Acepromazine Threshold Study

Scott D. Stanley, PhD
Professor
K.L. Maddy Equine Analytical Chemistry Laboratory
University of California - Davis

RMTC Acepromazine Study

- Introduction
  - Sponsor: Racing Medication and Testing Consortium
  - Title: Pharmacokinetics of Acepromazine after Single Dose Intravenous Administration to Conditioned TB Horses
  - Purpose: To determine the pharmacokinetics of Acepromazine and to estimate the withdrawal time of Acepromazine after single intravenous administration of Acepromazine at a clinically relevant dose to athletically conditioned Thoroughbred horses
  - Lead investigator: Dr. Scott D. Stanley
  - Preliminary Data: RMTC study conducted in 2009 and 2010 at UC-Davis

RMTC Acepromazine Study

- Animal Studies:
  - Study Center: UF Pharmacokinetics Laboratory in the Equine Performance Laboratory at the University of Florida
  - Study Director: Dr. Patrick Colahan
  - Animal care and use protocol: approved by University of Florida IACUC
  - Facilities: Inspected twice annually by university IACUC team
  - Subjects: Thoroughbred horses housed at UF College of Veterinary Medicine
  - Number of subjects: 20
  - Conditioning: Treadmill exercise three days per week except on dose administration days – able to run one mile in two minutes under conditions required for participation in study

RMTC Acepromazine Study

- Drug Administration Phase
  - Drug: Acepromazine (Generic)
  - Source: Webster
  - Formulation: Solution for injection – 10 mg/mL
  - Dose: 0.05 mg/kg of body weight
  - Route: Intravenous; Rapid bolus
  - Frequency: Once
  - Sample collections: individual venipuncture at each collection point into partially-evacuated glass tubes containing Li heparin
  - Sample handling and storage: Plasma was harvested and stored at -80°C until analyzed (less than two months)

RMTC Acepromazine Study

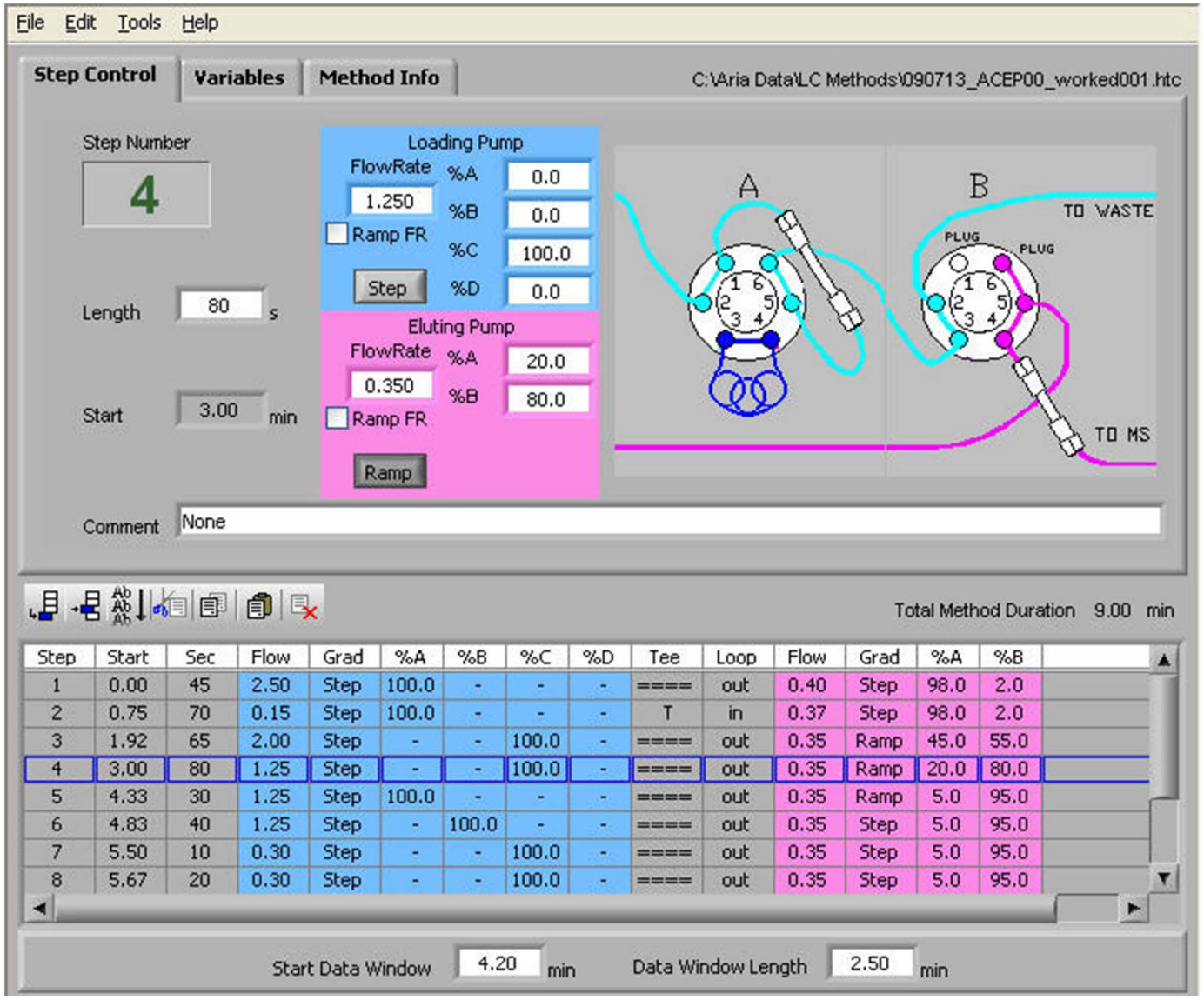
- Method Development and Validation Phase
  - Method Development Laboratory: K.L. Maddy Equine Analytical Chemistry Laboratory, University of California-Davis
  - Method Development Phase Director: Dr. Scott Stanley
  - Analyst: Daniel McKemie
  - Reference: US FDA Guidance Document for Method Validation
  - Method: Turbulent Flow Chromatography extraction followed by liquid chromatographic-mass spectral analysis of Acepromazine and HEPS using stable isotope labeled analogues of the analytes as internal standards.
  - Standards:
    - Acepromazine : U.S. Pharmacopeia
    - HEPS: Neogen Corp.
    - HEPS-d_4: Frontier Biopharm

RMTC Acepromazine Study

- Analytical Phase
  - Method: TFC LC-MS/MS
  - Calibrators: Nine calibrators (0.01, 0.05, 0.1, 0.25, 0.5, 1, 5, 10, and 50 ng/mL) in matrix-matched control plasma in duplicate; prepared fresh on day of analysis. Calibrators contained Acepromazine and HEPS.
  - Controls: Two positive control samples (100 and 250 pg/mL) in duplicate prepared before start of sample analyses and stored under same conditions as study samples. Control samples contained Acepromazine and HEPS.
  - Samples: Samples were analyzed in duplicate. Samples containing HEPS at concentrations greater than 50 ng/mL were diluted with plasma before analysis.

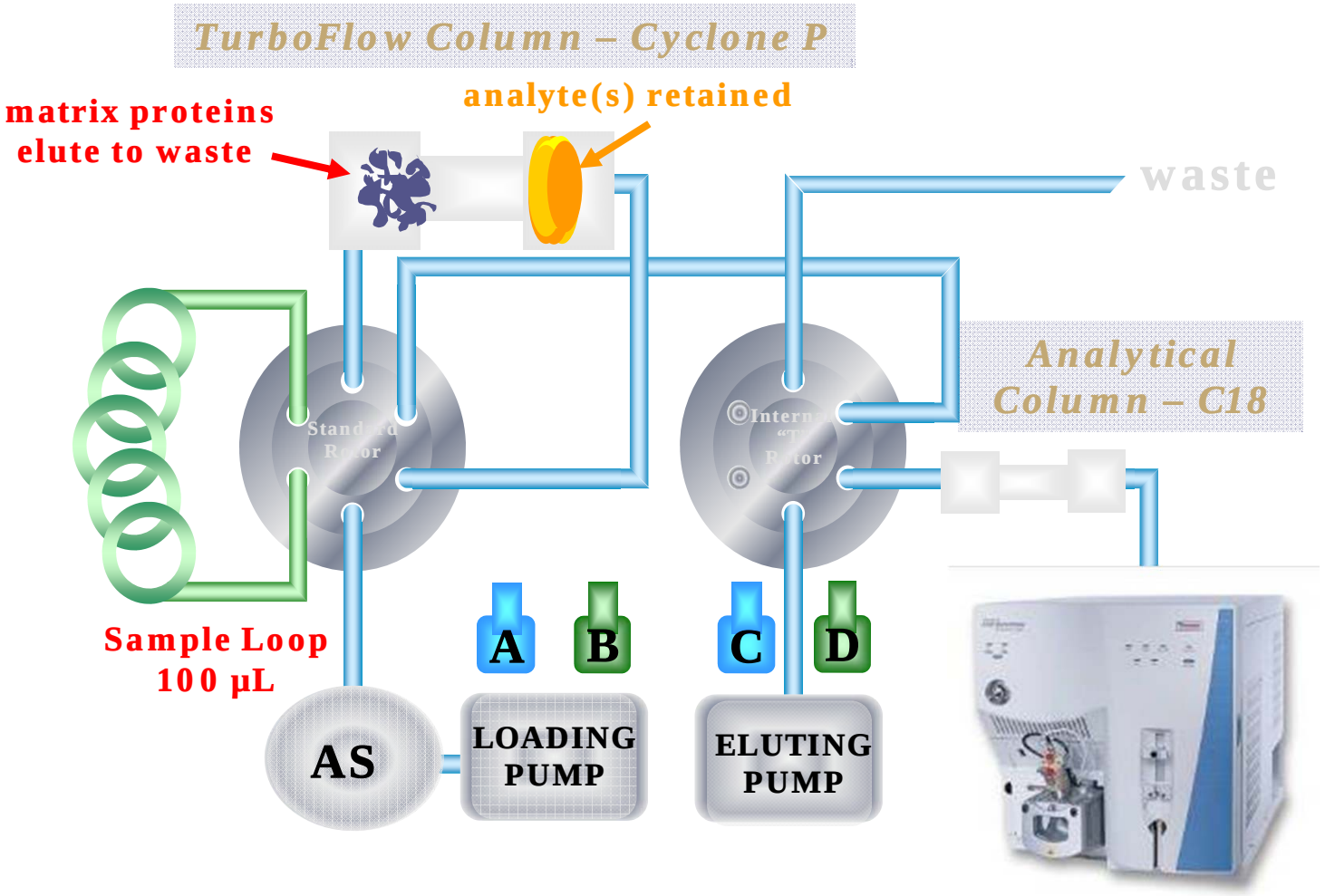
Acepromazine Study

- Analytical Conditions
  - Column
    - Type: ACE 3 C18 Analytical Column
    - Dimension: 2.1 mm × 100 mm
    - Particle size: 3.0 µm
    - Temperature: 40 °C
  - Guard Column
    - Type: ACE 3 C18 Pre-column
    - Dimension: 2.1 mm x 10 mm
    - Particle size: 3.0 µm
  - TFC Column
    - Type: Cyclone P; Dimension: 0.5 mm x 50 mm
  - Mobile Phase
    - Mobile phase A: 0.2% formic acid in water
    - Mobile phase B: 0.2% formic acid in ACN
    - Flow rate: 350 µL/min
  - Injection Volume: 50 µL



Step	Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	0.00	45	2.50	Step	100.0	-	-	-	====	out	0.40	Step	98.0	2.0
2	0.75	70	0.15	Step	100.0	-	-	-	T	in	0.37	Step	98.0	2.0
3	1.92	65	2.00	Step	-	-	100.0	-	====	out	0.35	Ramp	45.0	55.0
4	3.00	80	1.25	Step	-	-	100.0	-	====	out	0.35	Ramp	20.0	80.0
5	4.33	30	1.25	Step	100.0	-	-	-	====	out	0.35	Ramp	5.0	95.0
6	4.83	40	1.25	Step	-	100.0	-	-	====	out	0.35	Step	5.0	95.0
7	5.50	10	0.30	Step	-	-	100.0	-	====	out	0.35	Step	5.0	95.0
8	5.67	20	0.30	Step	-	-	100.0	-	====	out	0.35	Step	5.0	95.0

2-Dimensional HPLC Setup



Acepromazine Study

MS Method Parameters:

- Sample Processing: Turbulent Flow Chromatography
- Chromatography: 2-D HPLC
- MS: Thermo TSQ Vantage Triple Quadrupole
- Ionization: Heated Electrospray Ionization (HESI)
- HESI Temp: 200°C
- Scan Mode: HSRM
  - Q1 – 0.1 *m/z* at FWHM
  - Q3 – 0.7 *m/z* at FWHM
- Scan Width: 0.01 *m/z*
- Scan Time: 65 millisecond

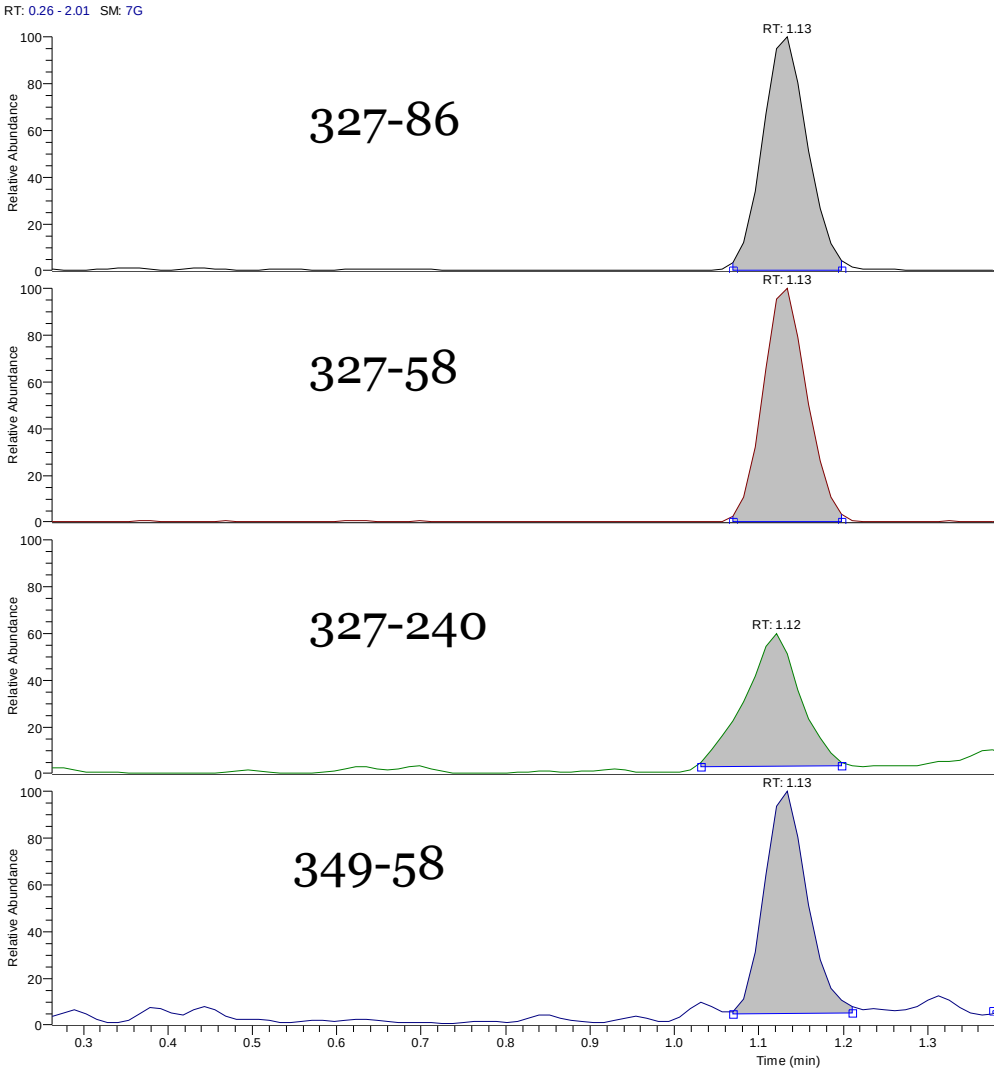
SRM Acquisition Parameters

SRM acquisition parameters for Acepromazine and HEPS and their deuterated analogues.

Analyte	Precursor Mass	Product Ion Mass	Scan Time	Collision Energy
Acepromazine	327.2	58.1	0.065	35
Acepromazine	327.2	86.1	0.065	20
Acepromazine	327.2	240.1	0.065	18
Acepromazine -d_4	331.2	254.1	0.065	23

Analyte	Precursor Mass	Product Ion Mass	Scan Time	Collision Energy
HEPS	345.2	58.1	0.20	35
HEPS	345.2	86.1	0.20	28
HEPS	345.2	242.1	0.20	34
HEPS-d_4	349.2	246.1	0.20	28

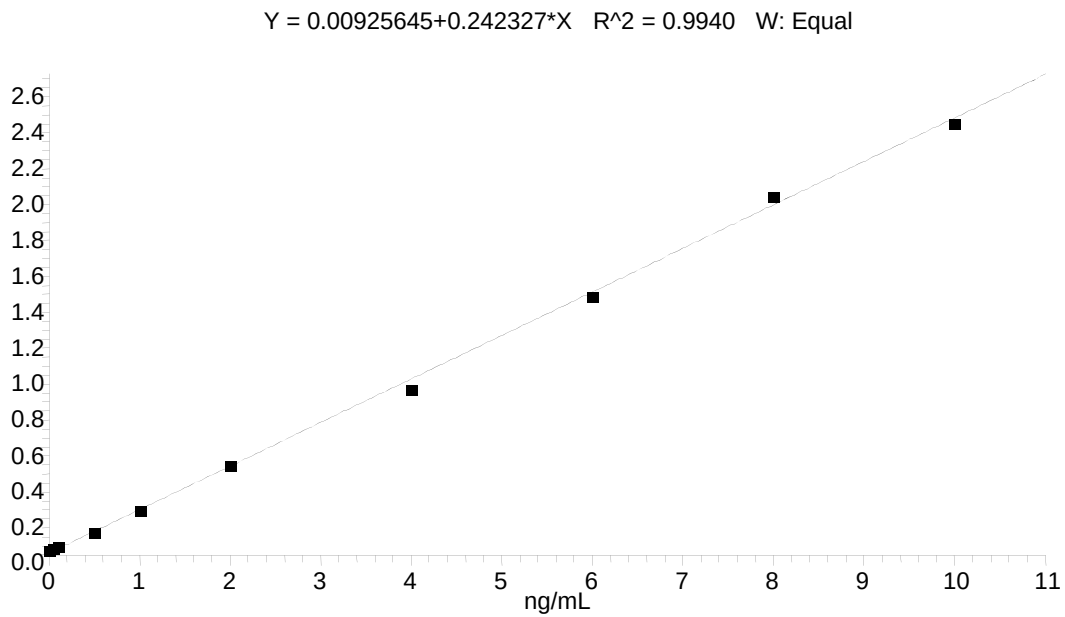
Acepromazine 0.2 ng/mL Calibrator



Ion chromatograms

Total ion chromatogram (upper trace) and ion chromatograms for quantifier ion (m/z 58) and qualifier ions (m/z 86 and 240) for Acepromazine and quantifier ion for HEPS-d_4 (m/z 58) (bottom trace).

Acepromazine Determination



Calibration curve for determination of HEPS from horse plasma by LC-MS (TFC liquid chromatograph and Quantum Vantage triple stage quadrupole mass spectrometer with HESI) using HEPS-d_4 as internal standard. The calibration curve ranged from 0.1 ng/mL to 10 ng/mL of plasma and was not weighted.

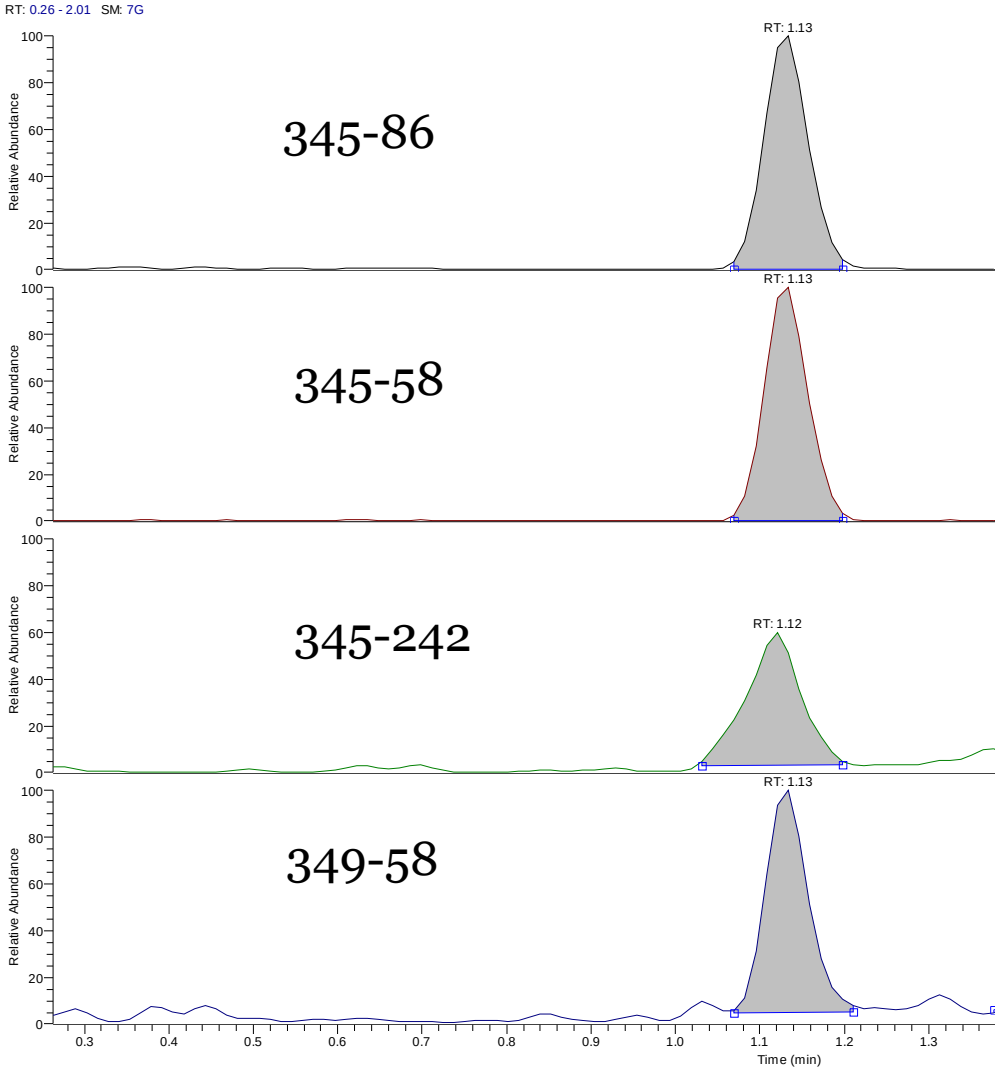
Acepromazine in Horse Plasma

- Range 0.01-10 ng/mL
- Weighted 1/x linear fit

Calibration Data

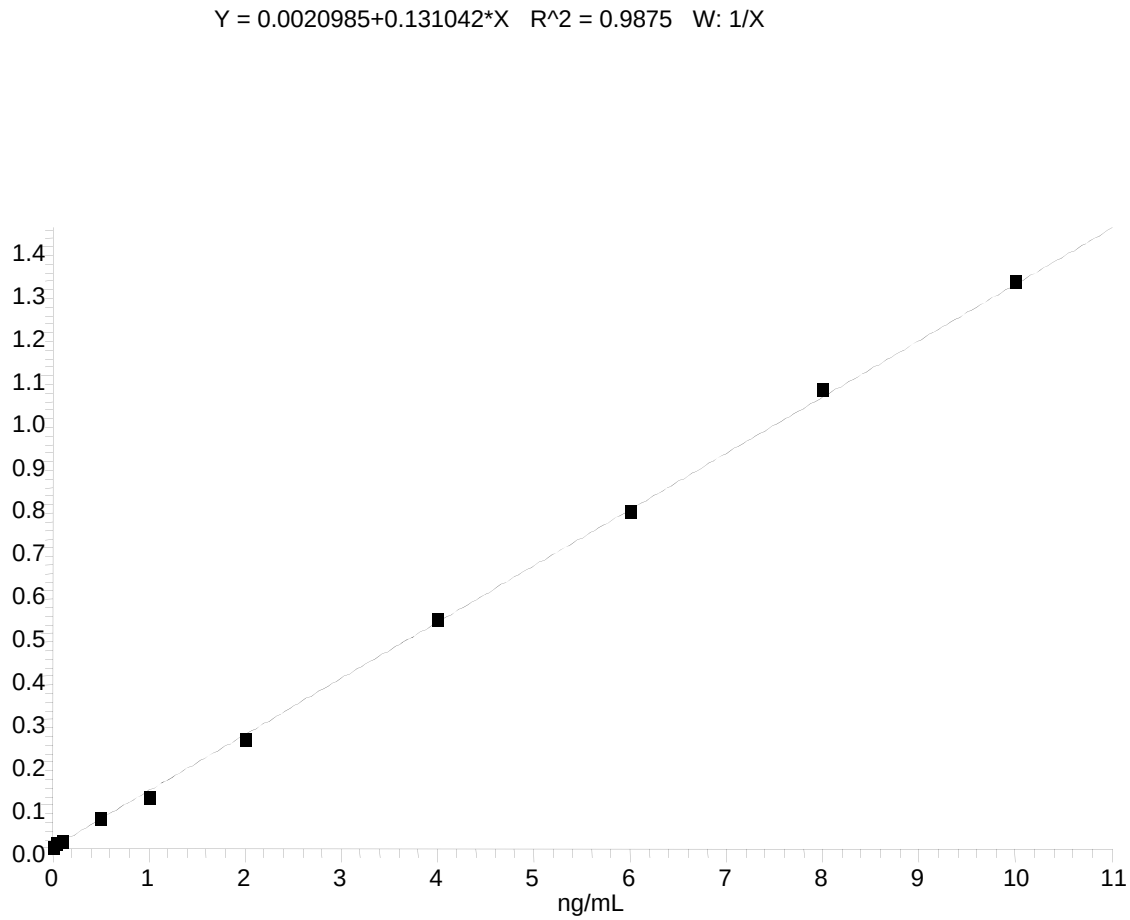
Run	Date	Y-intercept	Slope	R^2
Set 1	9/28/09	-0.008345	0.328707	0.9917
Set 2	9/29/09	0.00925645	0.242327	0.994
Set 3	7/29/10	-0.164921	0.082095	0.9955
Set 4	7/30/10	-0.094500	0.0701814	0.9791

HEPS 0.1 ng/mL C_0 Calibrator



Ion chromatograms

Total ion chromatogram (upper trace) and ion chromatograms for quantifier ion (m/z 58) and qualifier ions (m/z 86 and 242) for HEPS and quantifier ion for HEPS-d_4 (m/z 58) (bottom trace). The ion chromatograms were obtained at a HEPS plasma concentration of 0.1 ng/mL.



HEPS Determination

Calibration curve for determination of HEPS from horse plasma by LC-MS (TFC liquid chromatograph and Quantum Vantage triple stage quadrupole mass spectrometer with HESI) using HEPS-d_4 as internal standard. The calibration curve ranged from 0.01 ng/mL to 10 ng/mL of plasma and was not weighted.

HEPS in Horse Plasma

- Range 0.01-10 ng/mL
- Weighted 1/x linear fit

Calibration Data

Run	Date	Y-intercept	Slope	R^2
Set 1	9/28/09	-0.000181	0.13497	0.9972
Set 2	9/29/09	0.0020985	0.13104	0.9875
Set 3	7/29/10	-0.006985	0.0116	0.9985
Set4	7/30/10	-0.004902	0.00816	0.9983

Assay Precision

Intra assay (n=6)		Acepromazine	HEPS
QC level 1	Average (pg/mL)	92	94
(100 pg/mL)	%CV	11.1	9.1
QC level 2	Average (pg/mL)	250	247
(250 pg/mL)	%CV	5.4	6.2

Inter assay (n=33)		Acepromazine	HEPS
QC level 1	Average (pg/mL)	91	94
(100 pg/mL)	%CV	13.3	12.4
QC level 2	Average (pg/mL)	249	244
(250 pg/mL)	%CV	8.9	7.8

Assay Accuracy

Intra assay (n=6)		Acepromazine	HEPS
QC level 1	Average (pg/mL)	92	94
(100 pg/mL)	%CV	94	96
QC level 2	Average (pg/mL)	249	247
(250 pg/mL)	%CV	96	97

Inter assay (n=33)		Acepromazine	HEPS
QC level 1	Average (pg/mL)	91	94
(100 pg/mL)	%CV	95	95
QC level 2	Average (pg/mL)	249	244
(250 pg/mL)	%CV	97	99

Acepromazine Summary

- Plasma HEPS concentrations were determined for 24 hours after intravenous administration of a single dose (0.05 mg/kg of body weight) of HEPS to each of twenty Thoroughbred horses.
- Plasma HEPS concentrations were determined by a validated LC-MS/MS method characterized by a method limit of detection of 5.0 pg/mL and a lower limit of quantitation of 10 pg/mL.
- The method was validated with regard to accuracy, precision, specificity, sensitivity, linearity, ruggedness, and transferability.
- Plasma HEPS concentrations at 72 hours after dosing (n=20) were analyzed and a tolerance limit of 10 pg/mL was determine.
- Plasma concentration versus time data in six horses were subjected to PK analysis and a two compartment open model was selected based on AIC and goodness of fit criteria.
- Parent acepromazine was not detected, beyond 480 min, in any sample collected after intravenous acepromazine administration.