

**SCREENING, QUANTIFICATION AND CONFIRMATION OF KETOPROFEN IN  
EQUINE PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-  
TANDEM MASS SPECTROMETRY**

**DEVELOPED BY**

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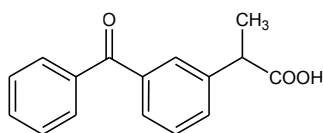
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## I. INTRODUCTION

Ketoprofen, 2-(3-benzoylphenyl) propanoic acid, is a non-steroidal anti-inflammatory drug (NSAID, Figure 1a), which has pharmacological actions similar to other drugs in this class, such as ibuprofen, flunixin, naproxen and phenylbutazone, to name a few. Ketoprofen is used in the management of acute and chronic rheumatoid arthritis and osteoarthritis. It functions as a dual inhibitor of cyclo-oxygenase and 5-lipoxygenase, which potentially broaden the anti-inflammatory effects of ketoprofen, making it a more effective NSAID than others. Ketoprofen are sometimes used in competing horses to mask signs of inflammation and pain. For this reason, it is classified by the Association of Racing Commissioners' International as a class 4 agent, and thus, its detection in post-race samples may lead to sanctions against the trainer(s) involved. It is for this reason that this Standard Operation Procedure was developed for screening, quantification and confirmation of ketoprofen in equine plasma to assist in the regulation of the use of ketoprofen in racehorses.

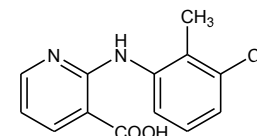
## II. SCOPE

This standard operating procedure comprises screening, quantification and confirmation procedures for the presence of ketoprofen in equine plasma sample using a Thermo Finigan TSQ Quantum Ultra triple-quadrupole mass spectrometry integrated with a Finnigan Accela LC system. The Racing Commissioners' International (RCI) has classified ketoprofen as a Class 4 substance, and as such has no pharmacologically tolerance concentration in plasma of any racehorse on the day of an officially sanctioned competition. Therefore, the reporting limit will be the limit of confirmation in equine plasma. Quantitation is included in this procedure for system and quality control, as well as for value-added quantitative estimates to further characterize the reported results and assist regulators in making valid administrative decisions.



$C_{16}H_{14}O_3$ , MW: 254.28

a. Ketoprofen



$C_{13}H_{11}ClN_2O_2$ , MW: 262.69

b. Clonixin

Figure 1. Chemical Structures of Ketoprofen and Clonixin (IS)

## III. PRINCIPLE OF METHODS

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The plasma sample is adjusted to pH 2~3 and subjected to liquid-liquid extraction using methyl tert-butyl ether. The extracts are reconstituted in LC mobile phase and the analyte is identified and confirmed by liquid-chromatography/ tandem mass spectrometry operated under electrospray ionization positive ion mode conditions. The concentration of ketoprofen is determined by the internal standard method using the peak area ratio. Clonixin (Figure 1b) is used as the internal standard. The limit of quantitation (LOQ) for ketoprofen in equine plasma by this method is 1~2 ng/mL. The limit of confirmation is 1~2 ng/mL. The qualifying ions for the confirmation of ketoprofen are  $m/z$  209, 194, and 103. The mass spectra of ketoprofen and clonixin (IS) are illustrated in Figure 2.

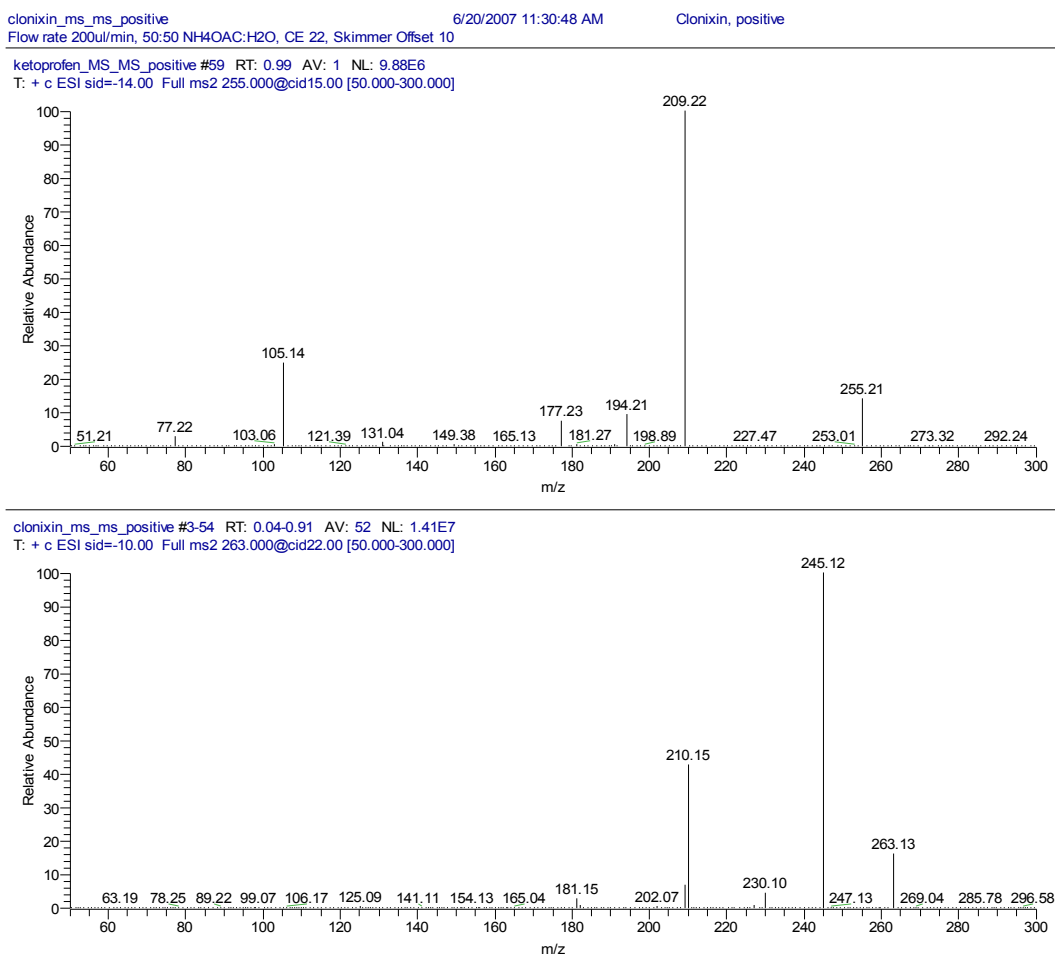


Figure 2. Mass spectra of ketoprofen (top panel) and clonixin (IS, bottom panel)

#### IV. PRIMARY DRUG AND INTERNAL STANDARD REFERENCE MATERIALS

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

Using the following reference power

Drug	QA Reference #	Lot #	Cat #	Source	Form
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Ketoprofen	S-KETP-1	128F0593	K-1751	Sigma	Powder
Clonixin	R-CLONX-1	NA	Sch-10304	Schering	Powder

### V. Preparation of primary reference stock solutions

1 mg/mL MeOH stock solution was prepared as follows:

**A. Ketoprofen**

Weigh X.xx mg ketoprofen powder into a glass bottle and label. Dilute to X.xx mL of HPLC grade (or better) methanol

**B: Clonixin**

Weigh Y.yy mg clonixin powder into a glass bottle and label. Dilute to Y.yy mL of HPLC grade (or better) methanol

Drug	Using	mg Used	mL Methanol
Ketoprofen	S-KETP-1	X.xx	X.xx
Clonixin	R-CLONX-1	Y.yy	Y.yy

Cap and mix until compound is completely dissolved in methanol.

**Complete the Balance Use Log and QA Primary Reference Standard Log for this process. Label the primary reference stock solutions with QA Primary Reference Log SR# (i.e. SR# xxx) and Primary Reference Powder Designation (i.e. S-KETP-1).**

### VI. PREPARATION OF FLOW INJECTION ANALYSIS (FIA) AND LIQUID CHROMATOGRAPHY (LC) COLUMN TEST SOLUTIONS

Material needed: 1 mg/mL MeOH stock solution

10 µg/mL FIA solutions in 50:50 (Water:MeOH) are prepared as follows:

Drug	Using	µL Used	mL 50:50
Ketoprofen	1 mg/mL stock solution	100	9.9
Clonixin	1 mg/mL stock solution	100	9.9

100 ng/mL LC column test solution comprises both ketoprofen and clonixin prepared in 90:10 (10 mM ammonium acetate:acetonitrile, v/v) as follows:

Drug	Using	µL Used	mL 90:10
Ketoprofen	10 µg/mL FIA solution	100	9.8
Clonixin	10 µg/mL FIA solution	100	

## VII. PREPARATION OF CALIBRATOR AND QC WORKING SOLUTIONS

Calibrator working solutions are prepared as follows:

Calibrator working solution (ng/mL)	Used for (ng/mL)	Solution used	µL Used	µL Optima Water
20	1	400 ng/mL working solution	500	9500
40	2	400 ng/mL working solution	1000	9000
100	5	2000 ng/mL working solution	500	9500
400	20	10 µg/mL FIA solution	400	9600
1000	50	10 µg/mL FIA solution	1000	9000
2000	100	10 µg/mL FIA solution	2000	8000
3000	150	10 µg/mL FIA solution	3000	7000
4000	200	10 µg/mL FIA solution	4000	6000

QC working solutions are prepared as follows:

QC working solution (ng/mL)	Used for (ng/mL)	Solution used	µL Used	µL Optima Water
100	5	2000 ng/mL working solution	500	9500
1000	50	10 µg/mL FIA solution	1000	9000
3000	150	10 µg/mL FIA solution	3000	7000

## VIII. PREPARATION OF 1.0 µg/mL CLONIXIN STANDARD WORKING SOLUTION

Drug	Using	mL Used	mL Optima Water Used
Clonixin (IS)	10 µg/mL FIA solution	1.00	9.00

## IX. CALIBRATOR AND QC SAMPLE PREPARATION:

### A. Calibrators

Eight calibrators are prepared from above calibrator working solution. A 25 µL aliquot of each calibrator working solution is spiked into 0.5 mL blank plasma, resulting in the calibrator concentrations being 1.0, 2.0, 5.0, 20, 50, 100, 150, 200 ng/mL

## B. QC samples

Three QC samples are prepared from the above QC working solution. A 25  $\mu\text{L}$  aliquot of each QC working solution is spiked into 0.5 mL blank plasma, resulting in QC concentrations of 5.0, 50, 150 ng/mL

## X. SAMPLE PREPARATION BY LIQUID-LIQUID EXTRACTION

### Safety Requirements: Lab coat, fume hood, eye protection

- A. 0.5 mL plasma sample (calibrators, QC, and samples) is transferred to a labeled 16 x 125 mm screw cap culture
- B. Add 25  $\mu\text{L}$  of each calibrator working solution and QC working solution into calibrator and QC plasma samples
- C. Add 10  $\mu\text{L}$  of 1  $\mu\text{g}/\text{mL}$  IS solution into each tube except NC sample (negative control without internal standard)
- D. Gently vortex all tubes
- E. Add 75  $\mu\text{L}$  of 1 M  $\text{H}_3\text{PO}_4$  solution into each tube
- F. Gently vortex all tubes
- G. Add 5 mL methyl tert-butyl ether into each tube, cap all screw-top tubes tightly and rotorack for 10 min.
- H. Centrifuge at 2, 500 ~ 3,000 rpm for 5 min (839-1409 x g).
- I. Decant the top organic layer into a labeled fresh test tube for each sample
- J. Dry sample under steady  $\text{N}_2$  stream at  $\sim 55^\circ\text{C}$
- K. Reconstitute the residues with 100  $\mu\text{L}$  of 90:10 (10 mM ammonium acetate:Acetonitrile, v/v)
- L. Transfer above solution in to a 200  $\mu\text{L}$  insert and load in the auto sampler vials. All the samples are now ready for analysis by LC-MS/MS

## XI. SAMPLE REQUIREMENTS FOR ANALYSIS

- A. Mobile phase blank
  1. Designate MB1.....MBn
- B. Negative (control) sample (with and without internal standard)
  1. Designate plasma NC (no internal standard) and NC-IS.
  2. Prepare negative (Control) samples using negative (blank) plasma.
- C. Positive control samples
  1. Designate plasma QCs.
  2. Prepare positive control samples as described in Section IX

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#### D. Calibrators

1. Prepare a set of calibrators for analysis of plasma samples.
2. Calibrator concentrations as designated in Section IX
3. Prepare plasma calibrators using negative control plasma from standard working solutions as described in Section IX

#### E. Column Test samples

1. Designated to use column test solution prepared in Section VI

## XII. LIQUID CHROMATOGRAPHIC/MASS SPECTROMETRIC OPERATING PARAMETERS

**Instrumentation:** LC-MS system consists of a Finnigan TSQ Quantum Ultra mass spectrometer equipped with heated electrospray ionization (H-ESI) probe, an Accela pump with an on-line degasser, and an Accela autosampler (ThermoFisher Scientific, San Jose, CA, USA). Xcalibur software (v. 2.2) is used for instrument control, data acquisition, and data processing.

**LC Column:** ACE 5 C<sub>8</sub> column (2.1 x 75 mm, 5 µm of particle size) with ACE 5 C<sub>8</sub> guard column and a 0.5 micrometer pre-column filter (MAC-MOD Analytical, Chadds Ford, PA, USA)

**Mobile Phase:** 10 mM NH<sub>4</sub>OAC (A) and Acetonitrile (B). Gradient HPLC program is used. Total analysis time is 9 minutes

Time (min.)	A%	B%	Flow rate (µL/min.)
0.00	90.0	10.0	100
2.00	50.0	50.0	200
5.00	50.0	50.0	200
5.50	20.0	80.0	200
7.50	20.0	80.0	200
8.00	90.0	10.0	200
9.00	90.0	10.0	200

**Injection Volume:** 10 µL

## XIII. SEQUENCE ORDER FOR ANALYSIS

The sequence order for screening and confirmation is the same, except for unknown samples. Screening samples are sequentially ordered by track and sample number, with blanks bracketing the individual tracks. 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 Plasma
5. Negative Control Plasma +Internal Standard
6. Blank
7. QC 5 ng/mL
8. QC 50 ng/mL
9. QC 150 ng/mL
10. Blank
11. Calibrator 1 ng/mL
12. Calibrator 2 ng/mL
13. Calibrator 5 ng/mL
14. Calibrator 20 ng/mL
15. Calibrator 50 ng/mL
16. Calibrator 100 ng/mL
17. Calibrator 150 ng/mL
18. Calibrator 200 ng/mL

19. Blank
  20. Track A, Samples 1 thru N
  21. Blank

Blank  
Track A, Sample X<sub>1</sub>  
Track A, Sample X<sub>2</sub>  
Track A, Sample X<sub>3</sub>  
Blank

Repeat as needed for the number of samples for confirmation

22. Repeat 19 thru 21 as needed
23. Blank
24. QC 5 ng/mL
25. QC 50 ng/mL
26. QC 150 ng/mL
27. Blank
28. Calibrator 1 ng/mL
29. Calibrator 5 ng/mL
30. Calibrator 20 ng/mL
31. Calibrator 50 ng/mL
32. Calibrator 75 ng/mL
33. Calibrator 100 ng/mL
34. Calibrator 150 ng/mL
35. Calibrator 200 ng/mL
36. Blank
37. Blank for instrument standby

#### XIV. IDENTIFICATION OF KETOPROFEN

The qualifying diagnostic ions for identification of ketoprofen as well as its retention time are shown in Table 1 and Figure 3.

**Table 1. Selected product ions for ketoprofen identification**

Analyte	Retention time	[M+H] <sup>+</sup>	Product ions (m/z)
Ketoprofen	3.5	255	209, 194, 103
Clonixin	3.9	263	245

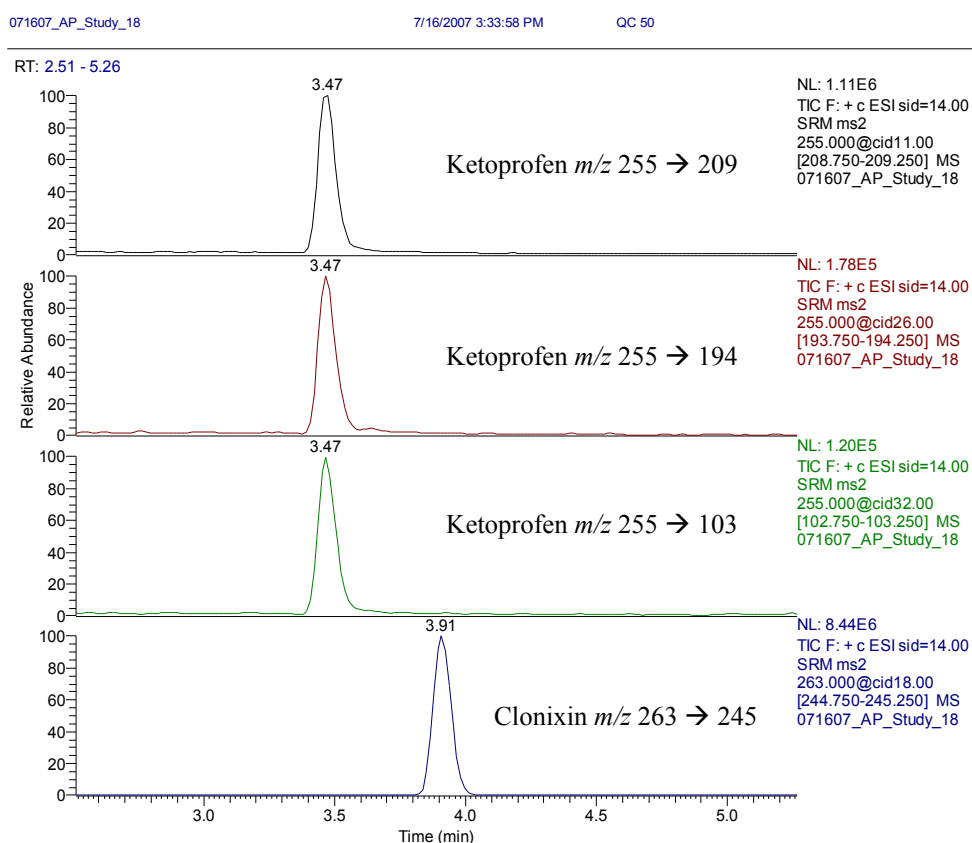


Figure 3: ESI(+)-LC-MS/MRM chromatograms of ketoprofen ( $m/z$  255  $\rightarrow$ 209;  $m/z$  255  $\rightarrow$ 194;  $m/z$  255  $\rightarrow$ 103) and clonixin ( $m/z$  263  $\rightarrow$  245) in equine plasma at 50 ng/mL.

All qualifying ions for ketoprofen are present in the MRM mode. The intensity ratios between the selected product ions and the predominant product ion are used as confirmation criteria (Table 2, Figure 4). Product ion with the largest intensity was used as the denominator in calculating ion intensity ratios. The ion intensity ratios listed in Table 2 are examples for understanding how the calculation was performed. Ion intensity ratios may vary depending on instrument type and parameter settings. The intensity ratio difference between unknown sample

and the calibrators + QCs should be lower than 20%. The retention time for the unknown sample, and calibrator/QC should agree to  $\pm 0.30$  minutes.

**Table 2. Intensity ratios of the product ions for confirmation of ketoprofen**

Ketoprofen product ion	Product ion intensity ratio(%)
209	100
103	$26.14 \pm 1.57$
194	$40.24 \pm 2.57$

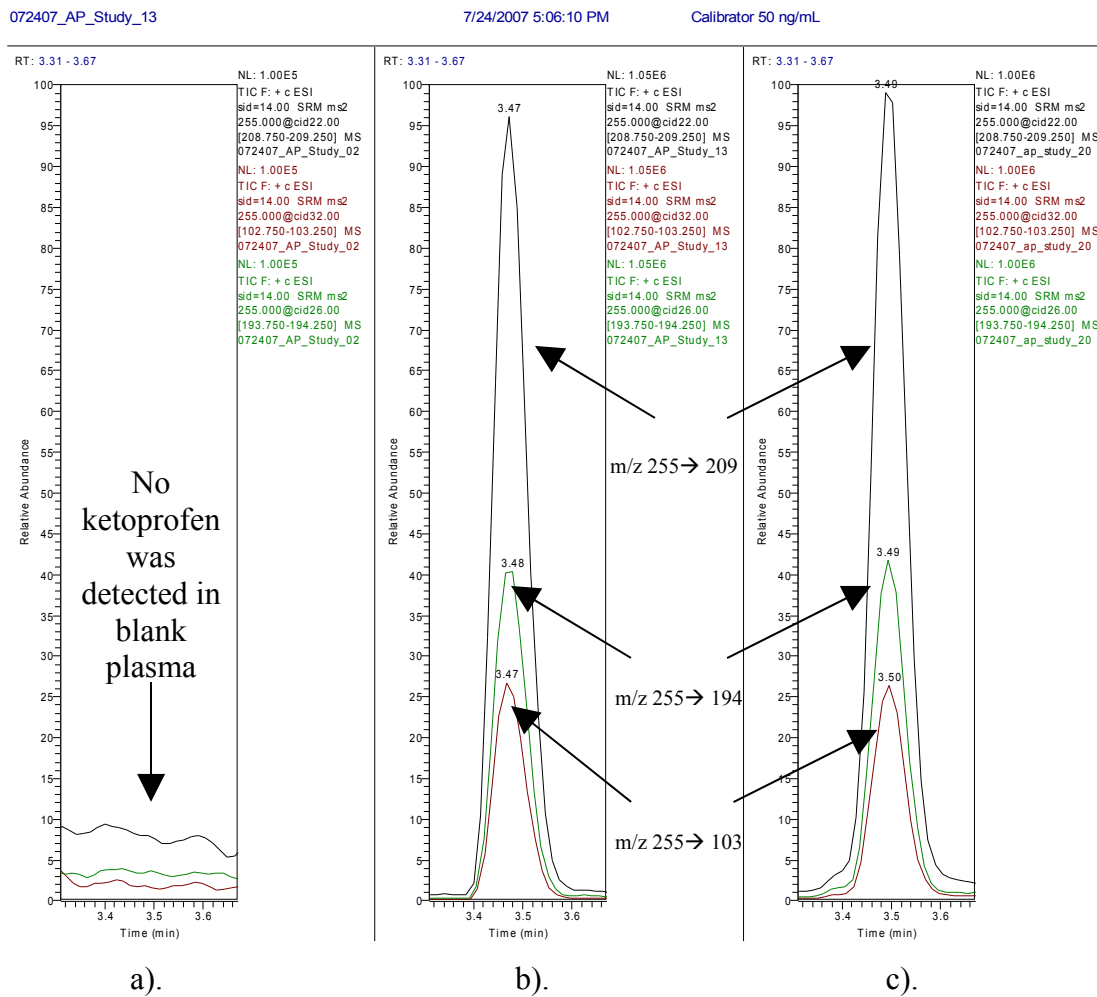


Figure 4. Product ion intensity comparison for ketoprofen in (a) blank plasma; (b). 50 ng/mL ketoprofen spiked in to blank plasma; (c). ketoprofen administration plasma sample.

## A. Identification of clonixin (IS)

The qualifying ion for the identification of the internal standard, clonixin, is m/z 245. Under LC-MS/MS conditions described, the diagnostic ions should be recognized at retention time  $\sim 3.9 \pm 0.3$  min (Figure 3).

## XV. CRITERIA FOR QUANTIFICATION OF KETOPROFEN IN EQUINE PLASMA

The product ions used for quantification of ketoprofen is m/z 209. The retention time of ketoprofen is  $3.5 \pm 0.3$  min (Figure 3)

The product ion of clonixin (IS) used for quantification of ketoprofen is m/z 245 with retention time  $3.9 \pm 0.3$  min (Figure 3)

Using the Xcalibur software, execute data processing method. Print the compound summary quantification report and calibration curve. The correlation coefficient should be greater than 0.995 (Figure 5).

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

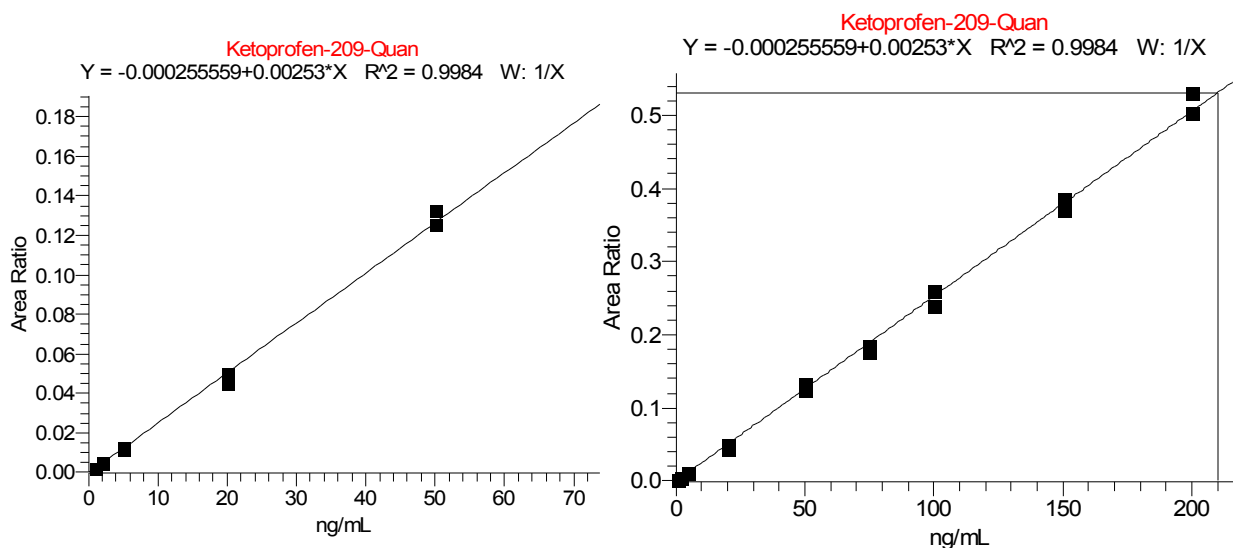


Figure 5. Calibration curves for ketoprofen in equine plasma

## XVI. Method Validation

### A. Extraction recovery

**Table 3. Liquid-liquid Extraction Recovery**

Conc. Spiked (ng/mL)		Extraction recovery (%) (n=6)	
		With H <sub>3</sub> PO <sub>4</sub>	Without H <sub>3</sub> PO <sub>4</sub>
Ketoprofen	5.0	40.6 ± 3.0	25.6 ± 1.3
	50	45.1 ± 1.5	25.0 ± 1.1
	150	51.0 ± 6.1	34.3 ± 1.3
Clonixin	50	93.4 ± 6.0	42.0 ± 3.8

Adding H<sub>3</sub>PO<sub>4</sub> significantly increase ketoprofen extraction efficiency.

### B. Matrix effect

**Table 4. Matrix effect on ionization of ketoprofen and clonixn (n=6)**

Analyte	Conc. Spiked (µg/mL)	Ion suppression or enhancement (%) <sup>a</sup>
Ketoprofen	5.0	-12.3 ± 7.3
	50	-13.8 ± 5.3
	150	-23.3 ± 4.0
Clonixin	50	-25.3 ± 4.8

<sup>a</sup>Ion suppression or enhancement (%) =  $(A_{\text{extract}} - A_{\text{solvent}}) / A_{\text{solvent}} \times 100$ , where  $A_{\text{solvent}}$  is the peak area of an analyte spiked in reconstitution solvent, and  $A_{\text{extract}}$  is the peak area of an analyte spiked in blank equine plasma extract. Negative value represents matrix suppression.

Negative values in the ion suppression/enhance column indicate that plasma matrix induced ion suppression effect on both ketoprofen and clonixin (IS). Ion suppression by equine plasma was less than 26% for both ketoprofen and clonixin (IS), indicating that matrix effect was not a major problem using liquid-liquid extraction. Thus, no further attempt was made to further minimize any ion suppression, since the precision and accuracy of the proposed method were within acceptable experimental error.

### C. Accuracy and precision

**Table 5. Intra-day and inter-day accuracy (bias %) and precision (RSD%) for quantification of ketoprofen in equine plasma (n=6)**

Conc. Spiked (ng/mL)	Intra-day			Inter-day		
	Conc. Determined (ng/mL)	Accuracy <sup>a</sup> (bias) (%)	Precision <sup>b</sup> (RSD) (%)	Conc. Determined (ng/mL)	Accuracy (bias) (%)	Precision (RSD) (%)
5.0	5.00	100.09	7.13	5.50	110.03	5.49
50	52.32	104.63	3.07	50.28	100.56	15.51
150	141.45	94.30	8.89	149.96	99.97	6.90

<sup>a</sup> Accuracy (bias %) = (conc. determined - conc. spiked)/conc. spiked x 100.

<sup>b</sup> Precision (RSD %) = standard deviation of conc. determined / conc. determined x 100.

m/z 237

## XVI. CRITERIA FOR REPORTING A SAMPLE POSITIVE FOR KETOPROFEN

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

- A. The test sample contains ketoprofen according to the chromatographic and mass spectrometric criteria.
- B. The signal-to-noise ratio of the least abundant qualifying ion for ketoprofen in each replicate of the test sample is greater than 10
- C. The LC retention times of the qualifying ion for ketoprofen in the unknown sample, QC control and calibrators are within  $\pm 0.30$  min. 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.
- D. The confirmation of ketoprofen is performed using the specific ion transitions and the intensity ion ratio of different product ions. The product ion intensity ratios are shown in Figure 4 and the qualifying ions and the ion intensity ratio for ketoprofen are listed in Table 1. Ratios may vary depending on instrument type and parameter settings. In practice, the intensity ratio of product ions for a positive sample should be  $\pm 20\%$  of the average value of those from the calibrators and QCs analyzed along with the positive sample in the same batch. (Figure 4).

- E. All blanks and negative control samples do not contain quantifiable ketoprofen concentration greater than 1 ng/mL (Figure 4).

## **XVII POSITIVE SAMPLE DATA PACKET ORDER OF ASSEMBLY**

1. SAMPLE TRANSFER SHEET (WS#32A)
2. SAMPLE USAGE SHEET (FORM #7)
3. COMPLETE SEQUENCE LIST
4. COMPLETE INSTRUMENT METHOD
5. CROMATOGRAM COMPARISON
6. MRM RATIO COMPARISON
7. MRM RATIO SUMMARY
8. COMPLETE QUANTIFICATION REPORT (INCLUDING CALIBRATION CURVE)
9. CONFIDENCE REPORT
10. HISTORICAL CONTROL CHART
11. COLUMN TEST

## **XXI OTHER REQUIRED DOCUMENTATION**

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

Sample list print-out that is maintained in the TSQ Quantum Ultra three ring binder Routine usage checklist completion (and maintenance log if needed). Sample Analysis logbook, indicating date, project, operator initials, and listing 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 REPORT
4. QUANTIFICATION REPORT

## **XXII. KETOPROFEN: PLASMA CLEARANCE**

Dose: 2.2 mg/kg

Administration route: Intravenous

Horse weight: 529 kg

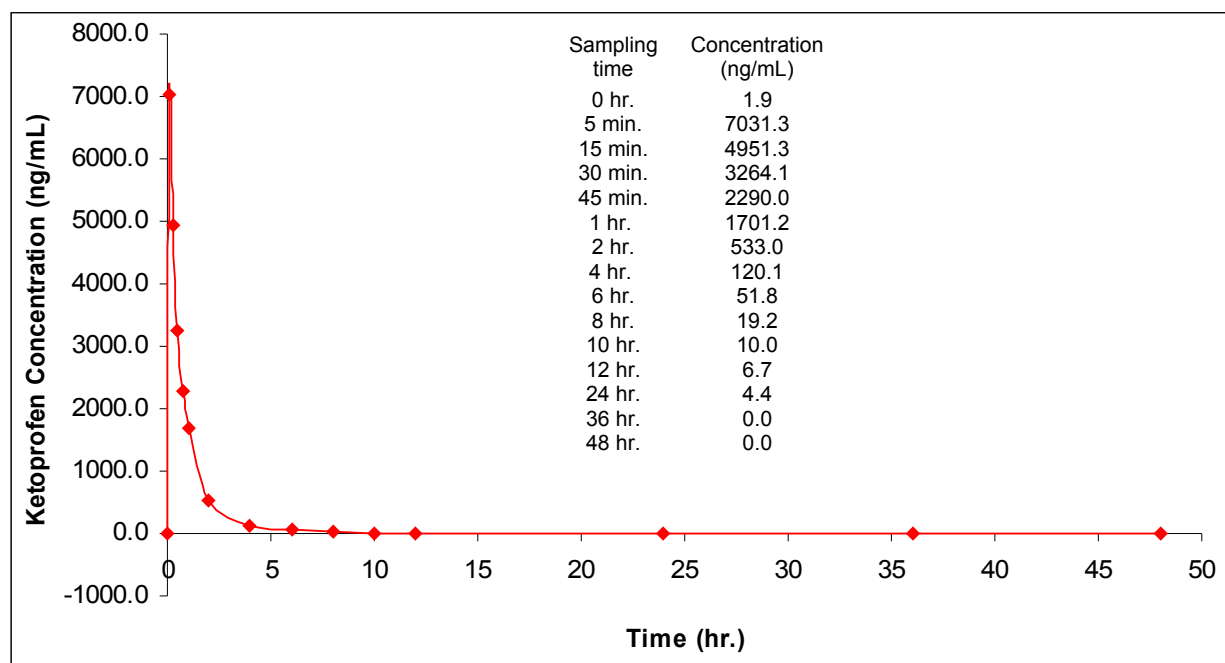


Figure 5. Ketoprofen concentration-time profiles following intravenous administration (dose: 2.2 mg/kg, horse weight: 529 kg). Ketoprofen attained peak concentrations in plasma within 5 min from time of administration, and the concentration of ketoprofen 24 hr post administration was detectable but was not at 36 hr.

## XXII. 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. Ammonium acetate, HPLC grade (Cat. No. A2149, Spectrum Chemical)
- E. Phosphoric acid, Baker Analyzed ACS reagent (Cat. No. 0260-3, J.T. Baker)

## XXIII. SOLUTIONS

### A. 1 M Ammonium acetate

- 1. Reagents
  - a) Ammonium acetate (HPLC grade)
  - b) Water (Optima grade)
- 2. Procedure

- a) Weigh 15.416 grams of ammonium acetate and dissolve it in 200 mL water (Optima) in a beaker. Transfer it into a labeled glass bottle and store at 4 °C.

3. Storage Requirements

- a) Store at 4 °C in refrigerator.

## **B. 1.0 M H<sub>3</sub>PO<sub>4</sub>**

1. Reagents

- a) Phosphoric acid (Baker Analyzed ACS reagent)
- b) Water (Optima grade)

2. Procedure

- a) Add ~ 100 mL water in 200 mL volumetric flask.
- b) Add 13.6 mL concentrated phosphoric acid into the 200 mL volumetric flask.
- c) Bring to 200 mL total volume by adding water.

3. Storage Requirements

- a) Transfer to glass bottle and store at ambient temperature.

## **A. 10 mM ammonium acetate solution**

4. Reagents

- a) 1.0 M ammonium acetate solution
- b) Water (Optima grade)

5. Procedure

- a) Add 10 mL 1.0 M ammonium acetate solution into 1L optima water

6. Storage Requirements

- a) Store at ambient temperature

## **C. 90:10 (10 mM ammonium acetate:acetonitrile, V/V)**

1. Reagents

- a) 10 mM ammonium acetate solution
- b) Acetonitrile (Optima grade)

2. Procedure

- a) Add 10 mL acetonitrile into 90 mL 10 mM ammonium acetate solution

3. Storage Requirements

- a) Store at ambient temperature

## **XXIV. MATERIALS**

- A. 16 × 100 mm test tubes.
- B. 16 × 125 mm screw-top test tubes.
- C. Polypropylene caps
- D. Test tube rack
- E. Pipettes and tips.
- F. Rotorack
- G. Centrifuge
- H. Vortex mixer (Scientific Industries, Inc. )
- E. Branson Ultrasonic Water Bath, 8510 ( Fisher Scientific or equivalent )
- F. Sample Concentrator ( Dri-Block DB-3, Techne )
- G. IEC HN-SII Centrifuge ( International Equipment Company )
- H. Rotorack ( Speci-Mix, Thermolyne )
- I. 2 mL autosampler vials
- J. 200 uL Insert (Target PP Polyspring, National Scientific Company )
- K. Balance (Mettler AT 261 Delta range, Mettler-Toledo Inc.)
- L. Eye protection and lab coat
- M. Gloves