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**TIP Approved SOP**  
**SEPARATION, QUANTIFICATION AND CONFIRMATION OF**  
**PEMOLINE IN EQUINE PLASMA BY HIGH PERFORMANCE LIQUID**  
**CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY**

**DEVELOPED BY**

**PA EQUINE TOXICOLOGY & RESEARCH LABORATORY**

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

Director: Dr. Cornelius Uboh  
E-mail: [ubohcorn@vet.upenn.edu](mailto:ubohcorn@vet.upenn.edu)  
[cuboh@state.pa.us](mailto:cuboh@state.pa.us)

Laboratory Manager: Jeffrey Rudy  
E-mail: [jeffrudy@comcast.net](mailto:jeffrudy@comcast.net)  
[jrudy@state.pa.us](mailto:jrudy@state.pa.us)

Method Development: Dr. Yi Luo  
E-mail: [yilo@vet.upenn.edu](mailto:yilo@vet.upenn.edu)

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](mailto:soma@vet.penn.edu)

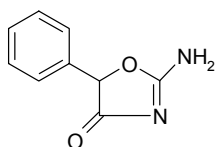
## SEPARATION, QUANTIFICATION AND CONFIRMATION OF PEMOLINE IN EQUINE PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

### INTRODUCTION

Pemoline is a stimulant to the dopaminergic system of the central nervous system<sup>1</sup>. The purpose of this study was to demonstrate that if a sample of equine plasma contained Pemoline, and was analyzed, there would be sufficient analytical data to demonstrate the presence of Pemoline, and to quantify and confirm the presence of pemoline in race horse samples. This SOP describes a simple, rapid, sensitive and reliable LC-MS-MS method to identify, quantify and confirm the presence or absence of pemoline in equine plasma samples.

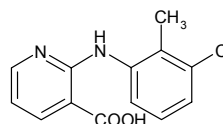
### SCOPE

This standard operating procedure describes the quantification and confirmation of pemoline in equine plasma. Liquid-liquid extraction of equine plasma is employed with subsequent analysis on an LC-MS (Thermo-Finnigan, San Jose, CA), consisting of a Surveyor MS pump with an on-line degasser, a Surveyor autosampler, and a TSQ Quantum mass spectrometer equipped with an electrospray ionization (ESI) probe. This method can be directly used as an instrumental screening method by appropriate alteration of the sample injection sequence. The scope of this work covers procedures to be used in isolating from plasma as the matrix, separating, detecting, quantifying and confirming the presence or absence of pemoline (Figure1) in equine plasma samples collected from racehorses. Reporting of a positive finding to the Racing Commission will be based solely on the results obtained by the LC/MS/MS methods described in this SOP. Any concentration of pemoline in equine plasma that does not meet the criteria presented by this SOP for reporting such a positive finding to the Racing Commission, will be considered a negative finding and will be so reported to the appropriate Racing Commission. Pemoline is a class 1 drug by the Association of Racing Commissioners International and therefore and concentration of pemoline detected and confirmed in plasma will be reported as a positive finding to the Racing Commission. Thus, there is no tolerance concentration of pemoline in plasma or urine at the time of participation of any racehorse in a sanctioned race in Pennsylvania.



MW: 176.18

Formula: C<sub>9</sub>H<sub>8</sub>N<sub>2</sub>O<sub>2</sub>



MW: 262.70

Formula: C<sub>13</sub>H<sub>11</sub>ClN<sub>2</sub>O<sub>2</sub>

Figure 1. Chemical structures of Pemoline and Clonixine (IS)

### PRINCIPLE OF METHOD

A rapid and sensitive method for high throughput (HTP) separation, identification, quantification and confirmation of pemoline in equine plasma by liquid chromatography coupled on-line with triple quadrupole Quantum tandem mass spectrometry (LC/TSQ-MS/MS; Thermo-Finnigan) is described. Analysis is performed by positive electrospray ionization. Efficiency for analyte recovery using various solvents for extraction and pH (2-12) is determined. Ethyl acetate (EtoAc)

is the best solvent for extraction and cleanliness of the extract. Plasma sample (0.5 mL) is augmented with pemoline (0.1-100 ng/mL), Clonixin is used as the internal standard. Plasma sample is mixed with 1.5 mL of 0.1 M phosphate buffer (pH 5.0), and is extracted using 5 mL EtoAc, and then its repeated and the extracts are pooled and concentrated at 60 °C (Techne Dri-Block Concentrator). The residue is reconstituted in 100 µL of mobile phase solvent (2 mM ammonium acetate: ACN, 5:95, v/v) and 10 µL was used for analysis. The retention time of pemoline under the described LC-MS conditions is 2.60 min and 2.85 minutes for clonixin (IS), respectively. Gradient LC programming procedure is performed. An Ace 5 C<sub>8</sub> column (2.1 x 80 mm; Mac-Mod, Chadds Ford, PA, USA) is used for analyte separation. Confirmation for the presence or absence of analyte is achieved by data dependent scan based on selecting the parent ion of the target analyte as basis for confirmation. The limit of detection (LOD) is 100 pg/mL (S/N ≥ 3), limit of quantification (LOQ) is 0.2 ng/mL with linear range of 0.2-100 ng/mL ( $r^2 > 0.995$ ), and limit of confirmation (LOC) is 1.0 ng/mL. Entire analysis is completed in 5 minutes. The method accuracy and precision are also determined using three doses: low, medium and high. The precision and accuracy at 0.5 ng/mL, 5 ng/mL and 50 ng/mL for pemoline during intra-day assay are 0.96% - 2.40% and 100.0% - 100.68%, respectively. The precision and accuracy at the same concentrations for inter-day assay are 0.72 - 2.72% and 101.40 - 102.00%, respectively. This method is fast, simple, sensitive and reliably reproducible.

#### **SAFETY REQUIREMENTS:**

A lab coat, protective goggles and gloves **MUST BE WORN** as the first line of protective measures. All chemicals and reagents **MUST** be used in a fume hood. Transportation of reagents from the storage room to the workbench **MUST** be accomplished with the use of a reagent rubber bucket. Always observe the “STOP” signs when coming out of the laboratory into the hallway. Foods and/or drinks are not allowed in the laboratory. All chemical wastes are to be properly transferred into waste containers pending waste removal by the contractor.

#### **PRIMARY REFERENCE STOCK SOLUTIONS**

##### **Primary Analytical Standard Reference Material**

Pemoline (Sigma, Lot# 38C0136, P-6260, R-PEM-1)

Formula Weight: 176.18. Pemoline is a Federal (USA) controlled substance and is kept in a locked safe, access of which requires the presence of two permanent employees (Chemist or higher in position) and signing of the accession log on the safe.

##### **Primary Analytical Internal Standard Reference Material**

Clonixin (Schering Research Division, Cat. #. Sch 10304).

Formula Weight: 262.70

*Obtain these materials from the QAO or Safe. Record accession of these materials on the pharmacy and Safe log sheets.*

#### **I. PREPARATION OF PRIMARY REFERENCE STOCK SOLUTIONS**

**Pemoline: 1 mg/ml in stock solution in methanol.**

##### **Materials**

Pemoline free base

Methanol

##### **Procedure**

Weigh out between 5 to 10 mg (X.xx mg) of pemoline standard powder into a glass bottle.  
 Dilute to volume using HPLC grade (or better) methanol (Volume Y.yy mL = X.xx).  
 Cap and mix until pemoline is completely dissolved in methanol.  
 The resulting concentration of pemoline is 1 mg/mL.  
 Store at approximately 4 °C (refrigerator).

*Complete Balance Use Log and QA Primary Reference Standard Log for this process.  
 Label the primary reference stock solutions with QA Primary Reference Log SR# 779 and Primary Reference Powder Designation (R-PEM-1).*

**Clonixin (IS):** 1 mg/mL of stock solution in methanol

**Materials**

Clonixin free base  
 Methanol

**Procedure**

Weigh out between 5 and 10 mg (X.xx mg) Clonixin into a glass bottle.  
 Dilute to volume using HPLC grade (or better) methanol (Volume Y.yy mL = X.xx).  
 Cap and mix until Clonixin is completely dissolved in methanol.  
 The resulting concentration of Clonixin is 1 mg/mL  
 Store at approximately 4 °C (refrigerator).

*Complete Balance Use Log and QA Primary Reference Standard Log for this process.  
 Label the primary reference stock solutions with QA Primary Reference Log SR# 754 and Primary Reference Powder Designation ( R-Clon-1).*

**II. PREPARATION OF SECONDARY REFERENCE STOCK SOLUTIONS**

Materials Needed: Pemoline Primary Reference Stock (1 mg/mL)  
 ACN:H<sub>2</sub>O:FA (50:50:0.1,v/v/v)

Prepare Pemoline Secondary Reference Stock solutions according to Table 1. All secondary reference working solution are prepared from 1 mg/mL stock solution (SR# 779).

Table 1. Standard working solution preparation of Pemoline

Target Conc. (µg/mL)	Made from stock solution (µg/mL)	Vol. Added (µL)	Vol. ACN/H <sub>2</sub> O/FA (50:50:0.1) (µL)	Conc. Added 10 uL in 1 mL of plasma (ng/mL)
10.0	1000.00	10	990	100
5.0	1000.00	5	995	50
1.0	10.00	100	900	10
0.5	5.00	100	900	5
0.1	1.00	100	900	1
0.05	0.50	100	900	0.5
0.01	0.10	100	900	0.1
0.005	0.05	100 4	900	0.05

Label secondary reference stock solutions with the Label designated in Table 1 and record preparation and labeling in the secondary preparation logbook in the appropriate Unit of the Laboratory.

**III. PREPARATION OF PEMOLINE QC WORKING SOLUTIONS**

Materials Needed: Pemoline Primary Reference Stock Solution  
 ACN:H2O:FA (50:50:0.1,v/v/v).

Prepare Pemoline QC Working solutions according to Table 2. All secondary reference QC working solution are prepared from 1 mg/mL stock solution (SR# 779).

Table 2. QC working solution preparation of Pemoline

Target Conc. (µg/mL)	Made from stock solution (µg/mL)	Vol. Added (µL)	Vol. ACN/H2O/FA (50:50:0.1) (µL)	Conc. Added 10 µL in 1 mL of plasma (ng/mL)
5.00	1000.00	5.0	995.0	50.0
0.50	5.00	100.0	900.0	5.0
0.05	0.50	100.0	900.0	0.5

*Label Pemoline QC working solutions with the Label designated in Table 2. Record the preparation and labeling in the secondary preparation logbook in the appropriate Unit of the Laboratory.*

**IV. PREPARATION OF PLASMA CALIBRATORS AND QUALITY CONTROLS**

The following calibrators are prepared in negative pooled equine plasma previously demonstrated by SOP to be negative for pemoline. All pemoline-spiked plasma calibrators and QCs are freshly prepared the same day as the sample analysis.

Table 3. Preparation of Pemoline equine plasma calibrators

Calibrator Code #	Target Conc. (ng/mL)	Working Solution (µg/mL)	Spike Working Solution (µL)	Volume of Plasma (mL)
MMDDYYPemoline0.05	0.05	0.005	5	0.5
MMDDYYPemoline0.1	0.10	0.01	5	0.5
MMDDYYPemoline0.5	0.50	0.05	5	0.5
MMDDYYPemoline1.0	1.00	0.1	5	0.5
MMDDYYPemoline5.0	5.00	0.5	5	0.5
MMDDYYPemoline10.0	10.00	1	5	0.5
MMDDYYPemoline50.0	50.00	5	5	0.5
MMDDYYPemoline100.0	100.00	10	5	0.5

Table 4. Preparation of Pemoline Equine Plasma Quality Control (QC) Sample

QC Code#	Target Conc. (ng/mL)	Working Solution (µg/mL)	Spike Working Solution (µL)	Volume of Plasma (mL)
Plasma NQP XX MMY*	0.0	0.0	0.0	0.5
MMDDYYPemoline 0.5 QC	0.5	0.05	5	0.5
MMDDYYPemoline 1.0 QC	1.0	0.10	5	0.5
MMDDYYPemoline 5 QC	5.0	0.50	5	0.5
MMDDYYPemoline 50 QC	50.0	5.00	5	0.5

\*: XX represents the batch No, MMY represents the month and year the negative plasma was collected.

- Record the preparation and labeling in the secondary preparation logbook in the appropriate Unit of the Laboratory.
- Prepare 16x125 mm screw cap culture tubes (12 of 12 types from Table 3 and 4.)
- Dispensing of pemoline negative plasma, adding 0.5 ml plasma into each labeled tube.
- Spiked 5 uL of each calibrator and QC in Table 3 and 4 into individual tube, respectively.
- Vortex and label each tube.

#### **V. PREPARATION OF CLONIXIN INTERNAL STANDARD WORKING SOLUTION**

Materials needed: 1 mg/mL of Clonixin primary reference stock  
 ACN:H<sub>2</sub>O:FA (50:50:0.1, v/v/v)

Procedure:

- Dilute 10 uL of 1.0 mg/mL of Clonixin standard stock solution in a glass vial to volume using 990 uL of 2 mM NH<sub>4</sub>AC:MeOH (50:50, v/v). Mix.
- The final concentration of Clonixin is 10 µg/mL.

Storage Requirements

- Store at approximately 4 °C (refrigerator).

#### **VI. SAMPLE REQUIREMENTS FOR ANALYSIS**

Prepare Calibrators, Quality Control samples (including negative control), and suspect samples (in triplicate) for each analysis performed.

## **VII. SAMPLE EXTRACTION BY LIQUID-LIQUID EXTRACTION**

Remove one set of previously prepared 0.5 mL calibrators and quality control samples from freezer storage and thaw.

1. Dispense 0.5 mL suspect sample (in triplicate) into individual clean, labeled 16x125 mm screw cap culture tubes.
2. Add 10  $\mu$ L of 10  $\mu$ g/mL clonixin into each tube, mix.
3. Add 1.5 mL of 0.1 M phosphate buffer, pH 5.0, into each tube, mix.
4. Add 5 mL of ethyl acetate (EtoAc) into each tube (calibrators, controls, and samples) and extract for 10 minutes by mixing on a rotorack. Centrifuge at 3000 rpm for 10 minutes.
5. Transfer each top EtoAc layer into a clean, labeled culture tube.
6. Repeat steps 4 to 5.
7. Combine the extracted solution.
8. Evaporate to dryness at 60 °C under steady stream of nitrogen or air.
9. Reconstitute the residues with 100  $\mu$ L of 2 mM NH<sub>4</sub>AC: ACN (5:95, v/v).
10. Transfer the above solution into labeled auto sampler vials fitted with limited volume inserts, cap. All the samples are now ready for LC/MS/MS analysis.

## **VIII. LIQUID CHROMATOGRAPHIC/MASS SPECTRAL IDENTIFICATION OF PEMOLINE**

### **Liquid Chromatographic and Mass Spectrometer Operating Parameters**

**Instrumentation:** Thermo-Finnigan TSQ Quantum mass spectrometer LC-MS system equipped with an electrospray ionization (ESI) probe. a Surveyor LC pump with an on-line degasser, a Surveyor autosampler. Xcalibur software is used for system control, acquisition, and data processing.

**LC Column:** MAC-MOD Ace C<sub>8</sub> column with guard column (Mac Modd, Chadds Ford, PA, USA)  
Length: 50 mm  
i.d. 2.1 mm  
Particle size: 5 micron  
Temperature: 27 ° C

**Mobile Phase:** A: 2 mM Ammonium acetate:acetonitrile:ammonium Hydroxide (NH<sub>4</sub>AC:ACN: NH<sub>4</sub>OH; 95:5:0.01, v/v/v; pH 4.70)  
B: 2mM (Ammonium acetate: Acetonitrile (NH<sub>4</sub>AC:ACN; 5:95, v/v)

**Gradient for Pemoline:**

Table 5. HPLC Condition for the Separation of Pemoline

Time (min)	A	B	Flow rate (µL/min)
0	100	0	250
1.00	100	0	250
1.01	20	80	250
3.50	20	80	250
3.51	100	0	250
5.00	100	0	250

Injection Volume: 10 µL

**Mass Spectrometric Parameters:**

Table 6. Mass spectrometric parameters of Pemoline

Analyte	Rt (min)	[M+H] <sup>+</sup>	BP product ion	Tube Lense Offset	CE	Spray voltage	Sheath gas pressure	Aux gas pressure	Capillary Temp (°C)	Collision pressure	Source CID
Pemoline	2.60	177.0	106.0	134	10	5000	47	25	285	1.5	20
Clonixin (IS)	2.85	263.0	245.0	94	18	4800	21	15	285	1.5	18

**IX. SAMPLE LIST SETUP FOR PEMOLINE ANALYSIS**

1. Mobile Phase Blank
2. Mobile Phase Blank
3. Column test1
4. Mobile Phase Blank
5. Mobile Phase Blank
6. Column test2
7. Mobile Phase Blank
8. Mobile Phase Blank
9. Column test3
10. Mobile Phase Blank

- 
11. Mobile Phase Blank
  12. Blank Plasma (QC Negative Control)
  13. Calibrator Series 1
  14. Mobile Phase Blank
  15. Mobile Phase Blank
  16. Quality Control Series 1 (Positive Controls)
  17. Mobile Phase Blank
  18. Mobile Phase Blank
  19. Sample 1, Replicate 1
  20. Sample 1, Replicate 2
  21. Sample 1, Replicate 3
  22. Repeat steps 17 through 21 for each additional sample
  23. Mobile Phase Blank
  24. Mobile Phase Blank
  25. Quality Control Series 2 (Positive Controls)
  26. Mobile Phase Blank
  27. Mobile Phase Blank
  28. Calibrator Series 2
  29. Mobile Phase Blank
  30. Mobile Phase Blank

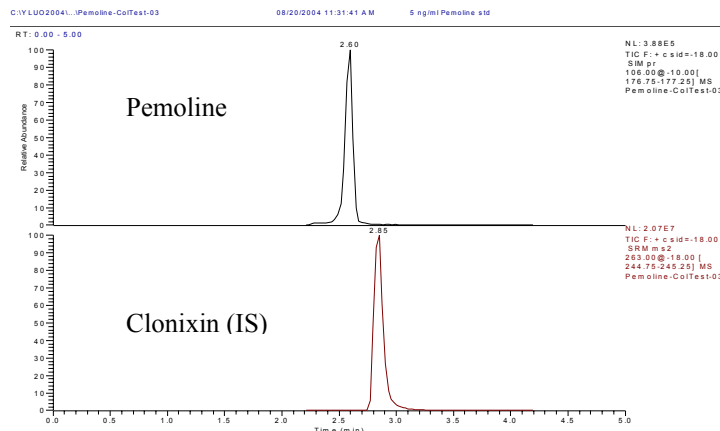


Figure 2. Data Dependent scan chromatograms of Pemoline and Clonixin (IS)

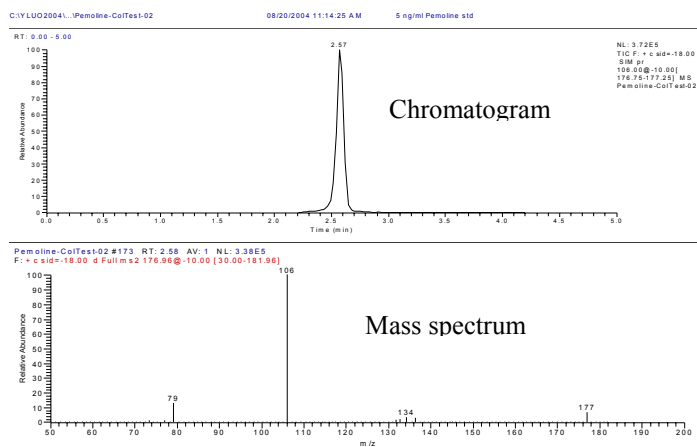


Figure 3. Data Dependent scan chromatogram and Mass Spectrum of Pemoline

## X. CRITERIA FOR IDENTIFICATION OF PEMOLINE FROM EQUINE PLASMA EXTRACT

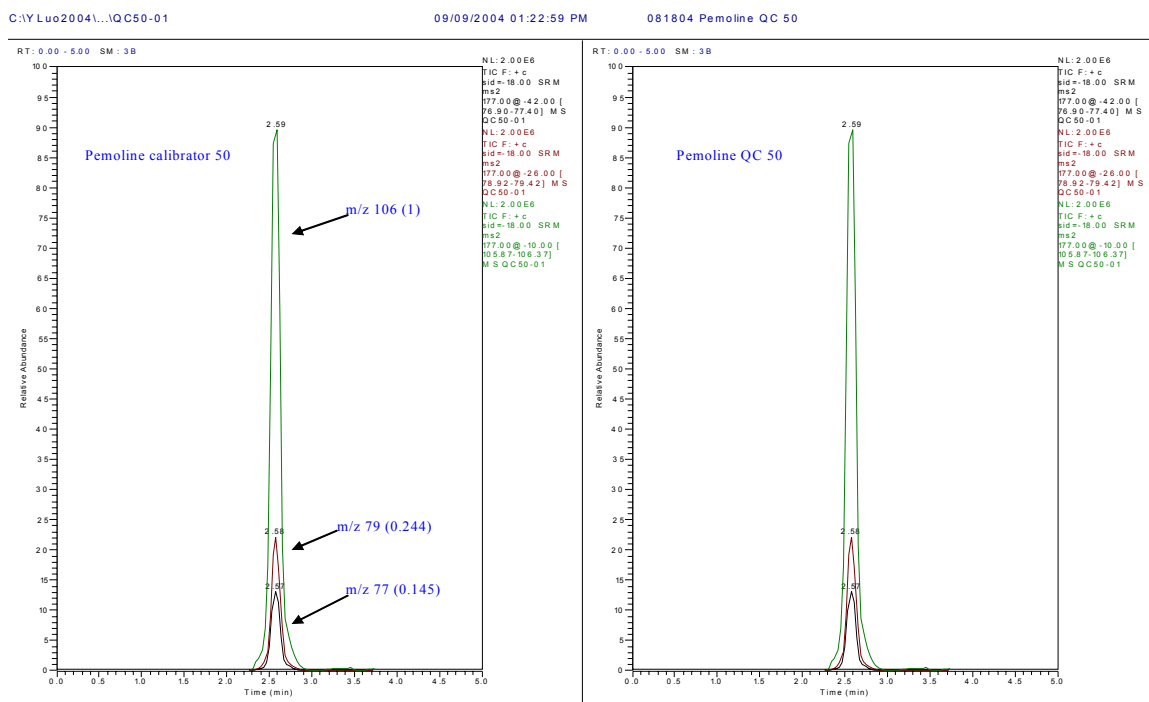
### **Identification of Pemoline**

The qualifying diagnostic ions for identification of Pemoline are  $m/z$  177  $[M+H]^+$ , 106 (BP), 79 and 77 (Figure 3, Bottom Panel).

All qualifying ions for Pemoline are present in the MRM chromatogram, the relative coefficient variation mass ratios should be less than 5% ( Table 7 ), and the retention time for the suspect sample, 5 ng/mL calibrator, and 5 ng/mL QC control agree to  $t_R \pm 0.15$  minutes for each individual analyte QC control, respectively (Figure 4).

**Table 7. Mass ratio determination of Pemoline MRM confirmation in Equine Plasma (n=5)**

Product ions	Determined mass ratios <sup>a</sup>	C.V.(%)
77	0.145 ± 0.002	1.38
79	0.244 ± 0.003	1.23
106	1.000	0.00



**Figure 4. MRM chromatograms of Pemoline in Plasma Calibrator and QC**

**Identification of Clonixin (IS)**

The qualifying and quantifying ions for the internal standard, Clonixin, are m/z 263 [M+H]<sup>+</sup> and 245 (BP). Under LC-MS/MS analytical conditions described, all the above diagnostic ions should be recognized at retention time of ~2.85 min.

**XI. CRITERIA FOR PEMOLINE QUANTIFICATION IN EQUINE PLASMA**

**Determination of Pemoline**

Using the Xcalibur Quantification software, execute quantification method\Pemoline\LC-MSmethod\Pemolinedatadependentscreening. Print the compound summary quantification report and calibration curve. The correlation should be greater than 0.995%.

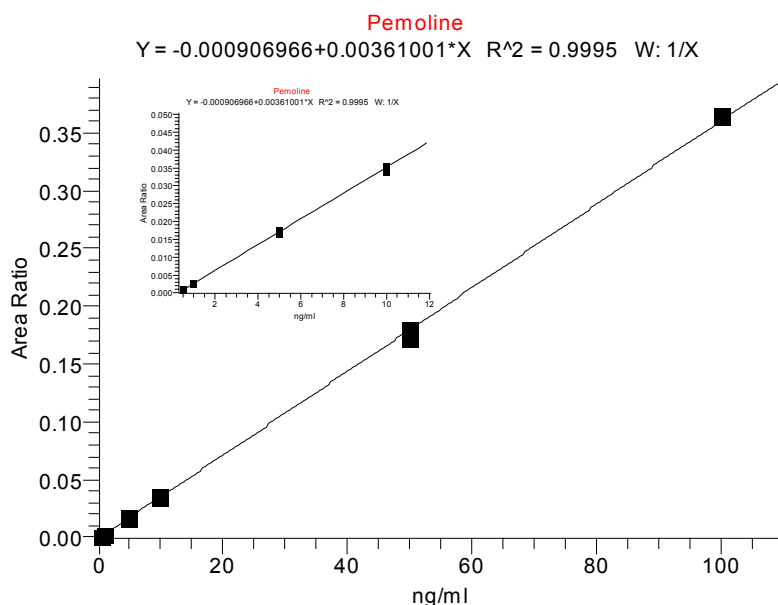


Figure 5. Calibration Curve of Pemoline in Equine Plasma. The insert represents calibration curve for the lower concentrations of pemoline

Examine the reported concentration for all samples. The accuracy of concentration of QC samples should be 80% - 120% for Pemoline.

The selected mass of Pemoline for quantification is m/z 106.

## **XII. CRITERIA FOR REPORTING A SAMPLE POSITIVE FOR PEMOLINE**

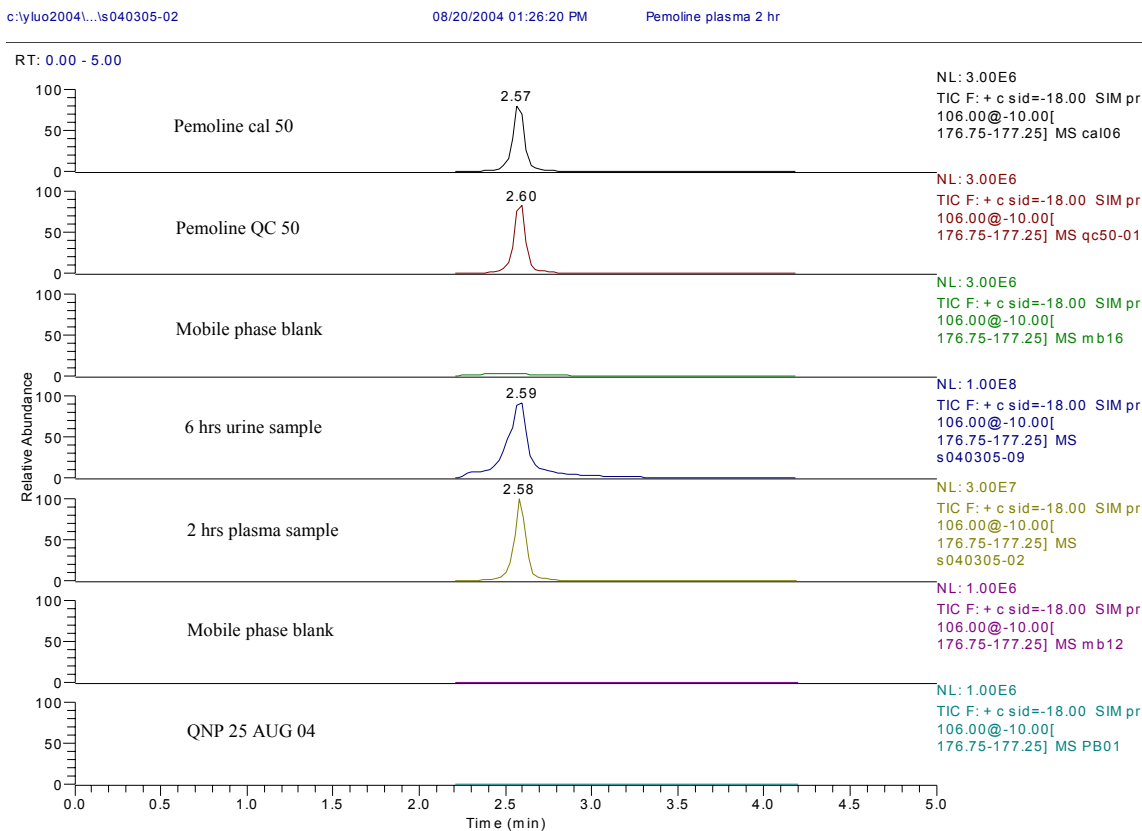
Report a test sample as positive per this standard operating procedure for Pemoline if ALL of the following criteria are met:

The test sample contains pemoline according to the chromatographic and data dependent mass spectra.

All confirmable suspect sample for MRM chromatograms should contain three selected ions of Pemoline (m/z 77, 79 and 106); for data dependant scan pemoline should contain m/z 79, 106 and 177).

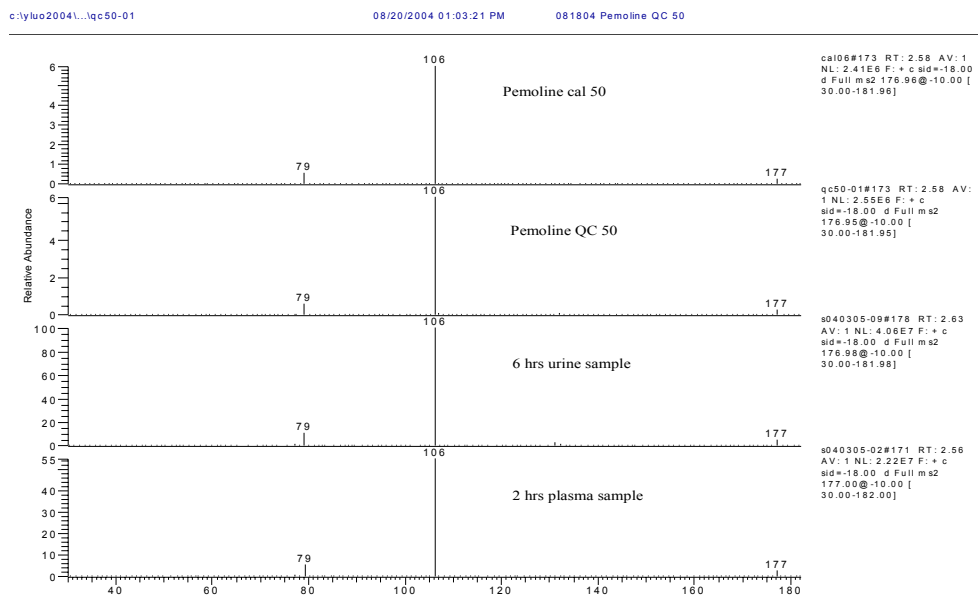
The LC retention times of the quantifying ion for Pemoline in the sample, 5 ng/mL QC control and the 5 ng/mL calibratorars within +/- 0.15 minutes. This is determined by inspection of the extracted ion chromatogram comparisons that are included in the analysis data packet. These chromatograms may be subtracted and/or smoothed.

The signal to noise ratio of the quantifying ions for Pemoline and internal standard (Clonixin) is greater than 5. This is determined by inspection of the data dependent scan mass spectra comparisons, which are included in the analysis data packet. These spectra should be averaged across the chromatographic peak at 20 % peak height. These spectra may be subtracted and/or smoothed.



**Figure 6. LC-MS chromatograms of Pemoline in Equine Plasma Post Pemoline ((500 mg, po) Administration to a Horse**

All blanks and Negative Plasma Control Samples should not contain quantifiable and confirmable pemoline concentration greater than 0.2 ng/mL (Figure 11).



**Figure 7. Data Dependent Mass Spectra of Pemoline in Equine Plasma Samples**

### **XIII. INTEGRATION**

The integration parameters of the quantifying method (\Pemoline\LC-MS\ have been set to produce consistent and reproducible integration from run-to-run and day-to-day. However, samples and conditions vary; therefore each chromatogram in the analysis panel must be individually inspected for proper integration. If improper integration is found, it may be manually corrected. With excessive manual corrections, the supervisor must be consulted to either:

Adjust the integration program parameters or

Investigate deteriorating conditions of:

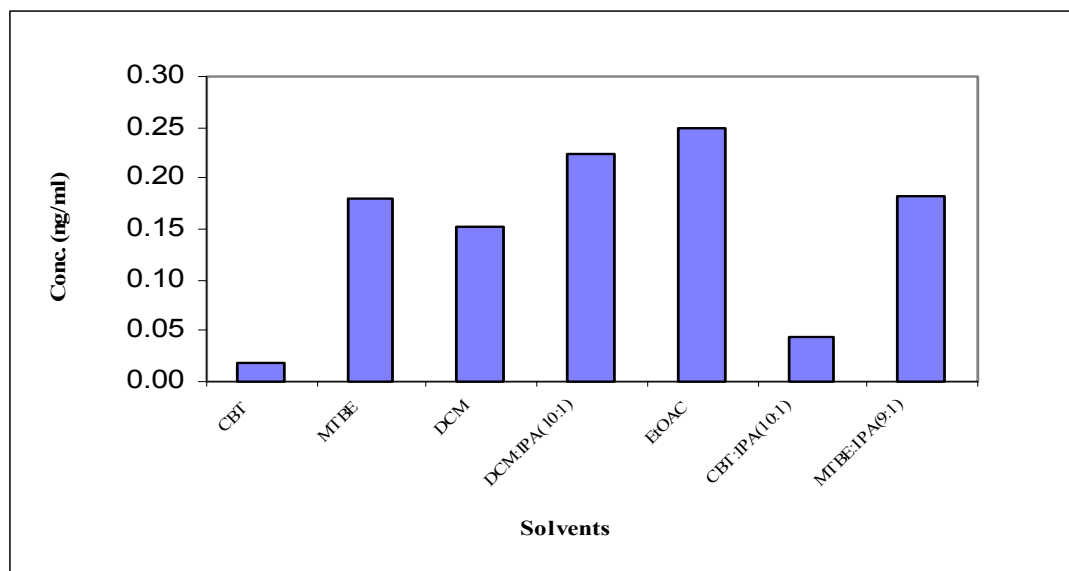
- The samples and standards
- Chromatographic system
- Mass spectrometer system

These are the only conditions that allow manual integration. Reproducible and systematic identification of deviations are an absolute requirement of a quantitative method to determine method and result precisions and confidence limits.

### **XIV. METHOD VALIDATION**

#### **Extraction Efficiency of Pemoline Using Various Organic Solvents**

The extraction efficiency of pemoline by different organic solvents is compared experimentally at pH 6.0. The results are listed in Figure 7. Of all the selected organic solvents used, ethyl acetate (EtOAc) is the most efficient extraction solvent for pemoline in plasma.



**Figure 8. Extraction efficiency of various organic solvents for pemoline (5 ng/mL) spiked into blank equine plasma ( n=5 ).**

Ethyl acetate (EtOAc) provided the best extraction efficiency than any other solvents, and thus it was used as the extraction solvent.

**Effect of Various pH Values on the Extraction of Pemoline from Equine Plasma**

The effect of various pH values on the extraction of pemoline from plasma was compared. Figure 8 shows the influence of pH 5 on the recovery of pemoline from plasma matrix. Sample at pH 5.0 provided the best recovery of pemoline from plasma matrix and thus all samples were adjusted to pH5.0 prior to extraction by ethyl acetate (see extraction procedure for pemoline on page 7).

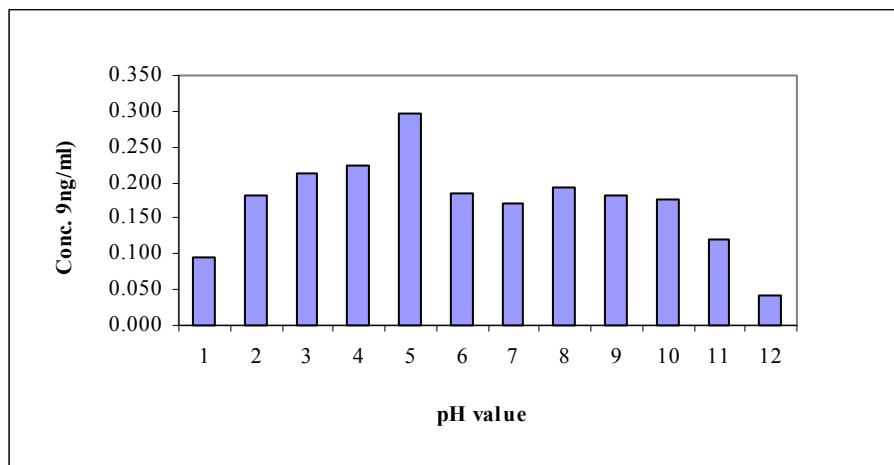


Figure 9. Influence of pH on the Extraction of Pemoline from Plasma (10 ng/mL spiked; n=5 ).

**Recovery of Pemoline from Equine Plasma**

All samples are adjusted to pH 5.0 and then extracted with EtoAc. The highest extraction efficiency is obtained for pemoline from equine plasma by ethyl acetate than all other solvents evaluated. The recovery of Pemoline by EtoAc from equine plasma at the concentration range of 0.5 ng/mL to 50 ng/mL is greater than 93% with coefficient of variation (CV %) of less than 9.5%. The recovery of varying concentrations of pemoline from equine plasma is listed in Table 7, page 10.

**Table 8. Recovery of Pemoline from Equine Plasma (n=6)**

Concentration spiked (ng/mL)	Concentration determined (ng/mL)	Recovery (%)	C.V. (%)*
0.5	0.47 ± 0.043	93.67 ± 8.55	9.13
5.0	4.96 ± 0.42	99.15 ± 8.44	8.51
50.0	52.50 ± 3.30	105.01 ± 6.61	6.29

\*C.V. (%) = standard deviation of the concentration detected/mean concentration detected x 100.

**METHOD VALIDATION**

The method is validated under the guidelines presented by Shah et al<sup>2</sup>. Twelve assays for validation are performed; six for within-run (intra-day assay) and six for between-run (inter-day) to assess precision, accuracy and specificity of the method.

**Intra-day and Inter-day Precision and Accuracy**

Inter-day and intra-day assay accuracy and precision are determined by analyzing two separate sets of eighteen validation samples at three concentrations for Pemoline (0.5, 5 and 50 ng/mL equine plasma) in six separate experiments, respectively. The concentrations of pemoline correspond to low, medium and high for constructing the calibration curves. Intra-day assay accuracy and precision are determined by analyzing six replicates of the three concentrations in each experiment. Inter-day assay accuracy and precision are determined by analyzing triplicates of the three concentrations in six different days, respectively. Accuracy is determined as the agreement between the concentration of the target analytes detected and that spiked into blank plasma. Precision of the assay is determined as the relative standard deviation expressed as a percentage of the standard deviation divided by the mean of observed concentration and is reported as percent coefficient of variation.

**Table 9. Intra-day and Inter-days Precision and Accuracy of Pemoline in Equine Plasma ( n= 6 )**

Pemoline added (ng/mL)	Intra-day			Inter-day		
	Pemoline determined	C.V. <sup>a</sup> (%)	AR <sup>b</sup> (%)	Pemoline determined	C.V. <sup>a</sup> (%)	AR <sup>b</sup> (%)
0.5	0.50 ± 0.012	2.40	100.00	0.51 ± 0.088	1.72	102.00
5.0	5.00 ± 0.12	2.40	100.00	5.07 ± 0.037	0.72	101.40
50.0	50.34 ± 0.48	0.96	100.68	50.77 ± 0.47	0.92	101.54

a: Coefficient of variation (C.V.%) = standard deviation of the quantity detected/mean quantity detected x 100.

b: Accuracy (AR%) = mean of detected quantity/spiked quantity x 100.

**EVALUATION OF STABILITY OF PEMOLINE IN EQUINE PLASMA**

Since the official race samples are not delivered to the laboratory for detection and determination on the same race day that the race is conducted, plasma samples are usually stored at -20 °C for a day or two after the blood is collected. For this reason, the effect of temperature on the stability of the target compounds in plasma is critical for maintaining the integrity of the samples so that accurate information can be obtained during analysis. Considering the fact that the samples may encounter different temperature conditions during transit and take some time to reach the laboratory during the sample delivery process, the stability experiment is designed to

accommodate or reflect the possible conditions the samples are likely to be exposed to from collection to delivery to the laboratory for analysis. In this SOP, pemoline, in three different concentrations (0.5 ng, 5.0 ng and 50 ng) is examined. Each concentration is spiked into 0.5 mL negative equine plasma. The stability of the analyte is evaluated at different storage temperatures (25 °C, 4 °C, – 20 °C and -70 °C). At room temperature (25 °C), 24 hr stability (0, 2, 4, 6, 8, 10 and 24) for the spiked plasma samples is evaluated. For short-term storage (4 °C), stability of the analyte is evaluated following storage for 10 days, and for long-term storage (– 20 °C and – 70 °C) stability of the analyte is evaluated after storage at these temperatures for five weeks (35 days). It should be noted that at each time period, samples are thawed, aliquot taken for analysis and the original samples are returned for storage at the previous temperature. This process allows evaluation of the effect of freeze-thaw cycles on the stability of the analyte. The peak areas averaged from triplicate samples are compared with those of the relevant 0 hr sample and other samples at different times and different temperatures, thus, the percent change of the analyte at the specified temperature is calculated.

**Table 10. Stability of Pemoline in equine plasma at various temperatures ( n=6)\***

Concentration added (ng/mL)	Concentration percentage determined at different conditions of sample storage				
	25 °C, 0 Hour	25 °C, 48 hours	4 °C, 10 Days	-20 °C, 35 Days	-70 °C, 35 Days
0.5	100	93.08 ± 01.43	94.40 ± 10.43	102.95 ± 5.99	100.94 ± 0.87
5.0	100	98.91 ± 7.25	96.70 ± 7.74	100.01 ± 1.06	100.01 ± 1.06
50.0	100	100.33 ± 4.09	98.80 ± 4.29	99.50 ± 2.27	99.50 ± 2.27

\*Stability is expressed as percent concentration (concentration detected divided by concentration added x 100).

**Demonstration of the Absence of Ionization Suppression or Enhancement**

Because parent-product ion LCMSMS is target compound specific, the determination of interfering substances can be only partially based on the purity of the product ion full scan mass spectrum. Co-eluting substances with parent ions differing from the target parent ion may still exert either enhancement or suppression of the ionization process, thus posing a severe challenge to the validity of quantitative results. Therefore, ionization stability is determined for the chromatographic and mass spectrometric conditions described in this SOP. This determination is for pemoline.

Pemoline is examined in three different concentrations (0.5 ng/mL, 5 ng/mL and 50 ng/mL). Each concentration is added to each dried extract of 0.5 mL negative equine plasma followed by

drying and dissolution in 100 µL LC sample solvent. The same concentration of each analyte in methanol is dried and dissolved in the same volume of the LC sample solvent. An aliquot of 10 µL is injected into LC-MS and analyzed. The chromatographic peak areas averaged from six sample duplicates for the standards in sample solvent and for the standards added to plasma extracts and reconstituted as described above are used for estimation of the contribution of the sample matrix to ion suppression or enhancement.

Endogenous compounds extracted from a sample matrix might suppress or enhance ionization of an analyte(s) recovered from that matrix resulting in the change of signal intensities of the analyte(s), especially when ESI mode is applied. Matrix effect may occur in any biological samples, including plasma and urine, etc. In this experiment, matrix effect on the compounds of interest is evaluated. As mentioned in the experimental section of this SOP, matrix effect is determined by comparing the chromatographic peak areas of each drug standard with those of the drug standard added to the extracts of 0.5 mL negative plasma, according to the following equation below:

**Table 11. Matrix Effect of Equine Plasma on Pemoline LC-MS Analysis (n=6)**

Pemoline spiked (ng/mL)	Blank plasma	Determined standard only (ng/mL)	Determined with blank plasma extract (ng/mL)	Martrix effect <sup>a</sup> (%)
0.5	0.00	0.50 ± 0.006	0.55 ± 0.013	10.00
5.0		4.97 ± 0.11	5.06 ± 0.15	1.81
50.0		50.02 ± 1.82	50.70 ± 1.13	2.03

$$\text{Ion suppression or enhancement (\%)} = (1 - A_{\text{extract}} / A_{\text{standard}}) \times 100$$

where  $A_{\text{standard}}$  is the concentration of a drug standard, and  $A_{\text{extrac}}$  is the concnetration of the same quantity of the drug standard added to the extract of a given plasma sample. As shown in Table 10, ion suppression or enhancement by plasma is less than 10% for all Pemoline results evaluated. The results show that the contribution by matrix effect on the analysis of pemoline is insignificant under the experimental conditions described.

**MEASUREMENT UNCERTAINTY**

The following statements define the estimation of Measurement Uncertainty (MU):

**Table 12. Estimation of Measurement Uncertainty in the Quantification of Pemoline in Plasma**

Symbol	Source of Uncertainty	Value Units (%)	Distribution	Divisor	Standard Uncertainty	Degrees of Freedom (n-1)	Other
U <sub>1</sub>	Intermediate precision	4.03	N	1	4.03	10	Pemoline 0.5 ng/ml
U <sub>2</sub>	Intermediate precision	3.02	N	1	3.02	10	Pemoline 5.0 ng/ml
U <sub>3</sub>	Intermediate precision	1.43	N	1	1.43	10	Pemoline 50.0
Combined Uncertainty		$(U_1^2)^{1/2} = 4.03; (U_2^2)^{1/2} = 3.20; (U_3^2)^{1/2} = 1.46$					
Expanded Uncertainty (k=2.3)		1: (4.03 x 2.3) = 9.27%; 2. (3.02 x 2.3) =6.95%; 3. (1.46 x 2.3) =3.36%;					

Method Uncertainty is initially established based on method validation.

1. The 95% confidence interval is expressed as +/- Standard Deviation x Coverage factor (k) (SD x k) for both unknown determinations as well as control values.
2. In using Laboratory Control Samples (LCS) or Quality Control Samples (QCS) for estimating MU, use of k= 2.3 (Coverage Factor) is recommended.
3. Control records and charts (5 ng/mL n=4) for method development and all analyses are created and maintained.

An Excel template is provided to perform several quality control functions. For every analysis, all samples will be entered into the template.

Automatic building and charting of historical control database with run and historical 95% confidence interval plotting will be maintained

Each entry is saved to the project (\\Xcalibur\Pemoline\Data) folder and is maintained as part of the analysis record archive.

**Demonstration of Specificity of the Method:**

Specificity of method is defined as the ability to measure the analyte of interest accurately and specifically in the presence of other components that could be expected to be present in the sample matrix. It is a measure of the degree of interference from things such as other active ingredients, impurities and unknown products. Specificity as an identification test ensures the identity of the analyte of interest by the method described. For this purpose, an administration of pemoline (250 mg; po) to a research horse was performed at New Bolton Center. Pre and post urine and plasma samples were collected. Post administration samples were collected at 2, 6, 8, 10 and 24 hr. The

concentration of pemoline in plasma and urine are shown in Table 13 and Figure 10 below. Since the concentrations of pemoline in plasma (172.45 ng/mL) and urine (1824.53 ng/mL) were the lowest at 24 hr compared to other time periods post pemoline administration, an attempt to confirm the presence of pemoline in urine and plasma samples was attempted (see Figures 11 and 12).

**TABLE 13: PEMOLINE CONCENTRATION IN PLASMA AND URINE POST ADMINISTRATION OF PEMOLINE**

Time (hr)	Plasma	Urine
0	0.00	0.00
2	423.63	4526.89
6	331.31	3152.99
8	309.94	2725.66
10	274.82	2388.52
24	172.45	1824.53

Figure 10 shows the plot of concentration of pemoline in plasma (black line) and urine (pink line) versus time. It is obvious that with a single oral dose of pemoline (250 mg) administration to a horse, the concentration of pemoline in urine is significantly higher than that in plasma and remains high into 24 hr post pemoline administration. Figures 11 and 12 show MRM chromatograms of pemoline in equine plasma and urine 24 hours post the administration of pemoline, respectively. The numbers in parentheses show the ratios of the most intense product ion (106 m/z) to the other product ions at the same retention time. These results indicate that by using the method described in this SOP, the presence of pemoline in both plasma and urine samples was confirmed. The method is therefore specific for the detection, identification, quantification and confirmation of pemoline in equine plasma and urine.

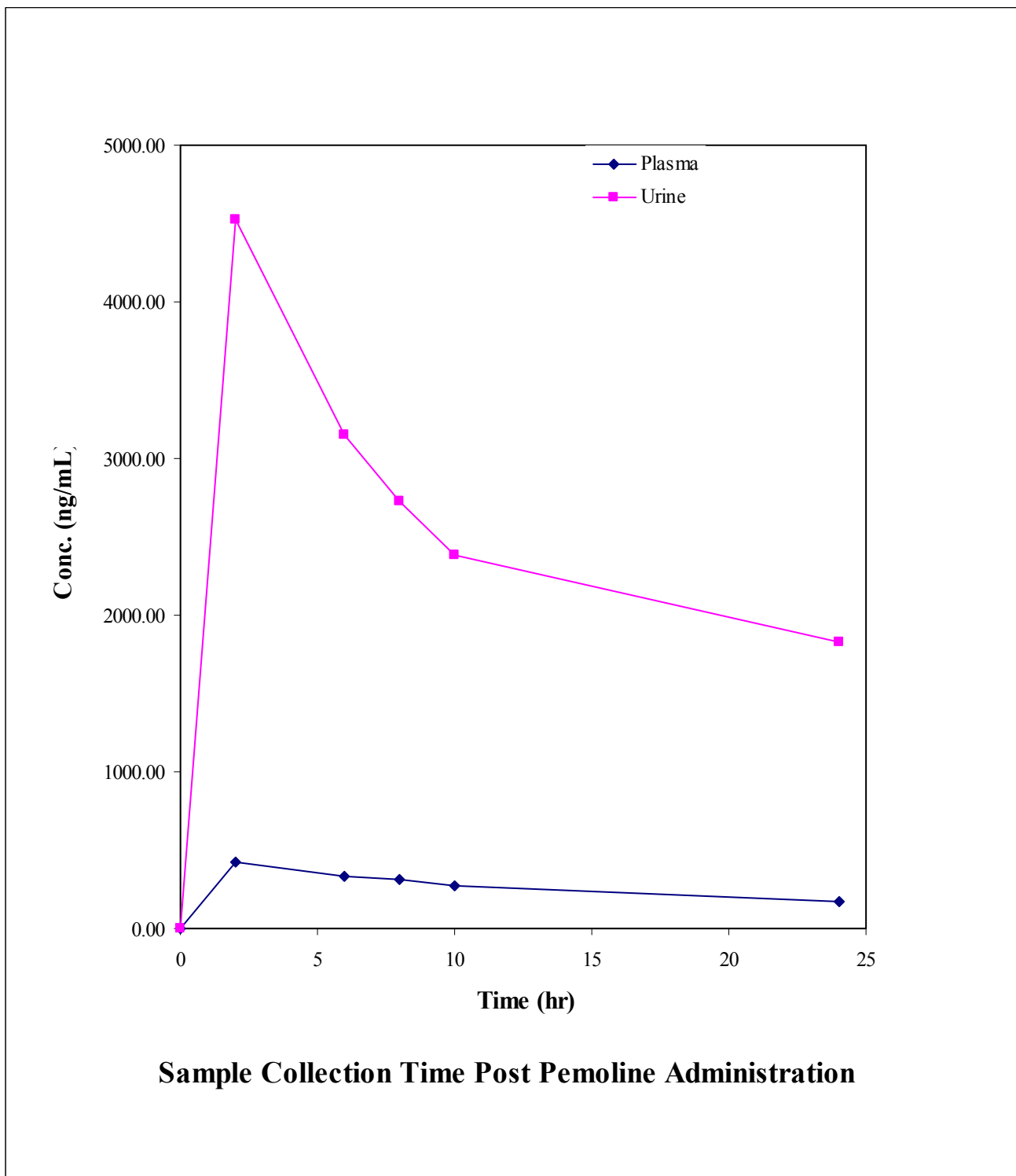


Figure 10: Concentration of pemoline in urine (pink) and plasma (blue) versus time of sample collection

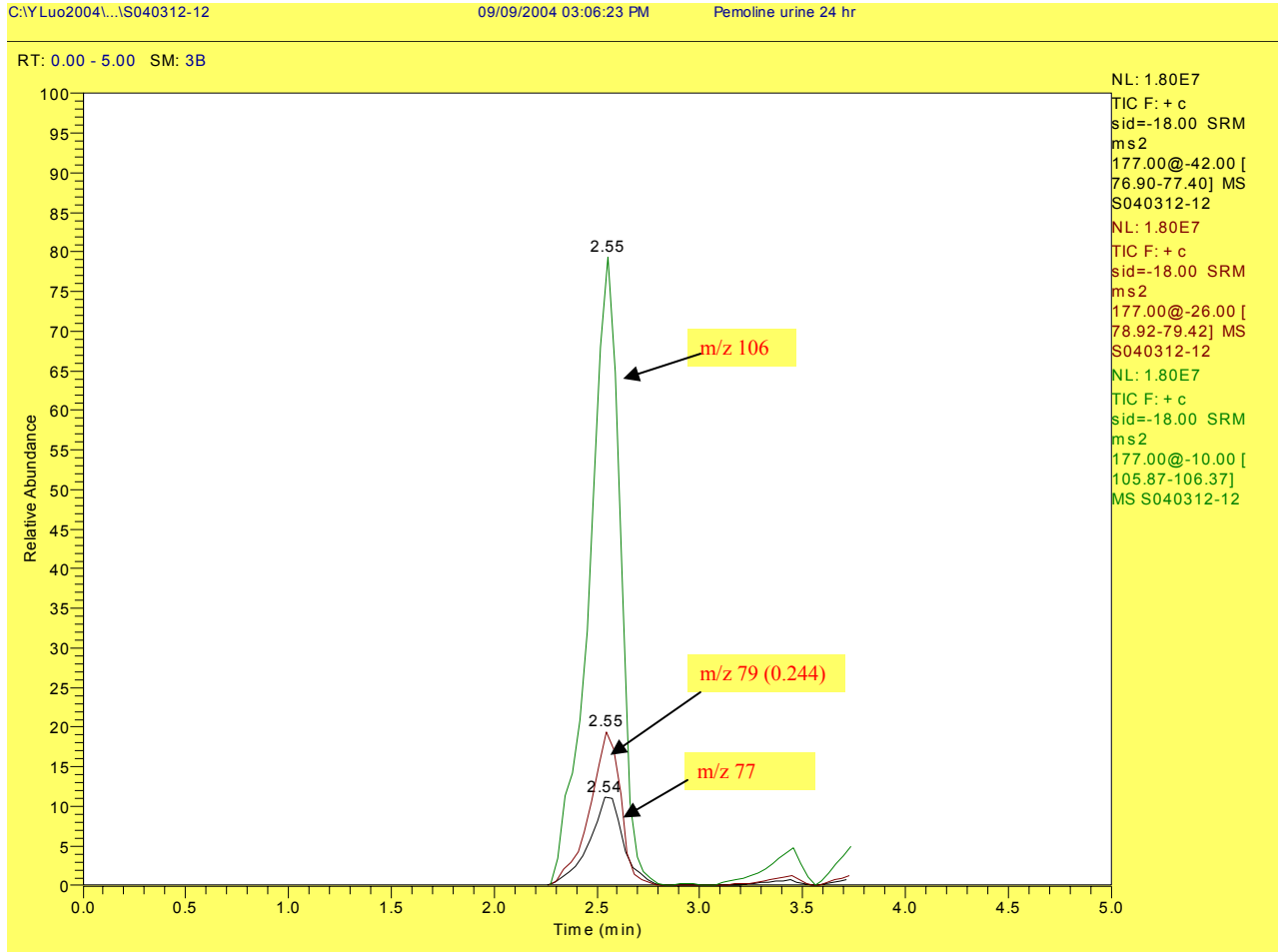


Figure11: MRM chromatogram of pemoline in equine urine sample collected 24 hr post administration of pemoline (250 mg, po) to a research horse.

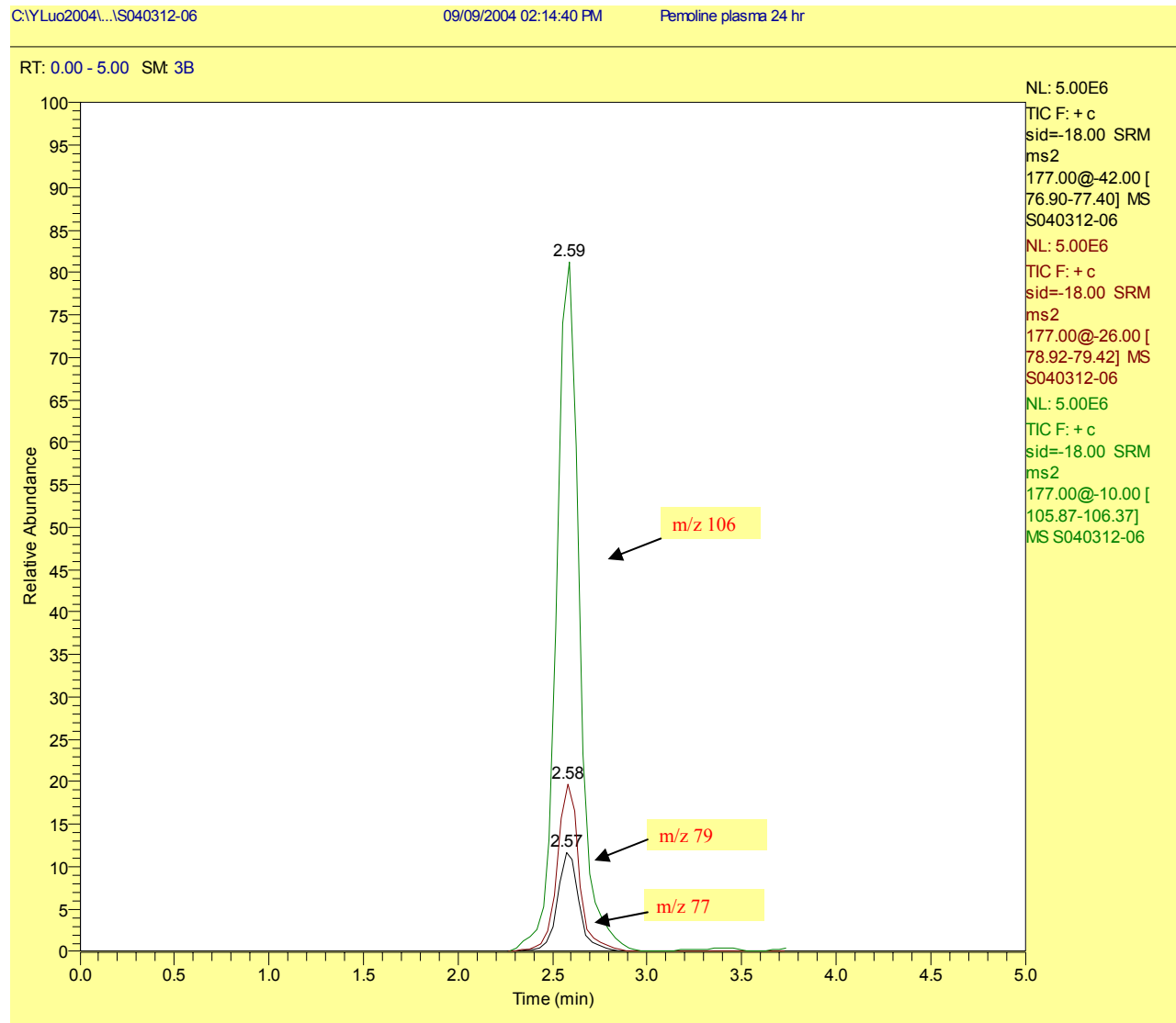


Figure 12: MRM chromatogram of pemoline in equine plasma sample collected 24 hr following the administration of pemoline (250 mg, po) to a research horse.

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## **XV. POSITIVE SAMPLE DATA PACKET ASSEMBLY ORDER**

1. SAMPLE TRANSFER SHEET (WS#32)
2. SAMPLE USAGE SHEET (FORM #7)
3. CONFIDENCE DETERMINATION REPORT
4. SAMPLE LIST
5. MASS CALIBRATION REPORT
6. TUNE PAGE SETTINGS
7. LC METHOD
8. MS METHOD
9. QUANTIFICATION REPORT
10. QUANTIFICATION CALIBRATION CURVE
11. COLUMN TEST CHROMATOGRAM
12. COLUMN TEST SPECTRUM
13. EXTRACTED ION CHROMATOGRAM COMPARISON
14. SPECTRA COMPARISON

### **Other required Documentation**

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

Sample list print-out that is maintained in the Quantum TSQ of three ring binder

Routine usage checklist completion (and maintenance log if needed)

Sample Analysis logbook, indicating date, project, operator initials, and description of official samples

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

1. SAMPLE TRANSFER SHEET (WS#32)
2. SAMPLE USAGE SHEET (FORM #7)
3. CONFIDENCE DETERMINATION REPORT
4. QUANTIFICATION REPORT

## **XVI. DATA ARCHIVING**

After successful assembly of required paper documentation for official analyses, whether positive or negative the data will be archived in the following manner:

On the Xcalibur workstation \\C: hard drive, create the following folder, if not already present:

\\C:\Project name (Drug name) for instance

C:\Yluo2004\Pemoline

Under this folder, create a folder with the following format:

DRUGmmdyy for instance:

C:\YLuo2004\Pemoline\data\ PemolineSample081704

Now copy ALL files from C:\Xcalibur\Project to the newly created archive folder.

Once all folders and files are successfully copied and verified, the files in C:\Xcalibur\Pemoline\Data may be deleted.

This manner of archiving is required by the Xcalibur software to allow the easiest and most complete reconstruction of all analysis scenarios.

In addition to this local archive, this hard drive is backed up on a weekly basis by our site Information Technologist.

## **Materials, Reagents, and Formulae**

### **I. REAGENTS**

- Methanol, HPLC grade (Cat. No. A 452-4, Fisher Scientific.)
- Acetonitrile, HPLC grade (Cat. No. A 452-4, Fisher Scientific.)
- Water, HPLC grade (Cat. No. W5-4, Fisher Scientific.)
- Ethyl Acetate (EtoAc), HPLC grade (Cat No. 9282-03, J.T. Baker)
- Ammonium Acetate, HPLC grade (Cat. No. CX0914-1, EM Science.)
- Ammonium Hydroxide, Certified A.C.S. PLUS (Cat. No. A669C-21, Fisher Scientific.)
- Phosphoric Acid, meets A.C.S. Specification (Cat. No. 0260-3, J.T. Baker Chemicals)
- Formic Acid, ACS reagent (EEC No. 200-5791, Sigma)
- Monobasic Potassium Phosphate, ACS reagent (Cat. No. P-0662, Sigma)

### **II. SOLUTIONS**

#### **0.1 M Phosphate Buffer ( pH 5.0)**

- Measure 100 mL of 1 M pH 5.0 Phosphate buffer
- Adjust to pH 5.0 with Ammonium hydroxide
- Bring to final volume of 1000 mL with HPLC grade water
- Mix thoroughly with stirring

#### **Acetonitrile:Water:Formic Acid ( 50:50:0.1 )**

##### **Procedure**

- a) Add 25 mL of acetonitrile to a liter glass container.
- b) Add 25 mL of water. Mix.
- c) Add 50 uL of formic acid. Mix.

##### **Storage Requirements**

Store at room temperature in a glass container. **Prepare fresh daily.**

#### **1 M Ammonium Acetate ( PH 5.0 )**

##### **Procedure**

- a) Weigh 74.1 g of ammonium acetate. Mix in 700 mL of water.
- b) Adjust pH to 5.0 with Acetic acid or Ammonium Hydroxide under stirring.
- c) Bring to 1000 mL with water and mix. The final pH value should be 5.0.

##### **Storage Requirements**

Store at 4 °C (refrigerator) in a glass container.

#### **HPLC Solvent A (2 mM Ammonium Acetate:Acetonitrile:Ammonium Hydroxide ( 95:5:0.01, v/v/v, pH 4.70 )**

##### **Procedure**

- a) Add 1 mL of 2 M ammonium acetate solution to 999 mL water. Mix.

- b) Add 950 mL to a 1000 mL glass bottle. Mix with 50 mL of Acetonitrile and 0.1 mL of ammonium hydroxide. Mix thoroughly before placing on HPLC. The final pH value should be 4.70, if not, adjust pH to 4.70 by using acetic acid or ammonium hydroxide while stirring.

**Storage Requirements**

Store at room temperature in a glass bottle.

**HPLC Solvent B (2 mM Ammonium Acetate:Acetonitrile, 5:95, v/v, pH  $\approx$  7.0)**

**Procedure**

- a) Add 950 mL of Acetonitrile into a glass bottle ( a liter ). Mix with 50 mL of 2 mM of ammonium acetate.  
b) Mix the solvent thoroughly before placing on HPLC.

**Storage Requirements**

Store at room temperature in a glass bottle.

**VII. MATERIALS**

16 x 100mm culture tubes.

16 x 125mm screw cap test tubes.

Pipettes and tips.

Vortex mixer (Scientific Industries, Inc. )

Branson Ultrasonic Water Bath, 8510 (Fisher Scientific or equivalent )

pH meter ( IQ Scientific Instruments )

Sample Concentrator ( Dri-Block DB-3, Techne )

IEC HN-SII Centrifuge (International Equipment Company )

Rotorack ( Specie-Mix, Thermolyne )

Kimwipes

2 mL auto sampler vials

200 uL Insert (Target PP Polyspring, National Scientific Company )

15 x 45 mm, 1 x 35 mm and 28 x 57 mm VWR brand vials

Balance (Mettler AT 261 Delta range, Mettler-Toledo Inc

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2. Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layoff I, Viswanathan CT, Cook CE, McDowall RD, Pittman KA, Spector S. Pharm Res. 1992; 9:588.