxyl as possibilities.

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- 2. Uniform Classification Guidelines for Foreign Substances and Recommended Penalties and Model Rule**, Rev January 3, 2003, Association of Racing Commissioners International Inc., RCI Drug Testing Standards and Practices Program
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