appendix

Determination of concentrations of ketoprofen, carprofen and flunixin in plasma of sheep following oral administration in a pain model conducted in sheep

Executive Summary

This report details the analytical component of MLA Project B.AWW.0230 – Development of assays and assessment of oral bioavailability of the NSAIDs ketoprofen, carprofen and flunixin, contracted to Pia Pharma Pty Ltd through the CSIRO Pastoral Research Lab Chiswick, Armidale, NSW. The objective of the analytical component was to develop analytical methodology to facilitate determination of concentrations of ketoprofen, carprofen and flunixin in plasma samples collected from test subjects following administration of investigative veterinary formulations containing the non-steroidal agents.

A sensitive, selective analytical method for the simultaneous determination of ketoprofen, carprofen, flunixin and metabolite 5-hydroxyflunixin in ovine plasma was developed and validated. This was achieved through a combination of an efficient extraction and sample clean up procedure using a mixed mode (C8 + Anion Exchange) Solid Phase Extraction sorbent; use of deuterated equivalents as internal standards; effective chromatographic separation and peak shape using Ultra High Pressure Liquid Chromatography; and negative electrospray ionisation and tandem mass spectrometry.

Bioanalytical Method Summary

Method Number	ATM018
Method Title	Determination of ketoprofen, carprofen, flunixin and metabolite 5-hydroxyflunixin in ovine by LCMS/MS
Method Type	Bioanalytical
Analyte/s	ketoprofen, carprofen, flunixin and metabolite 5-hydroxyflunixin
Test Matrix	Ovine plasma
Instrumental Determination	UHPLC/ -ve ESI MS/MS
Lower Limit of Quantitation (LLOQ)	10ng/mL ketoprofen 10ng/mL flunixin 20ng/mL 5-hydroxyflunixin 20ng/mL carprofen
Typical Range	10-2000ng/mL ketoprofen 10-10000ng/mL flunixin 20-2000ng/mL 5-hydroxyflunixin 20-3000ng/mL carprofen
Internal standards	Carprofen-d_3, Ketoprofen-$^{13}C,d_3$, Flunixin-d_3
Quantitation	Standard calibration curve prepared using an internal standard
Sample throughput	80-160 per batch
Analysis time	4-8 hours preparation 12 hours instrumental determination
Typical turn-around time	4-6 weeks

The analytical method for determination of flunixin, 5-hydroxyflunixin, ketoprofen and carprofen in ovine plasma was validated using an in-house protocol developed from procedures described in current EMA[2,3], APVMA[4], VICH[5,6] and FDA[7] guidance documents. Various tests were conducted, assessing core validation components; analyte identification, system suitability, chromatographic parameters, selectivity,

carry over, Lower Limit of Quantitation, calibration, accuracy and within-run precision. The data generated was evaluated against a set of pre-determined performance criteria, and an assessment of risk conducted. It was determined that data generated during the study met the validation criteria, and the method could produce results of definable integrity.

The assay range was established without knowledge of the concentrations likely to be encountered in treated animals in this particular study. It became evident that the cohorts receiving carprofen had µg/mL concentrations rather than ng/mL plasma concentrations, significantly exceeding the Upper Limit of Quantitation of the validated method. An amendment to the method was made in this case to extend the assay range and determine the concentrations of carprofen using UHPLC-UV, without changing the extraction methodology . Additional validation data was generated to support this amendment. The range for this assay was 1µg/mL-150µg/mL in ovine plasma.

The submission totalling 480 ovine plasma samples originated from the animal component of the bioavailability study conducted at the CSIRO Pastoral Research Lab Chiswick, Armidale NSW. Blood was obtained from study animals at designated time points following dose administration according to the trial protocol. Plasma was prepared, frozen and delivered to the laboratory.

Concentrations of the NSAIDS in plasma were determined using the validated methods. Calibration curves prepared using the ratio of analyte to deuterated internal standard, were used for analyte quantitation. Calibration curve correlation coefficients exceeded 0.99 for quantitative runs. Quality control activities for each analytical run were conducted and reviewed, and deemed to meet the in-house standard, prior to releasing results.

Concentrations of flunixin, 5-hydroxy flunxin and ketoprofen in treated and saline cohorts were reported in ng/mL. Carprofen was reported in µg/mL for the corresponding treated cohorts, and in ng/mL for the cohorts receiving saline.

The averages for each treated cohort were overlaid in a graphical presentation by analyte.

Determination of concentrations of ketoprofen, carprofen and flunixin in plasma of sheep following oral administration in a pain model conducted in sheep

Analytical services contracted to:	**CSIRO** **Pastoral Research Lab Chiswick**
Study Reference No.:	**B.AWW.0230**
Representative:	**Caroline Lee** CSIRO Pastoral Research Lab Chiswick New England Highway Armidale NSW 2350
Contractor Analytical Phase:	**Joe Pippia** **Managing Director** Pia Pharma Pty Ltd Suite 3.501, Level 3 126 Greville Street Chatswood West NSW 2067
Analytical Phase Reference:	**1309-066**

COMPLIANCE STATEMENT

Study Title:	Determination of concentrations of ketoprofen, carprofen and flunixin in plasma of sheep following oral administration in a pain model conducted in sheep
Component:	Analytical

Before commencement, the undersigned representative of Pia Pharma Pty Ltd discussed the requirements for analysis with the sponsor. Study objectives were established in order to deliver on expectations for assay sensitivity, sample integrity, data integrity, security, analysis timeframe and report quality.

The analytical component was conducted in accordance with APVMA Bioequivalence Guideline for Veterinary Chemical Products[1]. Evidence of the quality measures applied to method validation and subsequent sample analysis were provided in this report. Many of these measures were derived from relevant international guidance documents relating to conduct of bioequivalence studies[2] or use and validation of bioanalytical methods[3-7]. In addition, in-house Standard Operating Procedures (SOPs) provided specific guidance on various aspects of sample and data management.

By signing below, as a representative of Pia Pharma Pty Ltd, I declare that the analytical phase of this study was conducted under my supervision, and that the report is a complete and accurate representation of the analytical component.

Signed: _____ Date: 31/12/13

Mr Joe Pippia (B. App. Sc., M. Sci, Tech.)
Study Investigator

Table of Contents

Appendices

List of Tables and Figures

1 Introduction

1.1 Study objectives

The objective of the analytical component was:
- to develop analytical methods with capacity to determine relevant concentrations of ketoprofen, carprofen and flunixin in ovine plasma;
- To determine the concentrations of ketoprofen, carprofen and flunixin in plasma samples collected from test subjects following administration of the test formulations;
- To present analyte concentration data in order to facilitate an assessment of oral bioavailability of the test substances.

1.2 Scope

This was the analytical component of a study investigating the oral bioavailability of non-steroidal agents, ketoprofen, carprofen and flunixin in sheep administered an oral dose of a formulation containing one of these agents or a saline solution.

The determination of oral bioavailability required a sensitive analytical method offering Lower Limits of Quantitation (LLOQ) and a reasonable range, given that there was limited knowledge of the pharmacokinetic parameters of these non-steroidal agents in sheep. An assay facilitating the simultaneous determination of ketoprofen, carprofen and flunixin was advantageous for processing the large number of samples associated with the study.

A sensitive analytical method for the simultaneous determination of carprofen, ketoprofen, flunixin and its metabolite, 5-hydroxy flunixin is described within this report. This method relies on effective analyte extraction and matrix clean up using mixed-mode solid phase extraction; analyte resolution achieved with high quality analytical columns using Ultra High Pressure Liquid Chromatography; deuterated internal standards; and the selectivity and sensitivity achieved using tandem mass spectrometry in the negative ion mode, to achieve the objectives.

1.3 Schedule

Table 1. Key study dates

Samples received by laboratory	5.9.2013
Study initiation date	10.10.2013
Analytical component – Sample analysis	25.11.2013 – 6.12.2013
Final Analytical Study Report submitted	31.12.2013

2 Analytical Method

The sections below describe the analytical methodology for the determination of ketoprofen, carprofen, flunixin and 5-hydroxy flunixin in ovine plasma.

2.1 Materials and Reagents

2.1.1 Reference Materials

Analytical reference materials were sourced from reputable suppliers. The standards were assessed for conformance to Quality Assurance standards on receipt, and registered as part of incoming materials control procedures. The materials remained within their original containers, and were stored according to manufacturers' instructions. Certificates of analysis are provided in Appendix 1.

Table 2. Analytes –bioanalytical method

Material	Ketoprofen	
CAS	22071-15-4	
Molecular formula	$C_{16}H_{14}O_3$	
Molecular weight	254.28	
Source	Sigma (Vetranal)	
Lot No.	SZBA166XV	
Purity	99.9%w/w	
Expiry	15.6.2015	
Storage	Room Temp.	
Supplier	Sigma Aldrich	
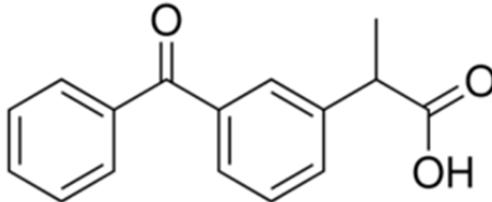
Material	Flunixin meglumine	
CAS	42461-84-7	
Molecular formula	$C_{14}H_{11}F_3N_2O_2 \cdot C_7H_{17}NO_5$	
Molecular weight	296.25 (flunixin) 491.46 (flunix meg)	
Source	Sigma Aldrich	
Lot No.	062M4730V	
Purity	60.28%w/w flunixin	
Expiry	20.6.2014	
Storage	-20°C	
Supplier	Sigma Aldrich	
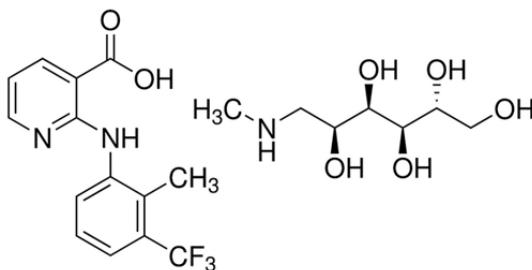
Material	**5-hydroxyflunixin**
CAS	75369-61-8
Molecular formula	$C_{14}H_{11}F_3N_2O_3$
Molecular weight	312.2
Source	Toronto Res. Chem
Lot No.	1-YSW-78-2
Purity	95%w/w (contains 5% flunixin)
Expiry	25.9.2014
Storage	-20°C
Supplier	Toronto Res. Chem

Material	**Carprofen**
CAS	53716-49-7
Molecular formula	$C_{15}H_{12}ClNO_2$
Molecular weight	273.71
Source	Sigma (Vetranal)
Lot No.	SZBB102XV
Purity	99.9%w/w
Expiry	12.4.2016
Storage	Room Temp.
Supplier	Sigma Aldrich
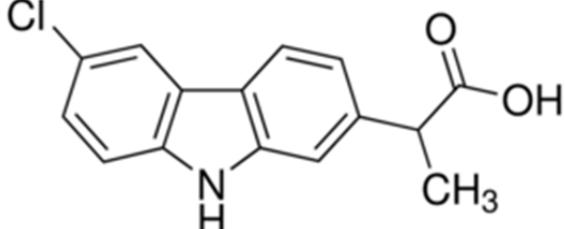
Material	**Ketoprofen-^{13}C,d$_3$**
CAS	
Molecular formula	$C_{15}{}^{13}CH_{11}D_3O_3$
Molecular weight	258.29
Source	Toronto Res. Chem
Lot No.	4-RUS-38-3
Purity	98%w/w
Expiry	25.9.2014
Storage	-20°C
Supplier	Toronto Res. Chem
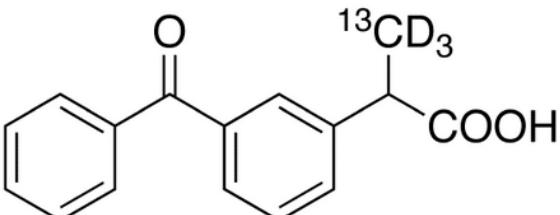
Material	Flunixin-d₃
CAS	1015856-60-6
Molecular formula	$C_{14}H_8D_3F_3N_2O_2$
Molecular weight	299.26
Source	Toronto Res. Chem
Lot No.	2-QFY-122-2
Purity	98%w/w
Expiry	25.9.2014
Storage	-20°C
Supplier	Toronto Res. Chem
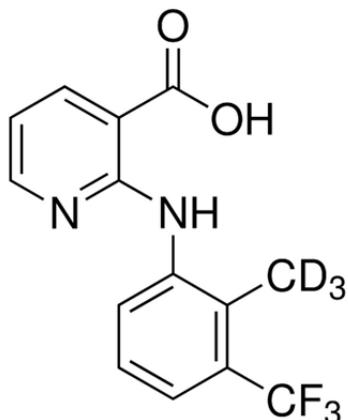
Material	Carprofen-d₃
CAS	1173019-42-5
Molecular formula	$C_{15}D_3H_9ClNO_2$
Molecular weight	276.73
Source	Toronto Res. Chem
Lot No.	1-JPI-35-1
Purity	98%w/w
Expiry	25.9.2014
Storage	-20°C
Supplier	Toronto Res. Chem
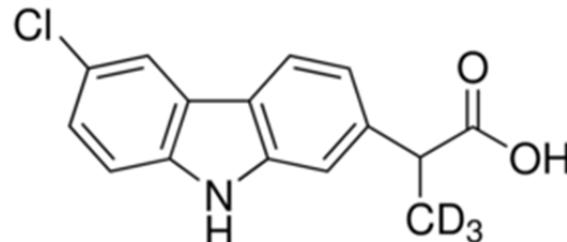
2.1.2 Reagents

The following reagents were used. Unless specified, reagents were of Analytical Reagent grade.

- Water – Type 1 water for UHPLC applications, Reverse osmosis or better for laboratory use.
- Methanol, HPLC Grade.
- Acetonitrile, HPLC Grade
- Glacial acetic acid, AR
- Potassium dihydrogen orthophosphate (KH_2PO_4), anhydrous AR Grade
- Formic acid (for LCMS)
- Trifluoroacetic acid (for HPLC)
- 100mM Phosphate buffer (pH 6.4) - Dissolve 2.72g KH_2PO_4 in RO water with continuous stirring. Adjust to pH 6.4 (±0.1) using 0.1M NaOH drop wise.
- 4% acetic acid in methanol – mix 4mL glacial acetic acid and 96mL methanol. Shake well.
- 0.1% formic acid (Mobile Phase A for LCMSMS) – add 1mL of formic acid to 1L of Type 1 water. Filter through a 0.45µm filter before use.
- 0.1% trifluoroacetic acid (Mobile Phase A fo UV system) – add 1mL trifluoroacetic acid to 1L of Type 1 water. Filter through a 0.45µm filter before use.
- Acetonitrile (Mobile Phase B) - Filter through a 0.45µm filter before use.

2.1.3 Standard Solutions

Two 0.2mg/mL stock solutions of each target analyte were prepared by dissolving 10mg reference material in 50mL methanol in separate, calibrated volumetric flasks. The stock solutions were assessed for conformity by HPLC. The acceptance limit was the check standard (Standard 2), must agree to within ±2% of the nominal value when calculated using the calibration curve of the primary stock reference standard (Standard 1).

Mixed intermediate standards (0.20, 0.40, 4.0 and 20.0µg/mL) containing ketoprofen, carprofen and flunixin were prepared in acetonitrile. Intermediate standard solutions of 5-hydroxy flunixin were prepared separately as the 5-hydroxyflunixin reference standard contained approximately 5% flunixin as an impurity. Fortified plasma samples and calibration standards were prepared from these mixed intermediate standards.

Stock and intermediate standards were stored in the refrigerator when not in use. They were given adequate time to equilibrate to room temperature prior to use. Stock and intermediate solutions were stable under these conditions for a minimum four weeks.

Calibration standards were prepared fresh each day.

2.1.4 Internal Standard Solutions

Deuterated internal standards, flunixin-d_3, ketoprofen-$^{13}C,d_3$ and carprofen-d_3, were dissolved in methanol to produce individual standards at 1000µg/mL. A mixed internal standard solution was prepared comprising 2.0µg/mL flunixin-d_3, 2.0µg/mL ketoprofen-$^{13}Cd_3$, and 4.0µg/mL carprofen-d_3. This internal standard solution was used for sample analysis and calibration standard preparation.

Internal standard stock solutions were stored in the freezer when not in use. Intermediate standards were stored in the refrigerator when not in use. They were given adequate time to equilibrate to room temperature prior to use.

2.2 Equipment

2.2.1 Consumables

- Centrifuge tubes, polypropylene, 15mL, 50mL
- Microcentrifuge tubes, polypropylene, 2mL (Eppendorf)
- 1mL/3mL polypropylene transfer pipettes
- 96 well collection plates (2mL Eppendorf)
- Cleanup® SPE plate, 96 well WSHQAX205 Sorbent: CUQAX2, Lot: 116050-PH, Expiry 19.11.2015 (UCT Inc., Bristol,PA)
- Autopipettor tips 200µL/ 1000µL (Eppendorf)
- HPLC vials, caps and septa (Phenomenex)

2.2.2 General Apparatus

- General Glassware - Grade B volumetric glassware or better.
- Analytical balance, Shimadzu AUW220D 0.00001g, range 0.01mg-220g
- Analytical balance Ohaus Pioneer PA214 0.0001g, range 0.1mg-210g
- Microcentrifuge, Hermle Z160M, 14000rpm max
- Vacuum pump, collection trap
- 96 well plate SPE manifold
- Autopipettors – Eppendorf 10-200µL, 100-1000µL
- 96 well plate shaker, IKA
- Pyrex weighing boat.
- Small stainless steel spatula.
- Volumetric flasks, clear borosilicate,10mL, 20mL, 50mL
- Ultrasonic bath
- 2mL sample vial holders
- Test tube racks, 16mm/60 tubes
- Reagent bottles, borosilicate 250, 500 and 1000mL

2.2.3 Chromatographic equipment

- Analytical HPLC column - Supelco Ascentis® Express 50x2.1mm, 2.7µm #USMD4769 – Sigma Aldrich (Pia Pharma Column #30)
- Eksigent® Ekspert™ ultraLC 100-XL Liquid Chromatograph UHPLC system comprising a binary pump system, in-line vacuum degasser, column oven, autosampler, control and data storage with Analyst (Ver1.6)
- Thermo Scientific Dionex Ultimate 3000 UHPLC system comprising a binary pump system, in-line vacuum degasser, column oven, autosampler, control and data storage with Chromeleon 7 Chromatography Data System
- AB Sciex API3200 tandem mass spectrometer with an Electrospray ionisation source.
- AB Sciex Multiquant (Ver. 2.1)

2.3 Methodology

ATM018 – Determination of non-steroidal anti-inflammatory agents; ketoprofen, carprofen, flunixin, and the metabolite 5-hydroxyflunixin in ovine plasma by LCMS/MS is documented and retained in Pia Pharma's Analytical Methods Manual. A summary of the method is provided in the following section.

2.3.1 Sample Preparation

Plasma samples were thawed completely and mixed for one minute using a gentle reciprocating motion, prior to obtaining the test portion.

2.3.2 Sample Extraction

A 500 µL aliquot of plasma representing the test portion was dispensed into a 2mL polypropylene centrifuge tube. 50µL of the combined, deuterated internal standard was added and the sample and mixed gently prior to addition of 500 µL acetonitrile. The sample was vortexed (1minute) to precipitate plasma protein. 1mL of phosphate buffer (pH6.4) was added to the extract and the mixture was centrifuged (5min, 13000rpm). prior to loading onto a prepared Solid Phase Extraction sorbent (Cleanup® WSHQAX205, UCT).

2.3.3 Solid Phase Extraction

The mixed mode 96 well SPE plate was placed on the extraction plate manifold and prepared by adding 1mL methanol, 1mL water and 1mL phosphate buffer (pH6.4) in succession, and allowing each to flow through the sorbent without the aid of vacuum.

Sample extracts were then loaded into designated wells, and permitted to flow through the sorbent without vacuum. When complete, 1mL water was used to wash the sorbent. The sorbent was then dried under vacuum for 15 minutes to remove excess water.

For the elution stage, a 96 well polypropylene reservoir plate (2mL) was placed within the manifold, under the SPE plate. 2 x 500µL aliquots of 4% acetic acid in methanol were transferred in succession, the first allowed to flow through each sorbent (without vacuum), followed by the second, combining in the reservoir plate below. The extract was mixed with gentle agitation using a 96 well plate shaker. The plate was sealed with a pierceable sealing mat prior to transferring to the autosampler for determination.

2.3.4 Instrumental Determination

An Eksigent® Ekspert™ ultraLC 100-XL Liquid Chromatograph was used for separation of the target analytes from any matrix interferences. 10µL of sample extract was injected into the system, and separation performed using a Supelco Ascentis® Express 50x2.1mm, 2.7µm analytical column maintained at 50°C. A gradient elution program, using 0.1% formic acid and acetonitrile as mobile phase constituents operating at 0.8 mL min^{-1}, resolved 5-hydroxy flunixin, ketoprofen, flunixin and carprofen from matrix interferences and endogenous sample components within a 4 minute run time. The deuterated internal standards eluted at the same retention time of the non-deuterated equivalent.

An AB Sciex API 3200 triple-quadrupole mass spectrometer was interfaced with the liquid chromatograph. The detector was configured with a proprietary turbo V source for desolvation and operated in negative electrospray ionisation (-ve ESI) mode for optimum analyte sensitivity. The mass spectrometer was operated in MS/MS mode, with transition masses identified and optimised for declustering potential, collision energy, and cell entry and exit potentials. Critical detector settings and transitions are provided in Table 4.

Table 3. UHPLC settings (for LCMSMS)

Instrument	Eksigent UHPLC system		
Ionisation	Negative Electrospray ionisation		
Detection system	Tandem Mass spectrometry		
Mobile phase	A: 0.1% Formic acid / B: acetonitrile		
Column	Supelco Ascentis® Express 50x2.1mm, 2.7µm #USMD4769 (#30)		
Column temperature	50°C		
Injection volume	10µL		
Flow rate	0.8mL min^{-1}		
Run time	4 minutes		
Solvent delivery (Gradient)	**Time (min)**	**% solvent A**	**% solvent B**
	0	95	5
	0.5	95	5
	2.0	40	60
	2.8	40	60
	2.9	95	5
	4.0	95	5

Table 4. Mass Spectrometer settings

Tandem mass spectrometer	API 3200 LCMS/MS (AB Sciex)			
Ionisation mode	Negative electrospray ionization in MRM mode			
Ion Spray Voltage	-4500V			
Desolvation Temperature	600˚C			
Analyte	**Q1 (m/z)**	**Q3 (m/z)**	**Declustering potential (V)**	**Collision energy (V)**
Carprofen #1 (Quantitative)	272.1	228.0	-70	-19
Carprofen #2 (Confirmatory)	272.1	226.0	-70	-41
Carprofen-d3 (ISTD)	275.0	231.0	-70	-23
Ketoprofen #1 (Confirmatory)	253.3	209.0	-18	-13
Ketoprofen #2 (Quantitative)	253.3	197.0	-18	-13
Ketoprofen-13C,d3 (ISTD)	257.2	197.1	-18	-13
Flunixin #1 (Confirmatory)	295.1	251.1	-40	-28
Flunixin#2 (Quantitative)	295.1	191.0	-40	-45
Flunixin-d3 (ISTD)	298.2	254.0	-55	-25
5-OH Flunixin#1 (Conf)	311.1	267.0	-56	-25
5-OH Flunixin#2 (Quant)	311.1	227.1	-56	-35

An amendment to the method was made during the study as it was considered necessary to extend the assay range of carprofen to obtain a higher Upper Limit of Quantitation (ULOQ). The variation used the same sample extraction and clean up procedure, however, the higher concentrations were determined using a UV detector at 240nm. Meloxicam was the internal standard. Instrumental parameters are detailed in Table 5.

Table 5. UHPLC-UV settings

Instrument	Thermo Scientific Dionex Ultimate 3000 UHPLC system		
Detection system	Diode Array Detector (DAD3000RS)		
Mobile phase	A: 0.1% Trifluoroacetic acid / B: acetonitrile		
Column	Supelco Ascentis® Express 50x2.1mm, 2.7μm #USMD4769 (#30)		
Column temperature	50°C		
Injection volume	2μL		
Flow rate	0.8mL min^{-1}		
Detector wavelength	240nm (carprofen), 355nm (meloxicam)		
Run time	4 minutes		
Solvent delivery (Gradient)	**Time (min)**	**% solvent A**	**% solvent B**
	0	99	1
	0.5	99	1
	2.0	1	99
	3.0	1	99
	3.1	99	1
	4.0	99	1

2.3.5 Quantitation

A minimum six calibration standard solutions were prepared at incremental concentrations spanning the relevant concentration range. A consistent, proportionate volume of internal standard was added to each standard, and the area ratio between target analyte and internal standard for each concentration was used to form the calibration curve. A linear detector response for each analyte ($r > 0.99$) was required for quantitation.

AB Sciex Multiquant (Ver. 2.1) software was used for data processing the LCMS runs. Chromeleon 7 software was used to process the data generated by the UHPLC-UV system. Concentrations of the analyte were calculated using the peak area ratio of target analyte detected in each sample to the corresponding internal standard, and the regression equation of the calibration curve. The internal standard for 5-hydroxyflunixin was flunixin-d_3.

2.4 Method Validation

The analytical method for determination of flunixin, 5-hydroxyflunixin, ketoprofen and carprofen in ovine plasma was validated using an in-house protocol developed from procedures described in current EMA[2,3], APVMA[4], VICH[5,6] and FDA[7] guidance documents. The core components of the validation protocol, expected outcomes and indication of compliance obtained, are summarised in Table 6. A summary of data generated during validation is presented in this section.

Table 6. Validation checklist

Validation Test	Description	Expected outcome	Satisfactory
Analyte Identification	Visual confirmation of presence of detector response at retention time of target analyte and internal standard.	A detector response that is sufficient to identify the target analyte and internal standard. Absence of response in blank matrix at concentrations >20% of the proposed LLOQ of the target analyte, and 5% of the internal standard.	Yes
System suitability	Repeatability of six injections of the same solution containing target analyte.	Area RSD <5% (LCMSMS) Retention time RSD <2%	Yes
Chromatographic parameters	Symmetry factor (EP)	Recommended Standard <1.5	Yes
	Resolution (EP)	Recommended Standard >1.5	Yes
	Number of theoretical plates (EP)	>3000	Yes
Selectivity	Analysis of a minimum six blank matrices from different sources.	Absence of analyte response and matrix interferences in extracts of blank matrices >20% of target analyte at LLOQ, and 5% of internal standard	Yes
Carryover	Residual of the analyte of interest, internal standard or matrix effects that appear within the chromatogram of the subsequent injection.	Carryover should not be greater than 20% of target analyte at LLOQ and 5% for the internal standard	Yes
Lower Limit of Quantitation (LLOQ)	Lowest concentration that can be consistently measured within accuracy and precision limits	Guide 1/20th Cmax or in accordance with study goals.	Yes
Calibration curve	Covers the range of concentrations of study samples	Calibration method justified. Calibration range determined adequate. Correlation coefficient (r) >0.99	Yes
Accuracy	Closeness of agreement of measured value to the true value – determined using fortified samples	Mean % accuracy of target analyte is within ±20% at LLOQ and ±15% at concentrations greater than LLOQ	Yes
Precision	Closeness of agreement of replicate determinations - based on %CV of fortified samples used for accuracy determination	%Coefficient of variation (%CV) of target analyte is within ±20% at LLOQ and ±15% at concentrations greater than LLOQ	Yes

2.4.1 Analyte Identification

The objective was to determine if the analytical methodology, instrumental technique and configuration could differentiate the analytes of interest from endogenous components of the matrix. This test was a visual examination of chromatograms of standards, internal standards, samples and blanks to confirm presence or absence of target analytes. A record of the comparison is provided in Table 7, and example chromatograms are provided in Appendix 2. Transitions selected for quantitation were used in this comparison.

Table 7. Analyte identification data

Molecule	5-OH Flunixin	Ketoprofen	Ketoprofen-^{13}C,d$_3$	Flunixin	Flunixin-d$_3$	Carprofen	Carprofen-d$_3$	Chrom. Ref.
Transition	311.1→227.1	253.3→197.0	257.2→197.1	295.1→191.0	298.2→254.0	272.1→228.0	275.0→231.0	
Retention Time (minutes)	2.49	2.53	2.53	2.64	2.64	2.81	2.81	
Mobile phase (0µL injection)	ND	ND	ND	ND	ND	ND	ND	Fig. 5
Reagent blank (4%methanolic acetic acid)	ND	ND	ND	ND	ND	ND	ND	Fig. 6
Flunixin standard [FLUNIX]	ND	ND	ND	2.66	ND	ND	ND	Fig. 7
5-hydroxyflunixin standard [5-OH FLU]	2.49	ND	ND	Small response (impurity)	ND	ND	ND	Fig.8
Ketoprofen standard [KETO]	ND	2.54	ND	ND	ND	ND	ND	Fig.9
Carprofen standard [CARP]	ND	ND	ND	ND	ND	2.81	ND	Fig. 10
Matrix blank	ND	ND	ND	ND	ND	ND	ND	Fig. 11
Matrix blank with internal standard	ND	ND	2.53	ND	2.65	ND	2.80	Fig. 12
Mixed Calibration standard*	ND	2.53	2.52	2.65	2.64	2.80	2.80	Fig. 13
Fortified sample (at LLOQ)*	ND	2.53	2.52	2.65	2.64	2.80	2.79	Fig. 14
Fortified sample (1000ng/mL)*	ND	2.52	2.52	2.64	2.64	2.79	2.79	Fig. 15

ND – no significant instrumental response detected at the retention time
*Note: fortified with flunixin, ketoprofen and carprofen only.

The presence of target analytes and internal standards at the respective transitions has been confirmed in samples where a detector response was anticipated. The peaks are well resolved from endogenous peaks.

2.4.2 System Suitability/ chromatographic parameters

To demonstrate that the instrument is stable prior to validation and that the settings and column configuration are capable of meeting a minimum in-house specification for peak shape, plate counts and resolution.

Table 8. System suitability and chromatographic parameters

Chromatographic parameter	Description	Evaluation criteria	Determined value			
			5-OH FLU	KETO	FLUNIX	CARP
Repeatability – retention time	Closeness of agreement of analyte retention time following six replicate injections	%Coefficient of variation <2%	0.33	0.32	0.20	0.18
Repeatability – peak area	Closeness of agreement of analyte peak area/ISTD area ratio following six replicate injections	%Coefficient of variation <5%	2.43	1.52	3.36	2.36
Symmetry Factor (EP)	Shape of the chromatographic peak 1 assumes Gaussian shape, <1 peak fronting, >1 peak tailing	Factor <1.5	1.17	1.17	1.13	1.17
Resolution (EP)	Degree of separation between two chromatographic peaks	Factor >1.5		0.36	2.36	2.54
Theoretical plates (EP)	Theoretical indication of column efficiency	Factor >3000	42254.20	31519.66	34951.29	52484.73

Chromatographic parameters are indicative of compliance with the in-house specifications. The resolution between 5-hydroxyflunixin and ketoprofen is not baseline, however, this is overcome due to the specificity of the MSMS transition selected.

2.4.3 Selectivity

The objective was to determine if the analytical method could differentiate the analytes of interest from endogenous components within the matrix.

Replicate aliquots (n=3) of plasma obtained from six individual subjects that had not received treatment with target analyte were analysed according to the method, including addition of the internal standard. The peak area ratio of the response detected at the retention time of the target analyte to the internal standard in the blank plasma was expressed as a percentage of peak area ratio of the corresponding plasma containing target analyte fortified at LLOQ.

The average % area ratio of the target analyte at LLOQ is presented in Table 9. The % peak area of the internal standard is presented in Table 10.

Table 9. Selectivity – average %area ratio of LLOQ

Blank Plasma	n	5-hydroxyflunixin	Ketoprofen	Flunixin	Carprofen
Transition		311.1→227.1	253.3→197.0	295.1→191.0	272.1→228.0
S5	3	0.00	7.25	0.61	18.74
S6	3	0.52	0.00	0.28	20.72
S7	3	1.36	10.95	0.46	6.73
S8	3	5.21	16.12	0.26	6.39
S9	3	2.20	0.00	0.13	2.75
S10	3	1.63	1.11	0.13	4.67

Table 10. Selectivity - %area of internal standard

Blank Plasma	Ketoprofen-^{13}C,d$_3$	Flunixin-d$_3$	Carprofen-d$_3$
Transition	257.2→197.1	298.2→254.0	275.0→231.0
S5	0.11	0.10	1.32
S6	0.06	0.11	1.21
S7	0.04	0.14	0.35
S8	0.15	0.19	1.07
S9	0.04	0.14	1.70
S10	0.05	0.11	0.80

All detected responses at the retention time and transition of target analytes are less than 20% of the LLOQ.

Responses at the retention time and transition of the internal standards are less than 5% of the peak area response of the internal standard.

The method is selective for ketoprofen, carprofen, flunixin and 5-hydroxyflunixin.

2.4.4 Carry over

The objective was to determine the degree and extent of carry-over. This was assessed by injecting a reagent blank after a high concentration sample or calibration standard at the upper limit of quantitation.

Table 11. Carry over data

Analyte	Retention time	Transition	%LLOQ	% internal std
5-hydroxyflunixin	2.49	311.1→227.1	20.0	-
Ketoprofen	2.54	253.3→197.0	13.8	-
Ketoprofen-^{13}C,d$_3$	2.54	257.2→197.1	-	0.06
Flunixin	2.61	295.1→191.0	6.8	-
Flunixin-d$_3$	2.61	298.2→254.0	-	0.17
Carprofen	2.81	272.1→228.0	15.0	-
Carprofen-d$_3$	2.81	275.0→231.0	-	0.19

The system configuration, including needle and injection loop wash step were considered adequate to prevent carry-over to the subsequent injection.

2.4.5 Lower Limit of Quantitation

The Lower Limit of Quantitation (LLOQ) is the lowest quantity of analyte that can be reliably and consistently determined within acceptable accuracy and precision limits. The objective was to determine the LLOQ, and if possible, achieve an LLOQ at or around $1/20^{th}$ C_{max} or in accordance with study goals.

Accuracy and precision limits for LLOQ establishment
Accuracy[1]: mean concentration within 20% of the nominal value
Precision[1]: %CV should not exceed 20%

Data relevant to the establishment of the LLOQ is presented in Table 13.

Table 12a. LLOQ determination (LCMSMS)

Analyte	Fortified Concentration ng/mL	Replicates n	Mean detected concentration ng/mL	Mean % accuracy	%CV	LLOQ ng/mL
5-hydroxyflunixin	20.0	4	21.05	105.2	15.1	20
Ketoprofen	9.94	6	10.07	101.3	12.2	10
Flunixin	9.84	6.	11.61	118.0	6.6	10
Carprofen	14.91	6	16.02	107.4	7.9	20

Table 12b. LLOQ determination (UV)

Analyte	Fortified Concentration µg/mL	Replicates n	Mean detected concentration µg/mL	Mean % accuracy	%CV	LLOQ µg/mL
1	2.176	4	1.978	90.9	14.7	1.0

The LLOQ are considered acceptable and meet study requirements.

2.4.6 Calibration Curve

The objective was to assess the instrumental response of the target analyte at a concentration range from LLOQ to an appropriate upper limit as determined by literature or study requirements. A minimum six concentrations of target analyte increasing incrementally over the desired range were prepared and analysed.

Example calibration curves for the quantitative transitions are provided in Appendix 3 and calibration parameters are summarised in Table 13.

Table 13. Calibration parameters

Analyte	Slope	Intercept	Weighting	Correlation coefficient (r)
5-hydroxyflunixin	5.0493×10^{-4}	8.86×10^{-5}	1/x	0.9998
Ketoprofen	0.00387	0.00382	1/x	0.9991
Flunixin	0.00215	-0.00216	1/x	0.9995
Carprofen	0.00251	0.00456	1/x	0.9987

The correlation coefficient (r) is consistent with the expectation of r>0.99 for the calibration curves produced using calibration standards. A 1/x weighting was applied

to the regression equation to improve accuracy and %CV at the sub-100ng/mL section of the curves.

2.4.7 Accuracy

The objective was to assess method accuracy by determining the closeness of the value determined by the method to the known concentration of the analyte.

A minimum four replicate fortified samples at four concentrations spanning the assay range were prepared and analysed. The concentrations were generally at LLOQ, three times LLOQ, 50% calibration range, 75% of the upper range.

Accuracy was assessed by comparing the determined value to the nominal (fortified) value and expressing as a percentage of the nominal value.

Detected concentration/Nominal value x 100= % accuracy.

Accuracy[1]: Four out of six accuracy determinations should be compliant with the following:
no more than ±20% from the nominal value at LLOQ, and no more than 15% for determinations of higher concentrations.

Method accuracy data obtained is summarised in Tables14a-e.

Table 14a. Accuracy determination – Ketoprofen

Sample	Fortified Conc. ng/mL	Replicates n	Mean detected concentration ng/mL	Mean % Accuracy
1	9.94	6	10.07	101.3
2	39.76	6	40.06	100.8
3	397.6	6	417.0	104.9
4	994.01	6	1063.8	107.2
5	1988.01	6	2083.9	104.8

Table 14b. Accuracy determination – Flunixin

Sample	Fortified Conc. ng/mL	Replicates n	Mean detected concentration ng/mL	Mean % Accuracy
1	9.84	6.	11.61	118.0
2	39.35	6	43.69	111.0
3	393.51	6	416.27	105.8
4	983.77	6	1078.8	109.7
5	1967.5	6	2151.4	109.4
6	9976.4	6	10095.1	101.2

Table 14c. Fortified sample accuracy determination – 5-hydroxyflunixin

Sample	Fortified Conc. ng/mL	Replicates n	Mean detected concentration ng/mL	Mean % Accuracy
1	20	4	21.05	105.2
2	2500	4	2806.3	112.3

Table 14d. Fortified sample accuracy determination – Carprofen (by LCMSMS)

Sample	Fortified Conc. ng/mL	Replicates n	Mean detected concentration ng/mL	Mean % Accuracy
1	14.91	6	16.02	107.4
2	59.62	6	68.57	115.0
3	596.2	6	670.36	112.4
4	1490.5	6	1619.2	108.6
5	2981.02	6	2959.07	99.26

Table 14e. Fortified sample accuracy determination – Carprofen (by UV)

Sample	Fortified Conc. µg/mL	Replicates n	Mean detected concentration µg/mL	Mean % Accuracy
1	2.176	4	1.978	90.9
2	8.703	4	8.959	102.9
3	13.055	4	12.821	98.2
4	21.758	4	21.055	96.8
5	78.33	6	74.624	95.3
6	156.66	6	157.97	100.8

Within-run accuracy data are compliant with the predetermined acceptance criteria.

2.4.8 Precision

The objective was to assess the closeness of repeated individual measures obtained by the method using fortified samples over a range of concentrations.

Within-run Precision[1]: Within-run CV should not exceed 15% for all concentrations except LLOQ where it should not exceed 20%.

Within-run precision data obtained is summarised in Tables 15a-e.

Table 15a. Precision (within-run) – Ketoprofen

Sample	Fortified Conc. ng/mL	Replicates n	Mean detected concentration ng/mL	%CV
1	9.94	6	10.07	12.2
2	39.76	6	40.06	6.9
3	397.6	6	417.0	2.7
4	994.01	6	1063.8	2.1
5	1988.01	6	2083.9	4.2

Table 15b. Precision (within-run) – Flunixin

Sample	Fortified Conc. ng/mL	Replicates n	Mean detected concentration ng/mL	%CV
1	9.84	6.	11.61	6.6
2	39.35	6	43.69	1.9
3	393.51	6	416.27	1.7
4	983.77	6	1078.8	2.8
5	1967.5	6	2151.4	2.6
6	9976.4	6	10095.1	8.1

Table 15c. Precision (within-run) – 5-hydroxyflunixin

Sample	Fortified Conc. ng/mL	Replicates n	Mean detected concentration ng/mL	%CV
1	20	4	21.05	15.1
2	2500	4	2806.3	3.0

Table 15d. Precision (within-run) – Carprofen (by LCMSMS)

Sample	Fortified Conc. ng/mL	Replicates n	Mean detected concentration ng/mL	%CV
1	14.91	6	16.02	7.9
2	59.62	6	68.57	6.3
3	596.2	6	670.36	2.8
4	1490.5	6	1619.2	5.5
5	2981.02	6	2959.07	3.8

Table 15e. Precision (within-run) – Carprofen (by UV)

Sample	Fortified Conc. µg/mL	Replicates n	Mean detected concentration µg/mL	%CV
1	2.176	4	1.978	14.7
2	8.703	4	8.959	3.5
3	13.055	4	12.821	2.0
4	21.758	4	21.055	1.8
5	78.33	6	74.624	0.8
6	156.66	6	157.97	7.9

Data generated for a within-run precision test using fortified samples was within acceptance limits.

3 Analysis of Study Samples

3.1 Sample Details

3.1.1 Description

Four hundred and eighty (480) plasma samples were received from the animal phase investigator on 5.9.2013. Samples were received in a frozen state, and immediately transferred to a Sanyo UMF-333 biomedical freezer maintained at -30°C. A sample schedule was provided by the animal phase investigator and is included in Appendix 4.

Sample management within Pia Pharma is controlled by an in-house SOP. For this submission, the samples were grouped by Cohort, treatment, animal number and sample time in the Pia Pharma Sample Receipt Spreadsheet. A unique laboratory identification number was assigned to each sample, and applied to each sample container for traceability purposes. The sample receipt table is provided in Appendix 5.

3.1.2 Sample Storage

Plasma samples were stored in a Sanyo UMF-333 biomedical freezer set at -30°C for the study duration.

3.1.3 Sample Preparation

On the day of analysis, samples representing the 'batch' were allowed to thaw completely at room temperature. Samples were mixed for one minute using a gentle reciprocating motion prior to obtaining the test portion. At completion of sampling, the sample containers were returned to the freezer. The time samples were held at room temperature was not permitted to exceed two hours.

3.1.4 Batch Analysis

On the day of analysis, all samples were prepared by the same analyst, without interruption, using the same reagents and under the same conditions.

Approximately 10% of the total samples analysed per batch were fortified samples for quality control purposes. Additionally, approximately 10% of study samples were repeated on the day of analysis, and the relative standard deviation of replicates assessed.

During the preparation stage, each batch of samples was processed in a series of smaller batches due to the centrifuge step being rate limiting. Each batch contained one fortified QC sample, ten test samples and one sample for repeat analysis.

For instrumental determination, the order of injection closely resembled the order of preparation, except fortified samples were injected at the start of the run immediately after the calibration standards.

3.2 Quality Control

This section details information regarding the quality control activities conducted during analysis of study samples in order to assess conformance with quality standards for a bioanalytical method.

3.2.1 Standard preparation

Duplicate stock solutions of each target analyte were prepared and compared by HPLC. Their agreement was determined to be within ±2%, indicating the method of standard preparation to be repeatable.

Mixed intermediate standard solutions of target analytes and internal standards were prepared prior to commencement and stored in the refrigerator when not in use during the analysis period. Intermediate standards were demonstrated to be stable for four weeks which exceeded the usage requirement.

3.2.2 Calibration

A minimum of six standard concentrations in 20% water/ 80% 4% acetic acid in methanol, spanning from LLOQ to the expected upper range, were prepared daily. Calibrators were injected at run commencement and at the end of the run. All injections were assessed during preparation of the calibration curve.

The calibration curve was prepared by plotting the nominal target analyte concentration (x axis) against the peak area ratio of target analyte and deuterated internal standard. This curve was used for determination of quality control samples and for quantitation of unknown concentrations in study samples.

A correlation co-efficient (r) greater than 0.99, was required for the calibration curve to be used for quantitative purposes. The back calculated concentrations of the calibrators were expected to be within 15% of the nominal value, except at LLOQ, were a deviation of 20% was acceptable.

3.2.3 Fortified plasma samples

Fortified QC samples were prepared daily, prior to commencement of batch analysis. Blank plasma was fortified with a nominal concentration of mixed intermediate solutions to produce a minimum four fortified plasma samples at concentrations from LLOQ to approximately 50-75% of the expected upper limit of quantitation. Replicates of each concentration were prepared and dispersed throughout the batch analysis. Generally, one fortified sample was analysed with every batch of ten samples.

Method accuracy was determined using the standard calibration curve. For the data set to be considered acceptable for release, a minimum four out of six fortified QC samples were expected to be within 15% of the nominal value, except at LLOQ where a limit of 20% was acceptable.

3.3   Quantitation of target analyte

3.3.1   Quantitation method

Data processing of chromatograms obtained by LCMSMS was conducted using AB Sciex Multiquant (Ver. 2.1) software. Data processing of chromatograms generated by UHPLC-UV was conducted using Chromeleon 7. Both software packages calculated the unknown concentration of target analyte in the sample extract by determining the peak area ratio of the target analyte and internal standard response, and using the regression equation of the standard calibration curve.

3.3.2   Acceptance of the analytical run

The quality control data generated with the batch was evaluated for conformance to pre-determined acceptance criteria prior to acceptance of the run. The criterion for acceptance of the analytical run was in accordance with quality standards derived from relevant guidelines.

At the conclusion of each analytical run, the analyst completed a Bioanalytical Quality Control Review (Form.011) which facilitated an assessment of conformance of the analytical run. Instrument reports and the assessment form were reviewed by both Analyst and Quality Assurance Officer in turn.   Acceptance of the run was co-signed by the Analyst and Quality Assurance Officer.   Deviations and corrective actions, if any, were identified, discussed and resolved during this process.

The system of double checking transcription of results from instrument report to Microsoft Excel spreadsheet was in accordance with in-house standard operating procedures. Analyst and Quality Assurance Officer co-signed the review form to confirm that the transfer was accurate.

3.4   Chromatograms

A selection of chromatograms generated during the study have been provided in Appendix 6.

3.5   Documentation

Electronic records of instrument raw data and reports were stored on the instrument hard drive under Project 1309-066. This data was backed up automatically to a second hard drive on the instrument.

An additional electronic copy of instrument reports in pdf and MS Excel was retained in the Pia Pharma project folder under Project 1309-066 and a back-up retained off-site.

All details pertaining to analyses were recorded on laboratory worksheets which were stored with hardcopies of analysis data and the Analytical Report in Project Folder 1309-066. The folder has been retained within a secured cabinet on the premises. Access to this cabinet is restricted to Authorised Persons only.

4 Results

4.1 Raw Data –

4.1.1 Flunixin

Concentrations of flunixin determined in plasma of treated animals within each cohort are presented in Table 16. Concentrations are reported in ng/mL.

Table 16. Analytical data – Flunixin ng/mL

Cohort 1 — Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
422	<10	6399.6	205.9	4082.4	690.8	4788.5	997.5	4908.3	647.3	8735.5	1128.6	10049.1
494	<10	3204.3	191.0	3632.1	96.3	5634.9	40.3	2798.6	35.8	2188.2	35.3	2468.5
498	<10	3877.9	131.2	4774.4	73.1	3359.2	77.9	2876.0	47.8	3258.6	41.0	2589.8
555	<10	2458.8	102.6	4351.8	179.3	4151.6	75.6	2695.2	77.7	3619.0	97.4	3796.2
572	<10	2307.1	107.3	1901.8	172.4	2701.8	96.3	2214.4	55.5	2382.9	41.2	2123.3
Average Flunixin Cohort 1	**0**	**3649.5**	**147.6**	**3748.5**	**242.4**	**4127.2**	**257.5**	**3098.5**	**172.8**	**4036.8**	**268.7**	**4205.4**

Cohort 2 — Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
302	<10	2943.0	141.8	3252.3	57.2	2923.6	49.7	2096.2	34.3	2380.2	43.4	2334.6
488	<10	2105.2	183.3	2650.5	194.7	2246.8	208.8	1976.1	76.9	1971.8	49.9	2310.5
492	<10	3211.8	122.0	2298.5	98.6	2461.1	74.4	2182.2	56.1	2729.3	54.9	2182.6
607	<10	4651.0	159.2	2956.2	113.9	4004.3	49.3	3125.2	35.2	3429.3	34.2	3614.6
769	<10	3122.1	212.1	3155.5	190.8	4052.8	50.3	3642.5	63.8	2923.7	118.6	2570.3
Average Flunixin Cohort 2	**0**	**3206.6**	**163.7**	**2862.6**	**131.0**	**3137.7**	**86.5**	**2604.4**	**53.3**	**2686.9**	**60.2**	**2602.5**

4.1.2 5-hydroxyflunixin

Concentrations of 5-hydroxyflunixin determined in plasma of treated animals within each cohort are presented in Table 17. Concentrations are reported in ng/mL.

Table 17. Analytical data – 5-hydroxyflunixin ng/mL

Cohort 1	Sampling time point (hours)											
Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
422	<20	457.0	34.8	453.7	81.3	454.7	203.5	<20	309.6	1171.9	1767.5	3085.3
494	<20	448.4	<20	278.2	<20	520.4	<20	131.7	<20	153.7	<20	153.8
498	<20	317.8	<20	322.7	<20	243.3	<20	255.3	<20	179.3	<20	376.0
555	<20	63.7	<20	120.8	<20	245.1	<20	125.7	<20	98.3	<20	199.3
572	<20	195.6	<20	105.0	<20	198.6	23.0	142.3	<20	129.7	21.6	93.7
Average 5-OH Flunixin Cohort 1	0	296.5	15.0	256.1	24.3	332.4	51.3	133.0	69.9	346.6	363.8	781.6

Cohort 2	Sampling time point (hours)											
Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
302	<20	186.1	<20	147.5	33.8	207.6	<20	182.9	<20	244.2	<20	145.7
488	<20	103.9	<20	104.2	<20	72.4	32.7	81.5	<20	166.3	<20	125.6
492	<20	394.0	<20	140.8	26.4	332.0	<20	326.4	<20	280.8	<20	341.8
607	<20	410.2	<20	123.7	<20	180.5	<20	191.4	<20	307.1	<20	225.4
769	<20	182.9	<20	22.2	<20	328.5	<20	309.7	<20	249.2	27.6	228.4
Average 5-OH Flunixin Cohort 2	0	255.4	10.0	107.7	18.0	224.2	14.5	218.4	10.0	249.5	13.5	213.4

Note: A value of 0.5 x LLOQ applied to values reported as <LLOQ for calculation of group average.

4.1.3 Ketoprofen

Concentrations of ketoprofen determined in plasma of treated animals within each cohort are presented in Table 18. Concentrations are reported in ng/mL.

Table18. Analytical data – ketoprofen ng/mL

Cohort 1   Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
311	<10	7409.1	13.6	6072.4	115.6	4467.8	<10	4931.3	<10	4619.3	<10	4050.1
437	<10	12536.6	25.7	11792.5	<10	8875.4	<10	6335.7	<10	5967.0	<10	5248.3
493	<10	7320.8	136.3	7196.3	107.2	6807.9	39.6	6207.3	14.2	5356.0	18.0	5870.9
730	<10	7108.4	20.2	9454.7	<10	7271.6	11.8	5737.3	12.2	5473.5	<10	4737.6
760	<10	8050.3	13.0	6510.8	<10	8346.0	<10	4101.3	<10	3829.2	<10	4304.1
Average Ketoprofen Cohort 1	0	8485.1	41.8	8205.3	47.6	7153.7	13.3	5462.6	8.3	5049.0	7.6	4842.2

Cohort 2   Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
306	<10	4456.9	17.2	5389.0	27.5	6882.3	11.9	4368.2	<10	7141.7	<10	6827.2
313	<10	3176.9	6.2	6922.6	<10	4971.3	<10	5081.1	<10	4404.6	<10	4723.9
436	<10	4196.3	19.2	3889.1	103.2	5710.3	<10	5009.0	<10	5542.9	<10	4949.4
520	<10	4125.1	85.4	4889.0	49.9	4618.3	10.8	3362.3	10.3	4315.0	13.9	4375.7
784	<10	6849.2	10.0	8796.5	5.0	7705.6	<10	6358.0	<10	12326.0	<10	6913.6
Average Ketoprofen Cohort 2	0	4560.9	27.6	5977.2	38.1	5977.6	7.5	4835.7	6.1	6746.1	6.8	5557.9

Note: A value of 0.5 x LLOQ applied to values reported as <LLOQ for calculation of group average.

4.1.4 Carprofen

Concentrations of carprofen determined in plasma of treated animals within each cohort are presented in Table 19. Concentrations are reported in µg/mL.

Table 19. Analytical data – carprofen µg/mL

Cohort 1 Carprofen   **Sampling time point (hours)**

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
312	<1.0	15.6	29.1	51.5	40.2	62.8	44.4	61.6	43.0	56.6	34.6	51.8
404	<1.0	41.1	36.6	59.1	54.4	78.6	55.9	78.5	47.8	67.7	49.3	69.6
558	<1.0	33.2	30.9	64.4	43.5	91.9	40.2	68.8	39.3	54.8	38.2	59.9
605	<1.0	56.4	27.9	88.6	49.7	102.6	39.3	37.4	66.0	59.4	29.2	57.8
675	<1.0	52.2	31.7	70.8	38.0	73.3	37.6	58.7	33.3	50.4	34.8	56.6
Average Carprofen Cohort 1	0	39.7	31.3	66.9	45.1	81.9	43.4	61.0	45.9	57.8	37.2	59.1

Cohort 2 Carprofen   **Sampling time point (hours)**

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
310	<1.0	20.2	34.5	59.5	46.9	59.1	42.3	64.6	38.6	54.3	35.2	51.2
502	<1.0	27.1	28.7	54.1	34.4	75.3	30.1	72.2	32.3	56.3	36.2	79.2
585	<1.0	57.1	30.7	60.1	41.9	57.5	37.5	55.2	34.5	54.0	33.0	53.3
640	<1.0	32.5	26.3	47.8	38.1	67.6	37.1	60.2	32.6	65.6	30.7	53.9
727	<1.0	23.7	34.8	55.8	44.2	67.6	40.3	60.9	37.5	55.9	36.5	56.3
Average Carprofen Cohort 2	0	32.1	31.0	55.5	41.1	65.4	37.5	62.6	35.1	57.2	34.3	58.8

4.1.5 Saline Group

The reported method was used to determine concentrations of flunixin, ketoprofen and carprofen in the plasma of animals treated with saline within each cohort. Concentrations are reported in ng/mL.

Table 20. Saline group analytical data –ng/mL

Cohort 1 Flunixin   Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
314	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
495	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
576	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
652	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
716	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Cohort 2 Flunixin   Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
301	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
340	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
458	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
559	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
698	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Cohort 1 Ketoprofen   Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
314	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
495	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
576	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
652	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
716	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Cohort 2
Ketoprofen Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
301	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
340	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
458	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
559	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10
698	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

Cohort 1 Carprofen Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
314	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
495	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
576	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
652	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
716	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20

Cohort 2 Carprofen Sampling time point (hours)

Animal No.	-24	-22	0	2	24	26	48	50	72	74	96	98
301	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
340	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
458	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
559	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20
698	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20

4.2 Graphical Presentation

A plot of average concentrations for each treated cohort are overlaid in Figures 1-4.
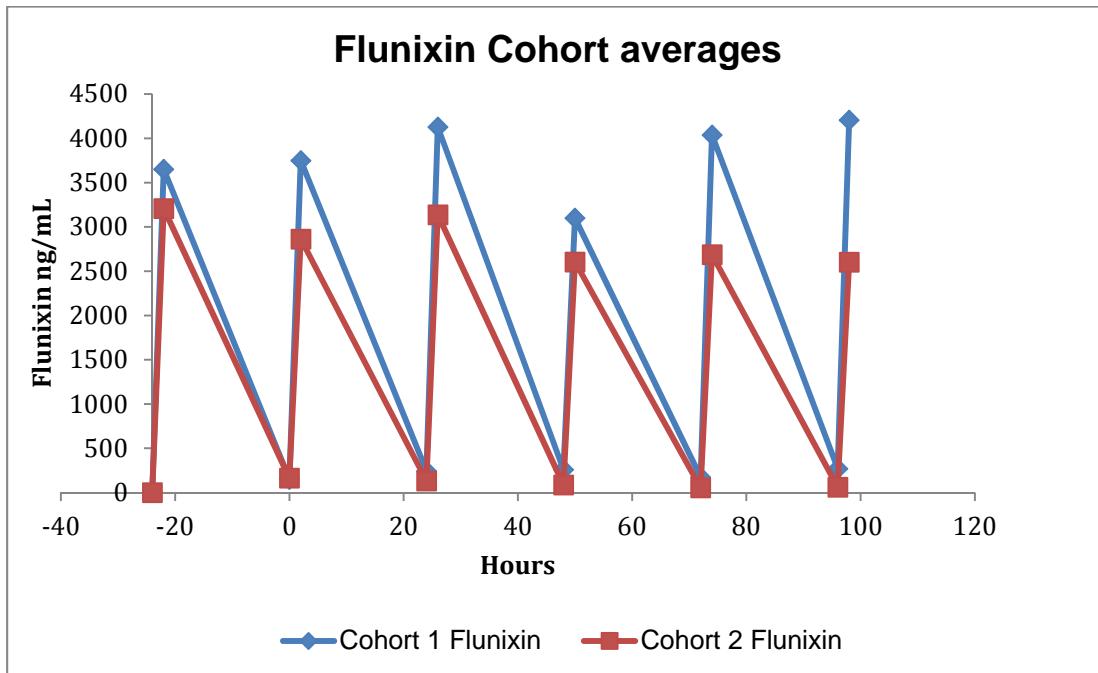
Figure 1. Average flunixin concentrations vs time – overlaid cohort data
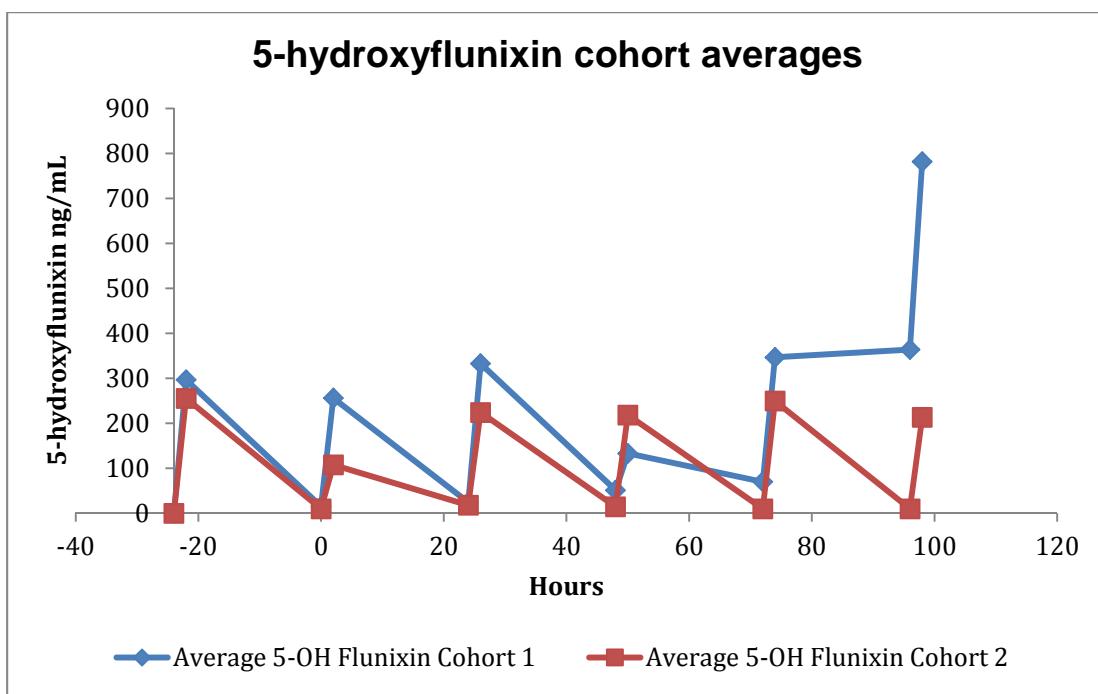
Figure 2. Average 5-hydroxyflunixin concentrations vs time – overlaid cohort data (animals administered flunixin)
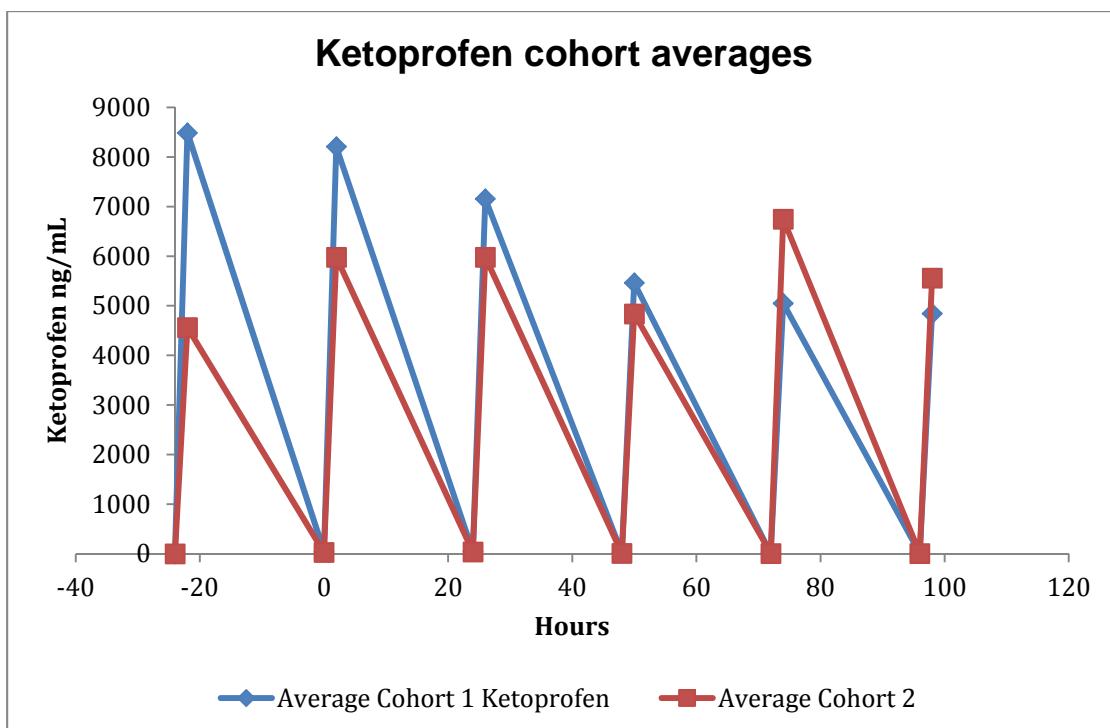
Figure 3. Average ketoprofen concentrations vs time – overlaid cohort data
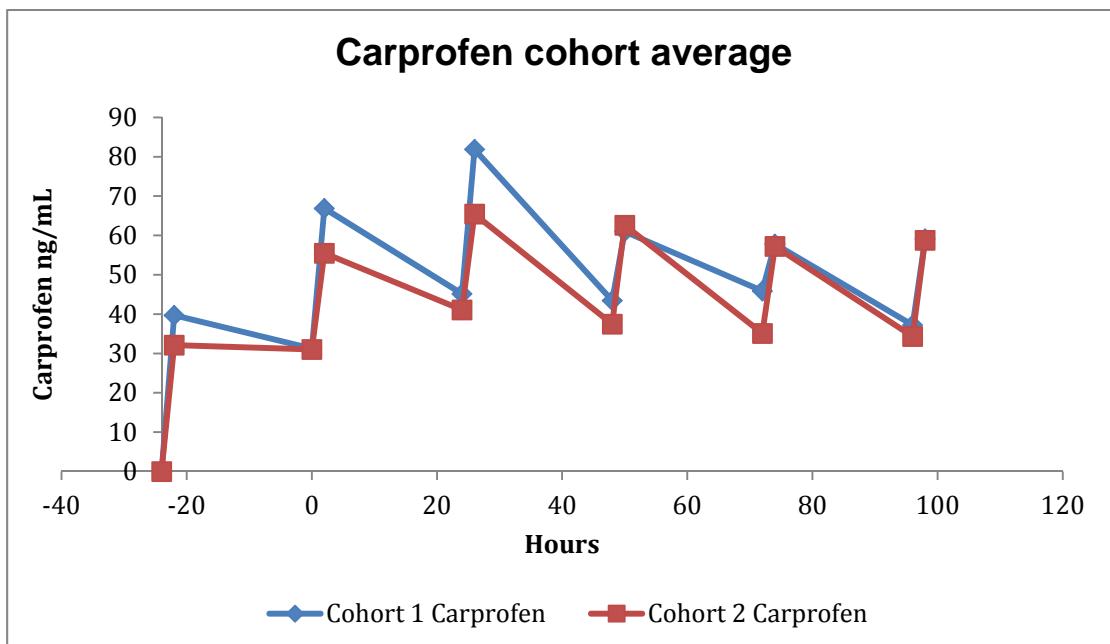
Figure 4. Average carprofen concentrations vs time – overlaid cohort data

5 Discussion

This report details concentrations flunixin, 5-hydroxyflunixin, ketoprofen and carprofen determined in plasma samples originating from an oral bioavailability study conducted in sheep.

Concentrations of flunixin, 5-hydroxyflunixin, ketoprofen and carprofen were determined using a validated analytical method based on liquid chromatography-positive electrospray ionisation- tandem mass spectrometry. Details of the methodology and outcomes of the supporting validation process have been reported. Specific control measures to assure the quality of the analyses conducted during this study have been presented.

The analytical data has been presented in tabular and graphical format.

6 Conclusion

The objectives of the analytical component;
- to develop and validate a sensitive and selective assay for the determination of flunixin, ketoprofen and carprofen in ovine plasma;
- to quantify and report concentrations of flunixin, ketoprofen and carprofen in ovine plasma;

were achieved.

7 References

1. APVMA Bioequivalence Guidelines for Veterinary Chemical Products
   http://www.apvma.gov.au/publications/guidelines/gl13_bioequivalence.php

2. Guideline on the conduct of bioequivalence studies for veterinary medicinal products Committee for Medicinal Products for Veterinary Use (CVMP) 11 April 2011 EMA/CVMP/016/00-Rev.2

3. Guideline on bioanalytical method validation (Feb 2012) Committee for Medicinal Products for Human Use (CHMP)EMEA/CHMP/EWP/192217/2009 http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf

4. APVMA Residue Guideline No. 26 - Veterinary Drug Residue Analytical Methods

5. VICH GL1 – Validation of Analytical Procedures – Definitions and Terminology (October 1998)

6. VICH GL2 - Validation of Analytical Procedures – Methodology (October 1998)

7. Guidance for Industry – Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2001

Appendix 1 – Certificates of analysis

CERTIFICATE OF ANALYSIS

Toronto Research Chemicals Inc.

Bringing you products for innovative research

2 Brisbane Road, North York, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:
75369-61-8

Catalogue Number:
H942420

Product:
5-Hydroxy Flunixin (Contain 5% Flunixin)

Synonyms:
5-Hydroxy-2-[[2-methyl-3-(trifluoromethyl)phenyl]amino]-3-pyridinecarboxylic Acid

Structure:
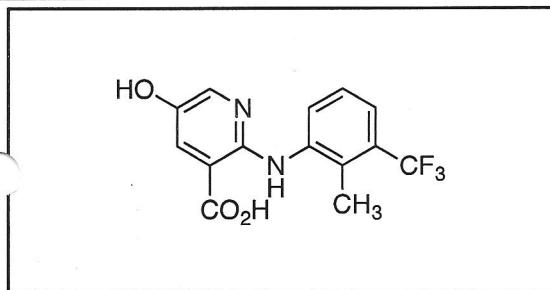
Molecular Formula:
$C_{14}H_{11}F_3N_2O_3$

Molecular Weight:
312.24

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
1-YSW-78-2

Melting Point:
>215°C (dec.)

Boiling Point:
N/A

Atmosphere:
Air

Appearance of Product:
Orange Solid

Solubility
DMSO, Methanol

Method for Determining Identity:
^{1}H NMR (DMSO-d_6) Spectroscopic and Mass Spectrometric Analysis

Stability
Not determined

Purity:
95%

Long Term Storage Condition:
-20°C Freezer

Additional Information:
TLC Conditions: SiO_2; Dichloromethane: Methanol = 9: 1; Visualized with UV and $KMnO_4$; Single spot; Rf=0.45.
^{1}H NMR and mass spectra conform to structure.
Contains 2.5% of Ethyl Acetate and 2% of Acetic acid.
Contain 5% Flunixin
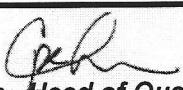
Philip Chan, Head of Quality Assurance

QC Test Date
March 6, 2012

Retest Date
March 6, 2015

PM Separations

Phone (07) 3390 1096
Email: customerservice@pmsep.com.au
www.pmsep.com.au

pm SEPARATIONS

TRC
Toronto Research Chemicals Inc.

Bringing you products for innovative research.

CERTIFICATE OF ANALYSIS

2 Brisbane Road, North York, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:

Product:
rac Ketoprofen-^{13}C,d3

Catalogue Number:
K200802

PM Separations

pm SEPARATIONS

Phone (07) 3390 1096
Email: customerservice@pmsep.com.au
www.pmsep.com.au

Synonyms:

3-Benzoyl-α-methyl-^{13}C,d3-benzeneacetic Acid; m-Benzoylhydratropic Acid-^{13}C,d3;
2-(3-Benzoylphenyl)propionic Acid-^{13}C,d3; RP-19583-^{13}C,d3; Alreumat-^{13}C,d3; Alrheumun-^{13}C,d3; Capisten-^{13}C,d3;
Epatec-^{13}C,d3; Fastum-^{13}C,d3; Ketofen-^{13}C,d3;

Structure:
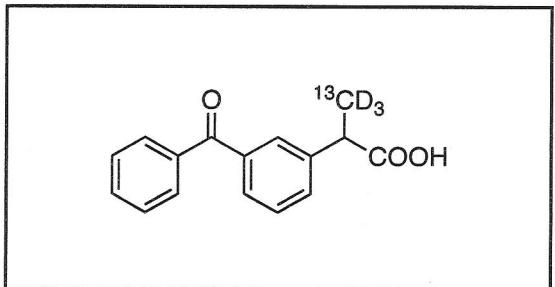
Molecular Formula:
$C_{15}{}^{13}CH_{11}D_3O_3$

Molecular Weight:
258.29

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
4-RUS-38-3

Melting Point:
93-95°C

Boiling Point:
N/A

Atmosphere:
Air

Appearance of Product:
White Solid

Solubility
Chloroform, Methanol

Method for Determining Identity:
^{1}H NMR (CDCl$_3$) Spectroscopic and Mass Spectrometric
Analysis

Stability
Not determined

Purity:
Chemical purity: 98%
Isotopic purity: 99.5%

Long Term Storage Condition:
-20°C Freezer

Additional Information:
TLC Conditions: SiO$_2$; Dichloromethane : Methanol = 9 : 1; Visualized with UV; Single spot, Rf=0.5.
^{1}H NMR and Mass spectra conform to structure.
Normalized intensity: $d_0 = 0.42\%$, $d_1 = 0.00\%$, $d_2 = 0.00\%$, $d_3 = 0.25\%$, $d_4 = 99.33\%$.
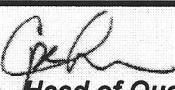
Philip Chan, Head of Quality Assurance

QC Test Date
March 30, 2012

Retest Date
March 30, 2015

SIGMA-ALDRICH®

CERTIFICATE OF ANALYSIS

Sigma-Aldrich Laborchemikalien GmbH D-30918 Seelze
Telefon: +49 5137 8238-150

Seelze, 06.12.2011/280708/10/12833

Order-No.:
Customer-No.:

Order-Code:

Quantity:

Production Date: 15.Jun.2010
Expiry Date: 15.Jun.2015

Article/Product: 34016   Batch : SZBA166XV

Ketoprofen VETRANAL

Reference Material (RM)

1. General Information

Formula: C16H14O3   Molar mass: 254.28 g/Mole
CAS-No.: [22071-15-4]   Recomm. storage temp.: roomtemp.
Usage   :

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5 % relative (confidence level = 95%, n= 6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	complying	
Assay (HPLC)	99.9	area %
Melting range	94.8-96.3	°C
Water (Karl Fischer)	0.05	%
Date of Analysis	29.Jun.2010	

3. Advice and Remarks

- The minimum shelf life is based on the current knowledge and holds only for proper storage conditions in the originally closed flasks/ packages.
- Whenever the container is opened for removal of aliqout portions of the substance, the person handling the substance must assure, that the integrity of the substance is maintained and proper records of all its handlings are kept. Special care has to be taken to avoid any contamination or adulteration of the substance.
- We herewith confirm that the delivery is effected according to the technical delivery conditions agreed.
- Particular properties of the products or the suitability for a particular area of application are not assured.
- We guarantee a proper quality within our General Conditions of Sales.

Sigma-Aldrich Laborchemikalien GmbH
Quality Management SA-LC

This document was produced electronically and is valid without a signature

HPLC-Method

Article	: Ketoprofen	
Article-No	: 34016	
Batch	: SZBA166XV	
Column	: L=250mm, ID=4,6mm; Supelcosil LC-18 5µm	
Eluent	: 50 % Acetonitrile	
	50 % Water + 0,1% Phosphoric acid	
Flow	: 1,4ml/min	
Detector	: UV-255nm	
Injection-Volume	: 10µl	
Sample-Preparation	: 0,2mg/ml	
Linearity	: checked	
Evaluation	: Normalisation (uncorrected)	
Operator	: Weinig	

Chromatogram
Ketoprofen C:\LabSolutions\Data\HPLC XIII\1012833-001.lcd
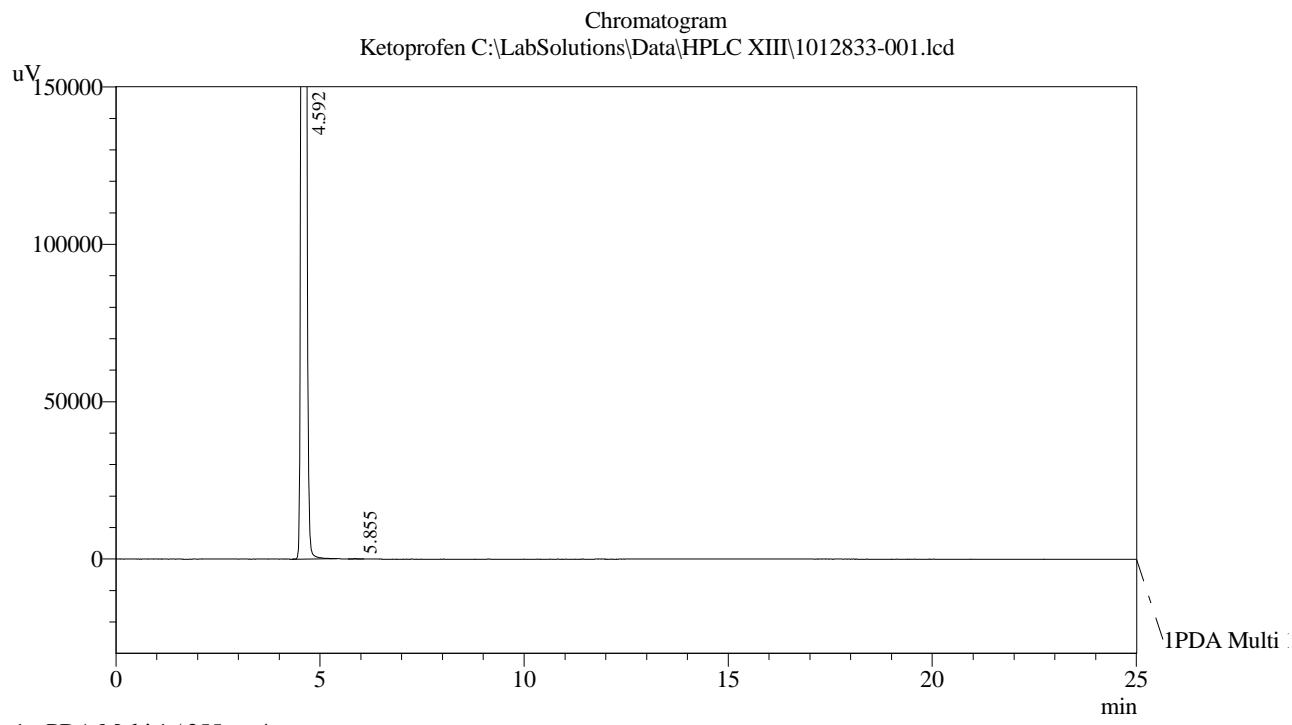
1   PDA Multi 1 / 255nm 4nm

PeakTable

PDA Ch1 255nm 4nm

Peak#	Ret. Time	Area	Area %
1	4.592	5235345	99.988
2	5.855	640	0.012
Total		5235985	100.000

TRC
Toronto Research Chemicals Inc.

Bringing you products for innovative research.

CERTIFICATE OF ANALYSIS

2 Brisbane Road, North York, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

1. Identification

CAS Number:

Product:

Flunixin-d3

Catalogue Number:

F455402

PM Separations

pm SEPARATIONS

Phone (07) 3390 1096
Email: customerservice@pmsep.com.au
www.pmsep.com.au

Synonyms:

2-[[2-(Methyl-d3)-3-(trifluoromethyl)phenyl]amino]-3-pyridinecarboxylic Acid; 2-(2-(Methyl-d3)-3-trifluoromethylanilino) nicotinic Acid; Sch 14714-d3;

Structure:
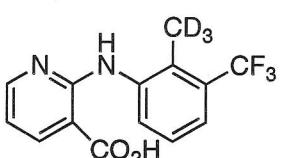
Molecular Formula:

$C_{14}H_8D_3F_3N_2O_2$

Molecular Weight:

299.26

Source of Product:

2. Analytical Information

Lot Number:
2-QFY-122-2

Melting Point:
225-227°C

Boiling Point:
N/A

Atmosphere:
Air

Appearance of Product:
Off-White Solid

Solubility
DMSO, Methanol

Method for Determining Identity:
^{1}H NMR (DMSO-d6) Spectroscopic and Mass Spectrometric Analysis

Stability
Not determined

Purity:
Chemical purity: 98%
Isotopic purity: 99%

Long Term Storage Condition:
-20°C Freezer

Additional Information:

TLC Conditions: SiO$_2$; Hexane: Ethyl Acetate=1: 1; Visualized with UV and AMCS; Sinlge spot; Rf=0.5.
^{1}H NMR and mass spectra conform to structure.
Normalized intensity: d3=-99.65%, d2=0.00%, d1=0.00%, d0=0.35%.

Philip Chan, Head of Quality Assurance

QC Test Date
May 19, 2011

Retest Date
May 19, 2014

SIGMA-ALDRICH®

Certificate of Analysis

Product Name:
Flunixin meglumine – ≥98% (HPLC)

Product Number:	**F0429**
Batch Number:	**062M4730V**
Brand:	SIGMA
CAS Number:	42461-84-7
MDL Number:	MFCD01725419
Formula:	C14H11F3N2O2·C7H17NO5
Formula Weight:	491.46 g/mol
Quality Release Date:	20 JUN 2012
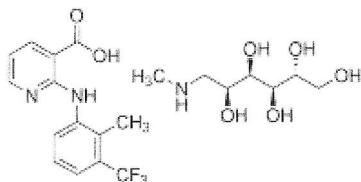
Test	Specification	Result
Appearance (Color)	White to Beige	White
Appearance (Form)	Powder	Powder
Elemental Composition C14H11N2O2F3 .C7H17NO5	Pass	Pass
Purity (HPLC)	> 98 %	100 %
Identity	Confirmed	Conforms

Brendan Nye, QC Team Leader
Quality Control
Natick, Massachusetts   US

MWT FLUNIXIN   296.25   x 100
MWT FLUNIXIN Meg.   491.46

= 60.28 %
ar.

Version Number: 1   Page 1 of 1

CERTIFICATE OF ANALYSIS

Toronto Research Chemicals Inc.

Bringing you products for innovative research.

2 Brisbane Road, North York, ON. M3J 2J8 Canada Tel: (416) 665-9696 Fax: (416) 665-4439
E-mail: orders@trc-canada.com Website: www.trc-canada.com

PM Separations

Phone (07) 3390 1096
Email: customerservice@pmsep.com.au
www.pmsep.com.au

pm SEPARATIONS

1. Identification

CAS Number:
1173019-42-5

Catalogue Number:
C184352

Product:
rac Carprofen-d3
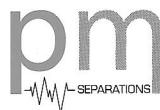
Synonyms:

6-Chloro-α-(methyl-d3)-9H-carbazole-2-acetic Acid; (dl)-6-Chloro-α-(methyl-d3)carbazole-2-acetic Acid; C 5720-d3; Carprodyl-d3; (+/-)-Carprofen-d3; Imadyl-d3; NSC 297935-d3; Rimadyl-d3; Ro 20-5720-d3;

Structure:
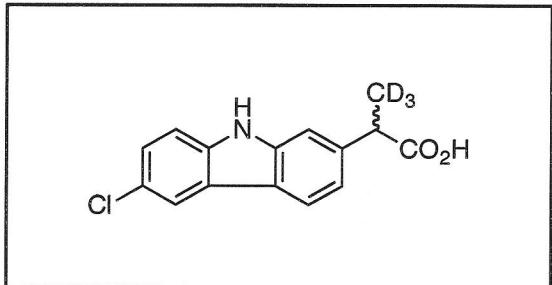
Molecular Formula:
$C_{15}H_9D_3ClNO_2$

Molecular Weight:
276.73

Source of Product:
Synthetic

2. Analytical Information

Lot Number:
1-JPI-35-1

Melting Point:
187-192°C

Boiling Point:
N/A

Atmosphere:
Inert Gas

Appearance of Product:
Brownish-Gray Solid

Solubility
DMSO, Methanol

Method for Determining Identity:
^{1}H NMR (DMSO-d$_6$) Spectroscopic and Mass Spectrometric Analysis

Stability

Purity:
Chemical purity: 98%
Isotopic purity: 99%

Long Term Storage Condition:
-20°C Freezer, Under Inert Atmosphere

Additional Information:
TLC conditions: SiO$_2$; Dichloromethane: Methanol = 9: 1; Visualized with UV and AMCS; Single spot; Rf=0.4.
^{1}H NMR and mass spectra conform to structure.
Normalized Intensity: d0 = 0.20%, d1 = 0.84%, d2 = 0.23%, d3 = 98.74%.
Contain 1.3% Acetic Acid.

Philip Chan, Head of Quality Assurance

QC Test Date
January 25, 2012

Retest Date
January 25, 2015

SIGMA-ALDRICH®

CERTIFICATE OF ANALYSIS

Sigma-Aldrich Laborchemikalien GmbH D-30918 Seelze
Telefon: +49 5137 8238-150

Seelze, 04.05.2011/186858/11/08939

Order-No.:
Customer-No.:

Order-Code:

Quantity:

Production Date: 12.Apr.2011
Expiry Date: 12.Apr.2016

Article/Product: 33975   Batch : SZBB102XV

Carprofen VETRANAL

Reference Material (RM)

1. General Information

Formula: C15H12ClNO2   Molar mass: 273.71 g/Mole
CAS-No.: [53716-49-7]   Recomm. storage temp.: roomtemp.
Usage   :

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5 % relative (confidence level = 95%, n= 6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)   complying
Assay (HPLC)   99.9   area %
Melting range   199.0-202.7 °C
Water (Karl Fischer)   0.07   %
Date of Analysis   03.May.2011

3. Advice and Remarks

- The minimum shelf life is based on the current knowledge and holds only for proper storage conditions in the originally closed flasks/ packages.
- Whenever the container is opened for removal of aliqout portions of the substance, the person handling the substance must assure, that the integrity of the substance is maintained and proper records of all its handlings are kept. Special care has to be taken to avoid any contamination or adulteration of the substance.
- We herewith confirm that the delivery is effected according to the technical delivery conditions agreed.
- Particular properties of the products or the suitability for a particular area of application are not assured.
- We guarantee a proper quality within our General Conditions of Sales.

Sigma-Aldrich Laborchemikalien GmbH
Quality Management SA-LC

This document was produced electronically and is valid without a signature

HPLC-Method

Article	: Carprofen
Article-No	: 33975
Batch	: SZBB102XV
Column	: L=250mm, ID=4,6mm; Supelcosil LC-18 5µm
Eluent	: 50 % Acetonitrile
	50 % Water + 0,1% Phosphoric acid
Flow	: 1,4ml/min
Detector	: UV-237nm
Injection-Volume	: 10µl
Sample-Preparation	: 0,1mg/ml
Linearity	: checked
Evaluation	: Normalisation (uncorrected)
Operator	: Schowe

Chromatogram
B102X C:\LabSolutions\Data\Project1\1108939.AIA\SIGNAL01.cdf
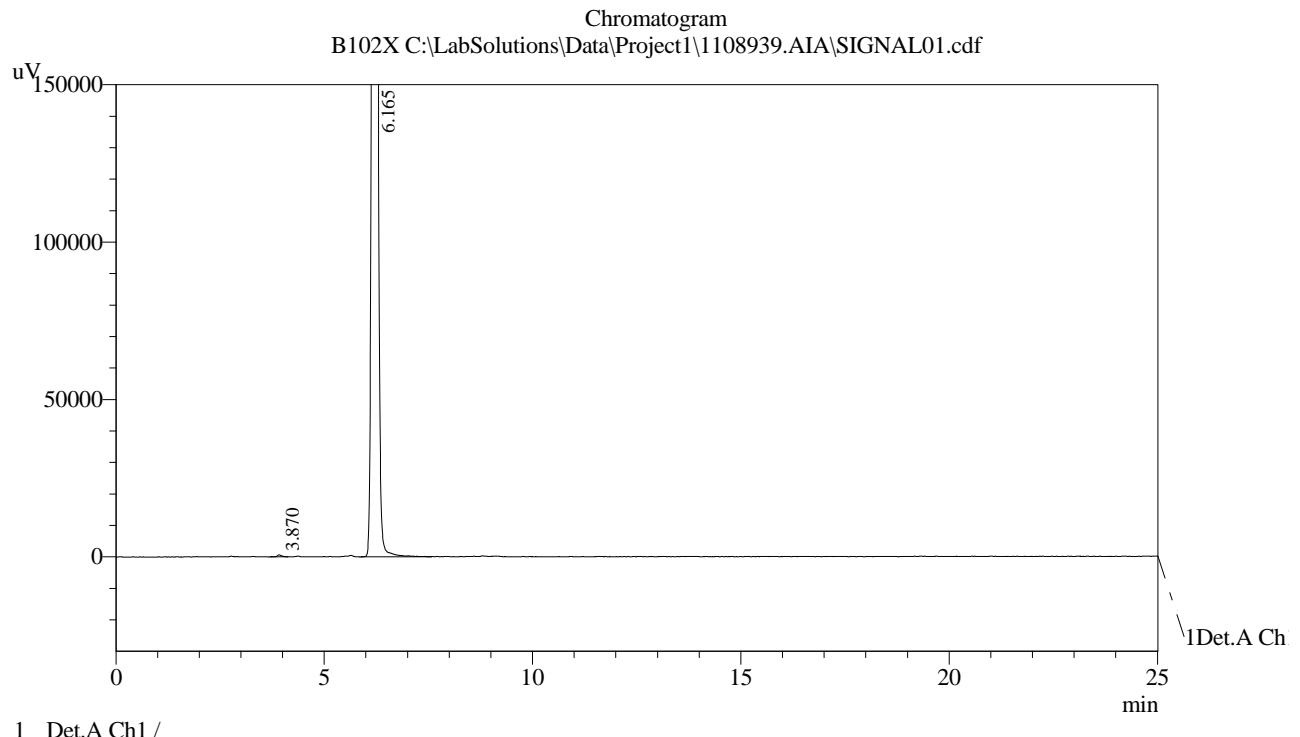
1   Det.A Ch1 /

PeakTable

Ch1

Peak#	Ret. Time	Area	Area %
1	3.870	4	0.085
2	6.165	5140	99.915
Total		5145	100.000

Appendix 2 – Analyte identification

Mobile phase
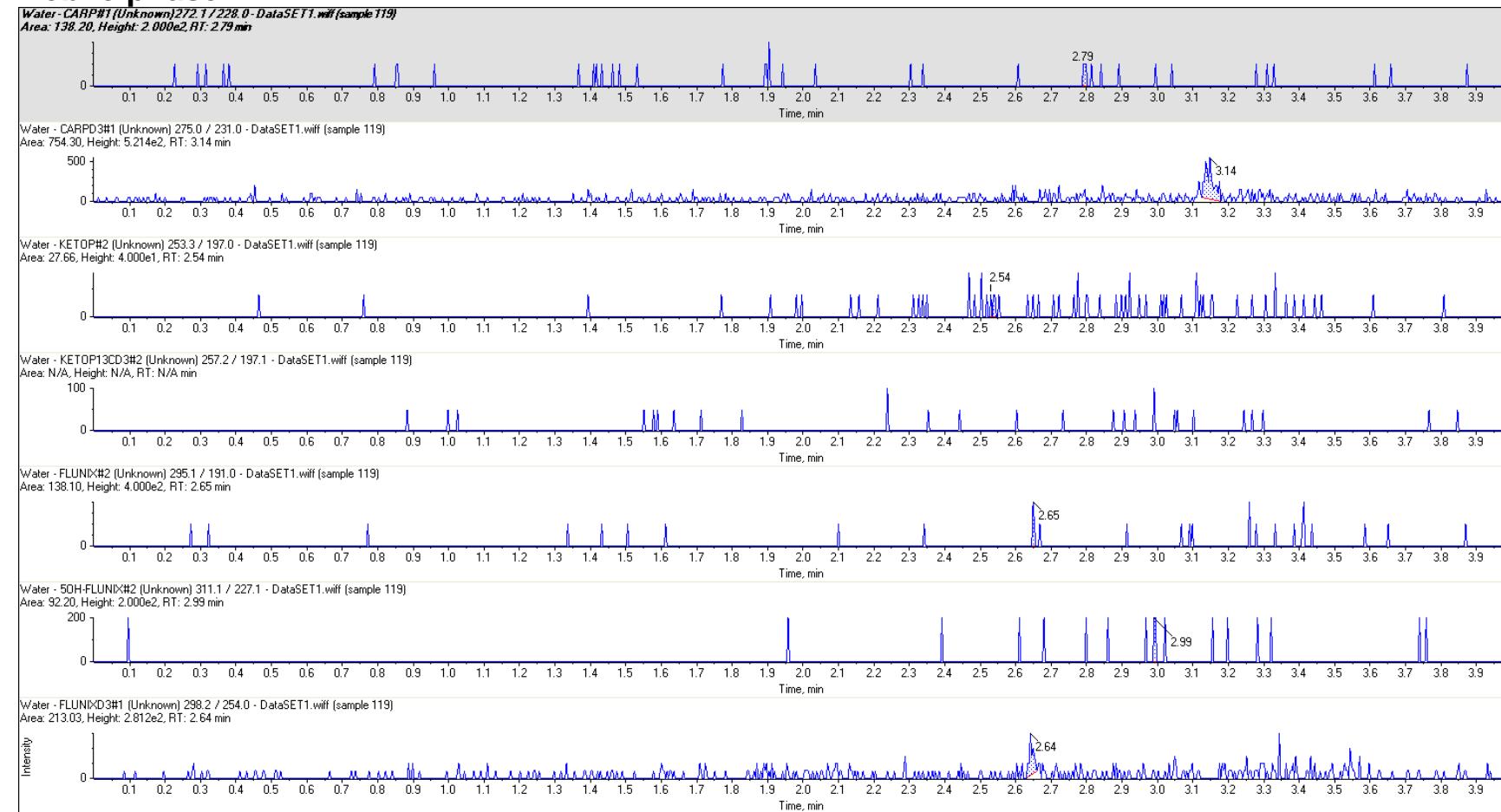
Figure 5: Chromatograms - Mobile phase only

Reagent Blank
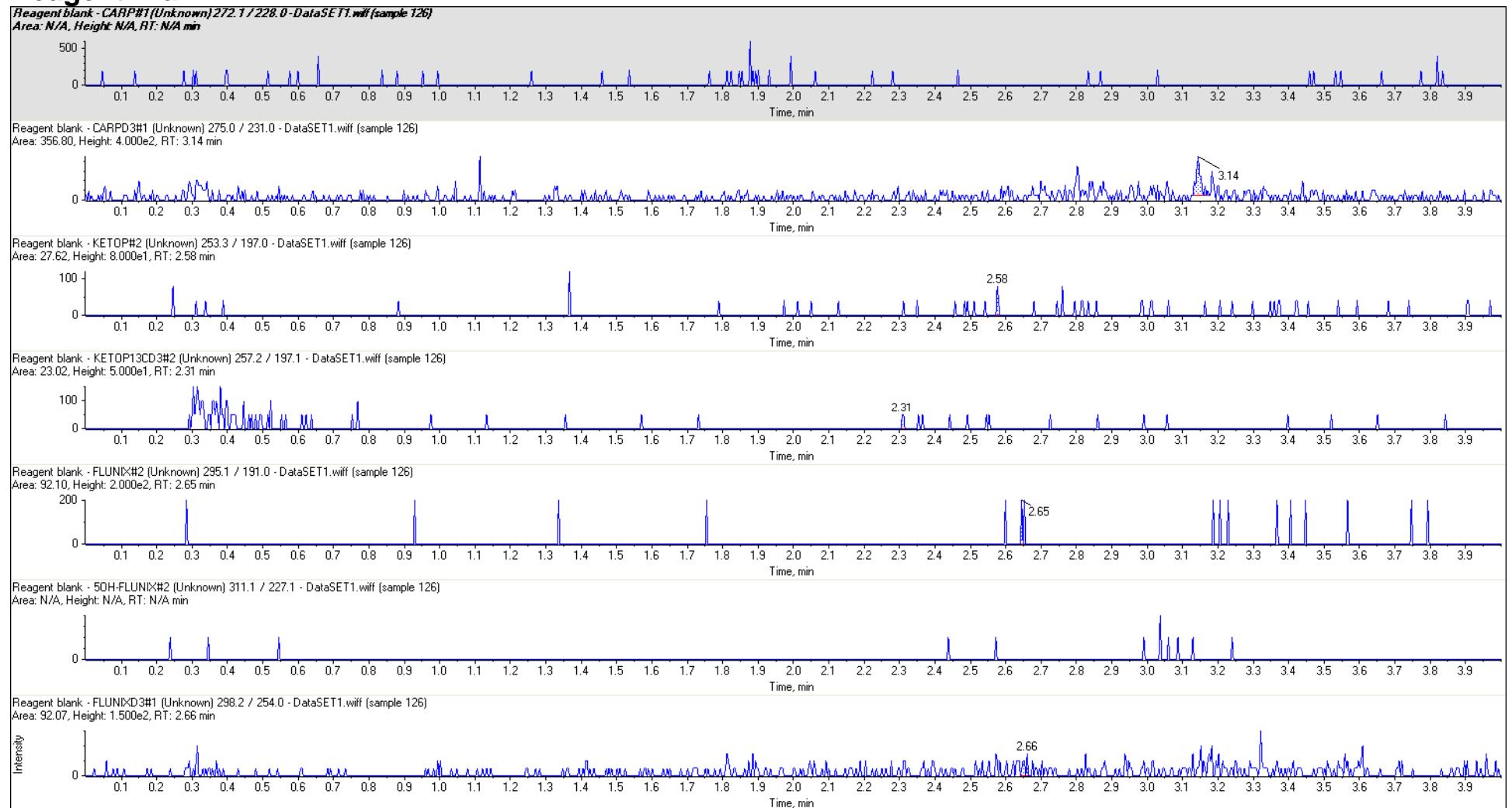
Figure 6: Chromatograms – reagent blank

Flunixin standard
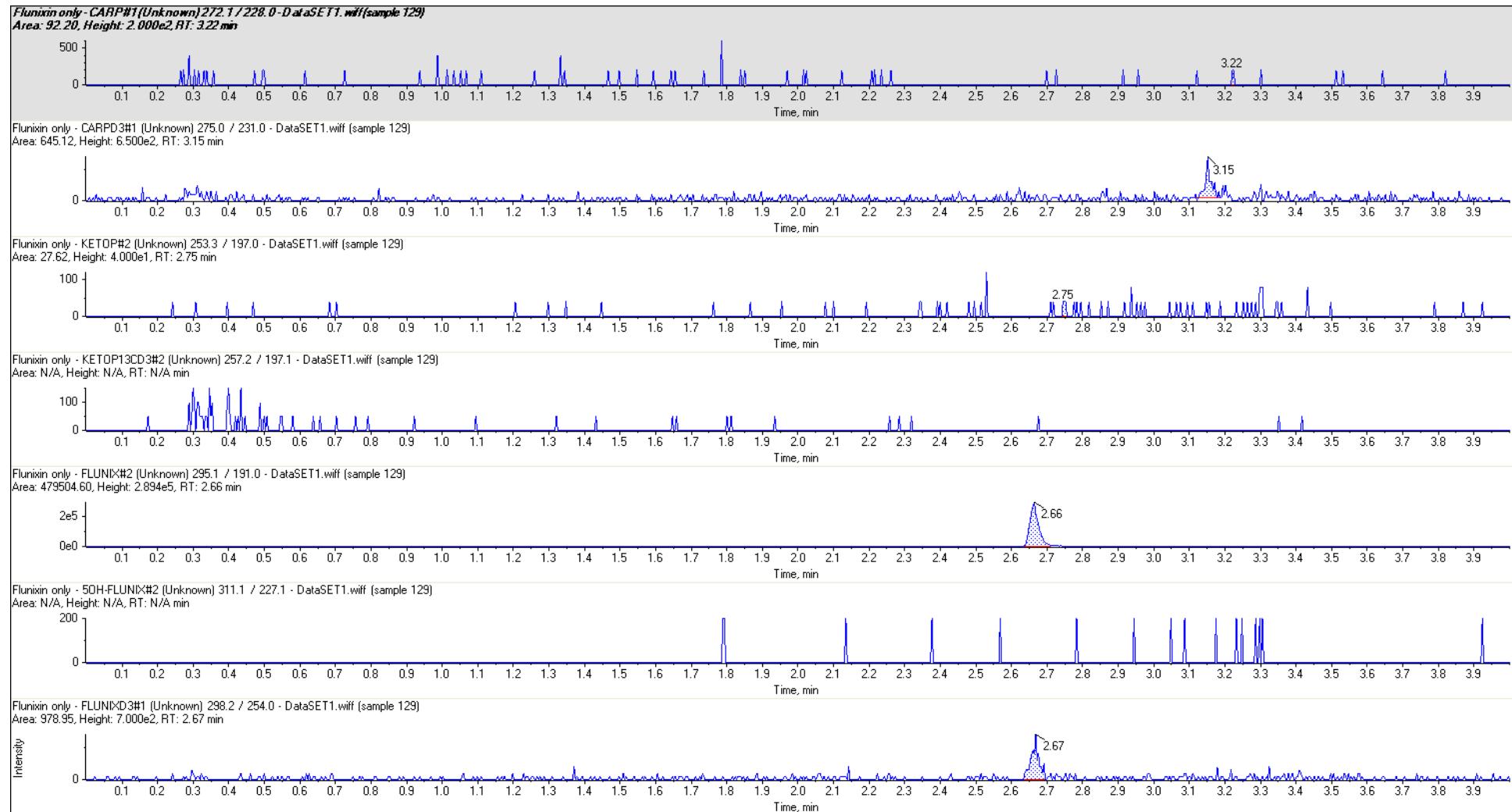
Figure 7: Chromatograms – flunixin standard solution

5-hydroxyflunixin standard
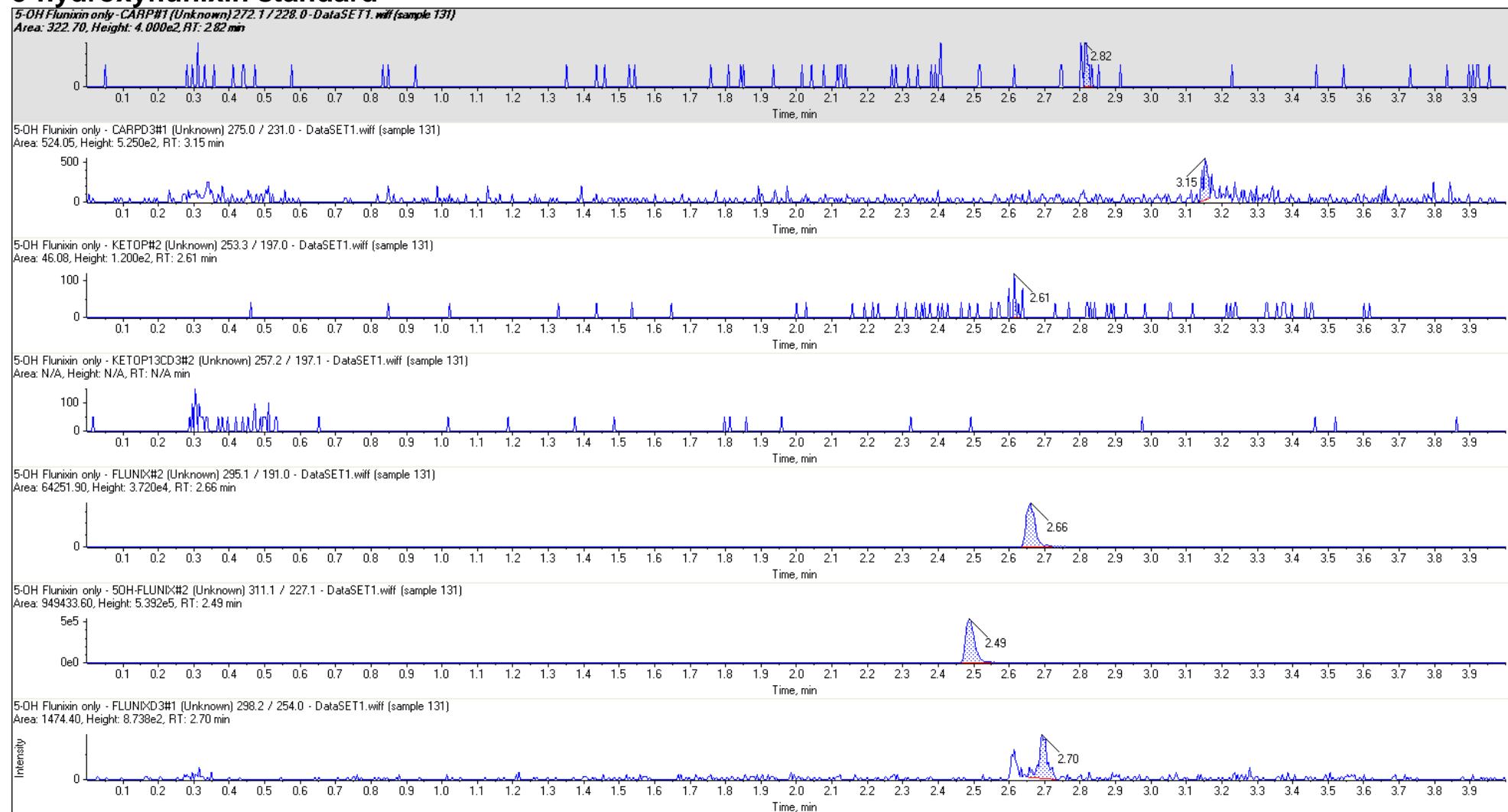
Figure 8: Chromatograms – 5-hydroxyflunixin standard solution

Ketoprofen standard
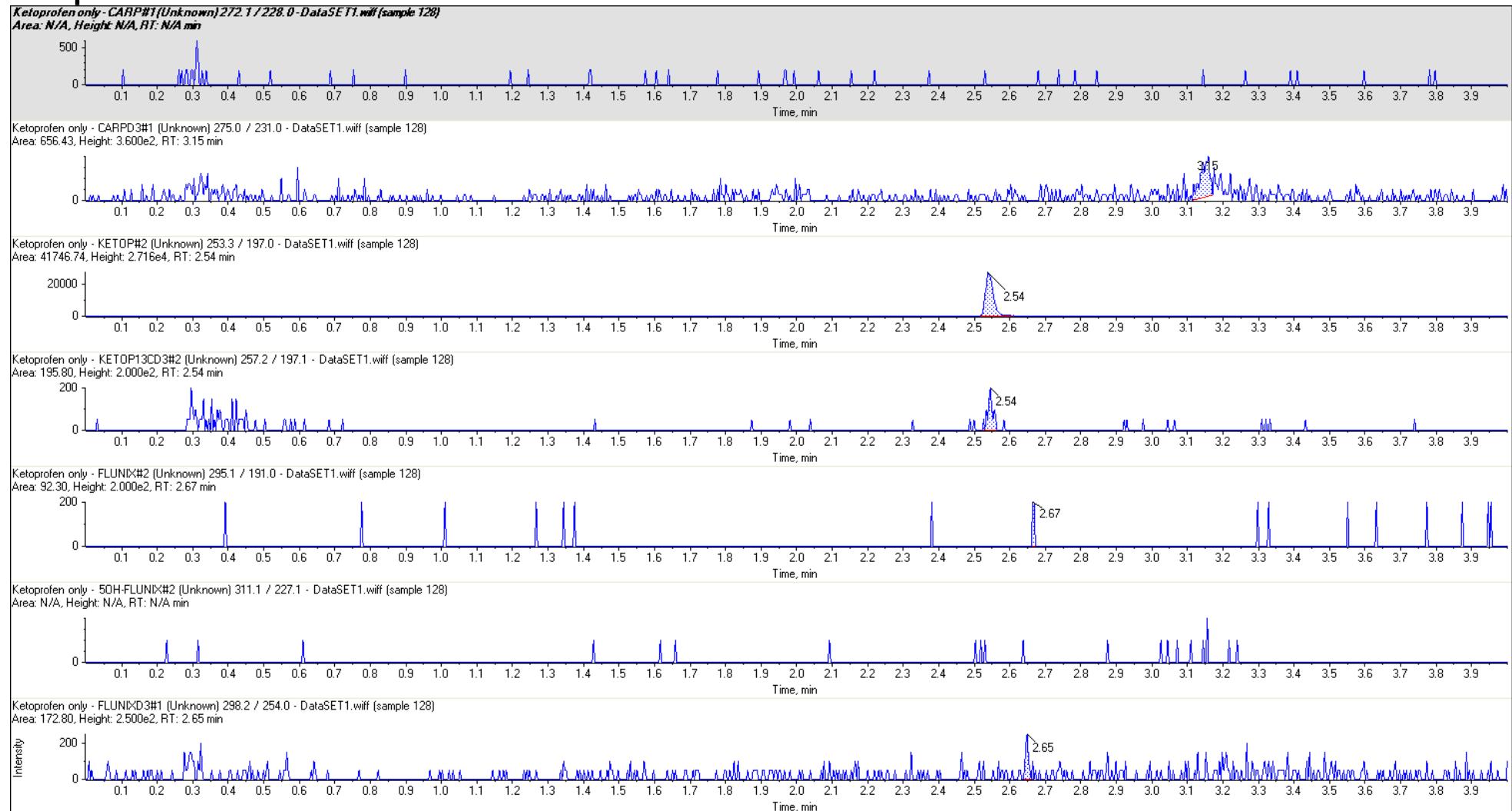
Figure 9: Chromatograms – ketoprofen standard solution

Carprofen standard
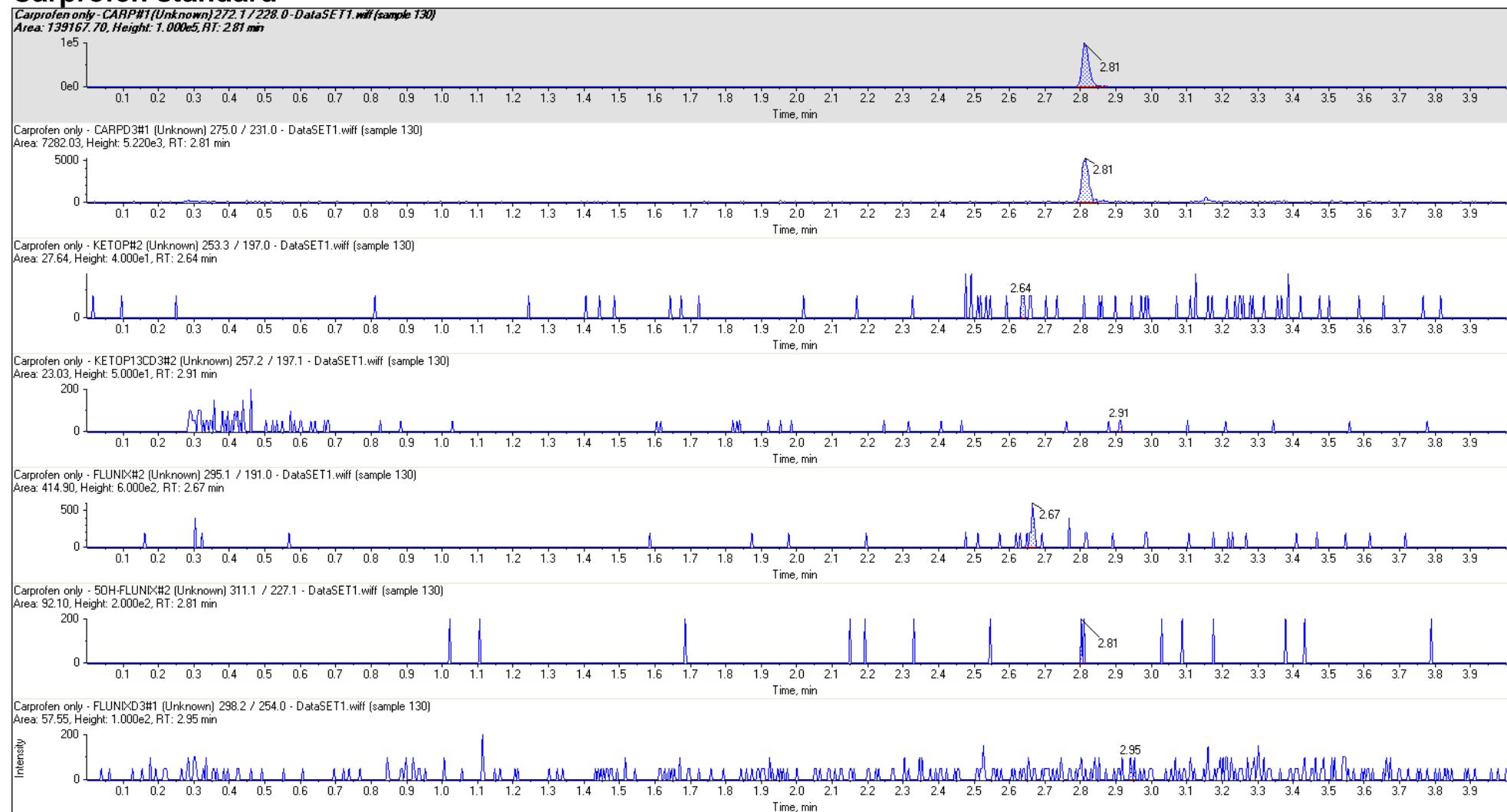
Figure 10: Chromatograms – carprofen standard solution

Matrix blank
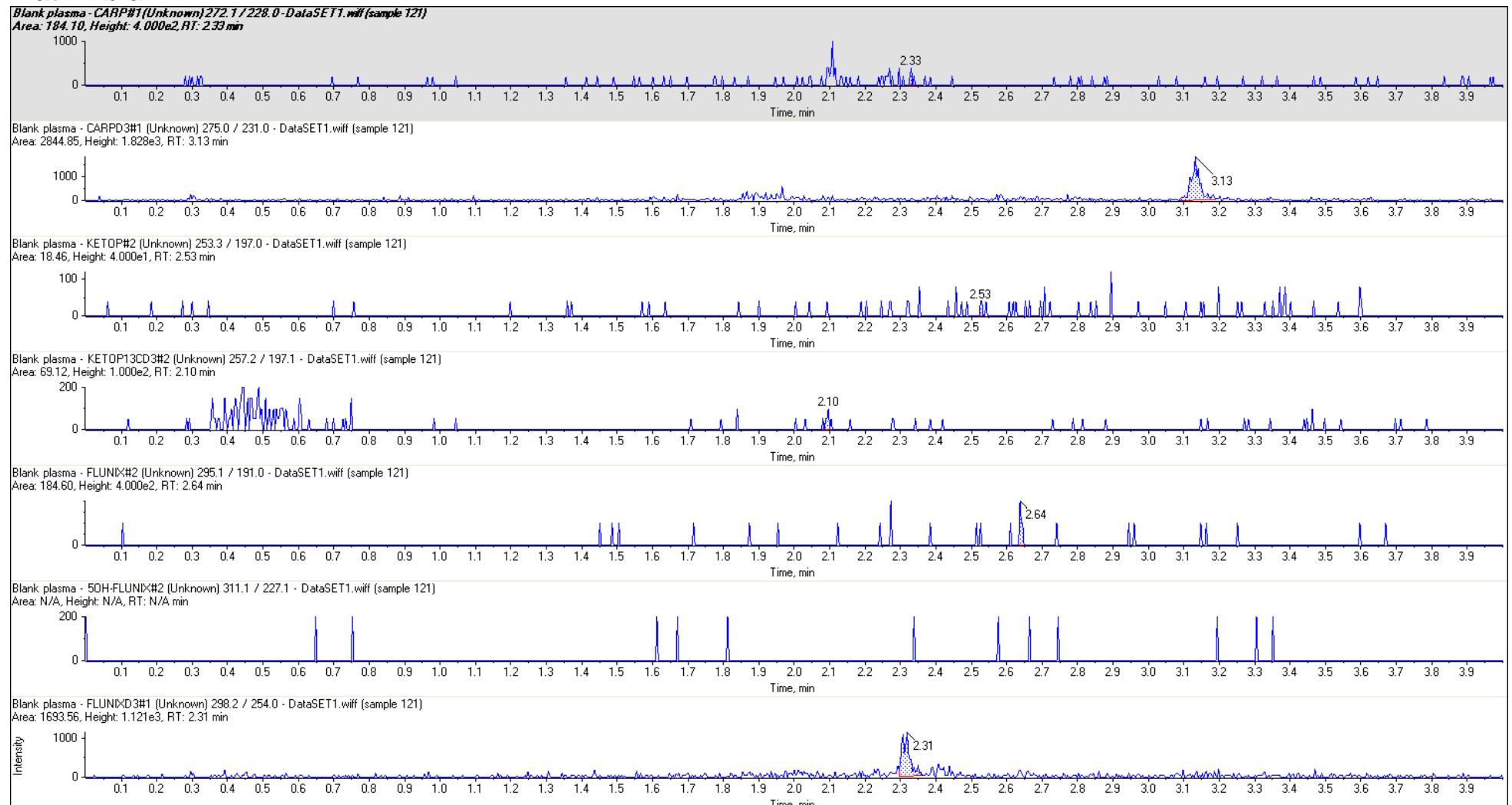
Figure 11: Chromatograms – blank plasma extract

Blank plasma with internal standards
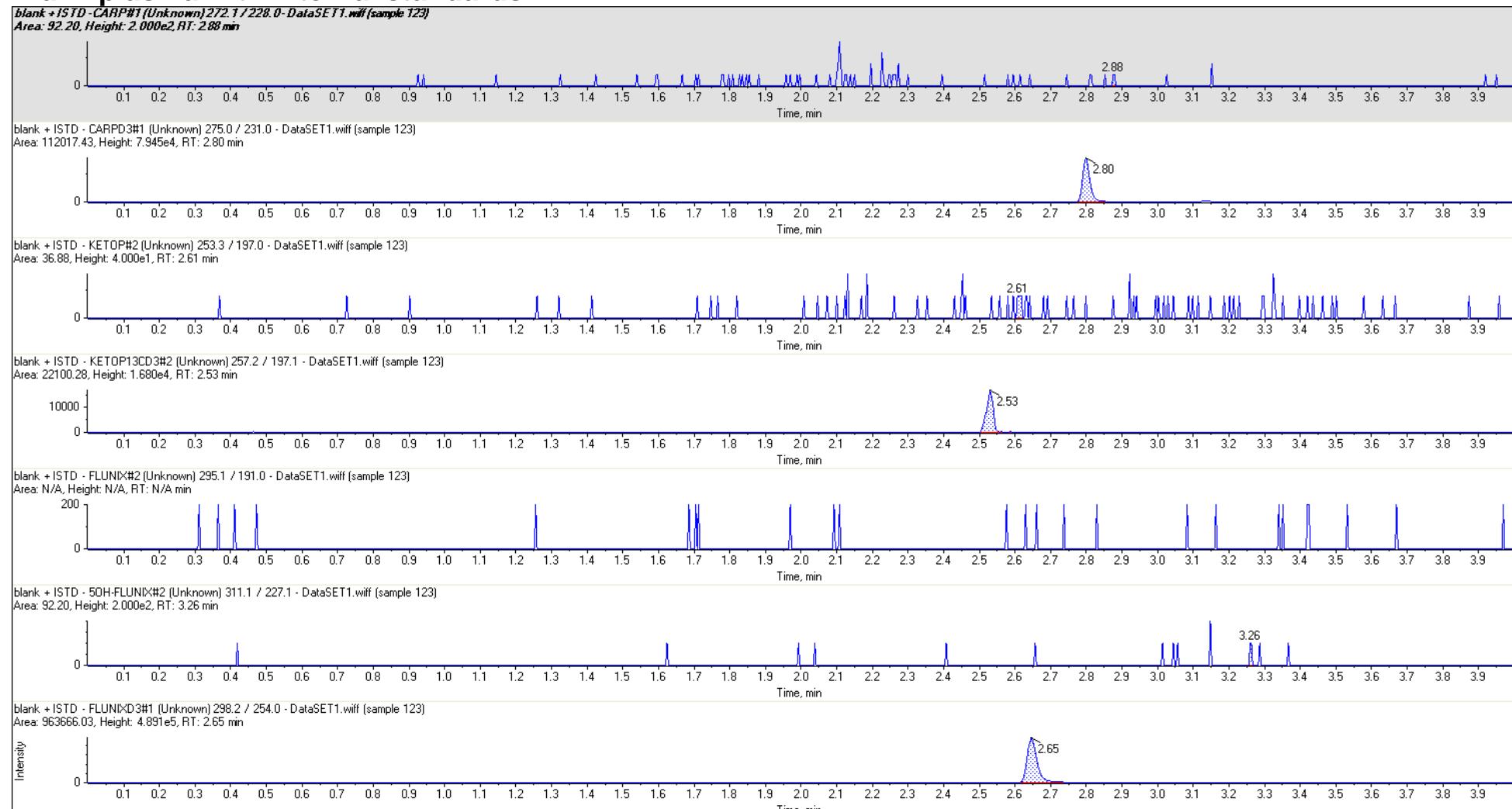
Figure 12: Chromatograms – Blank plasma extract containing internal standards

Calibration standard with internal standards
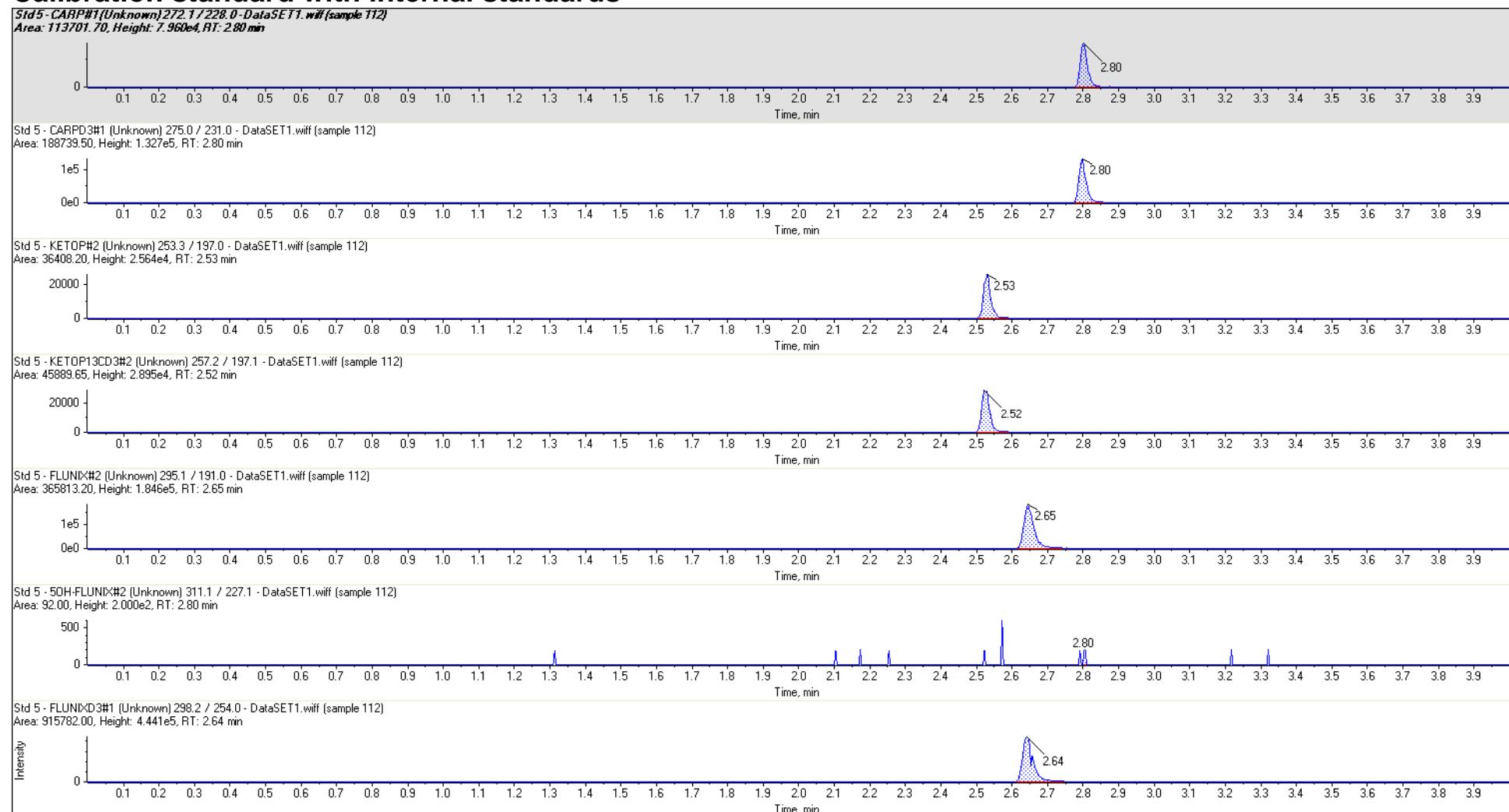
Figure 13: Chromatograms – mixed NSAID standard solution

Fortified plasma (LLOQ) with internal standards
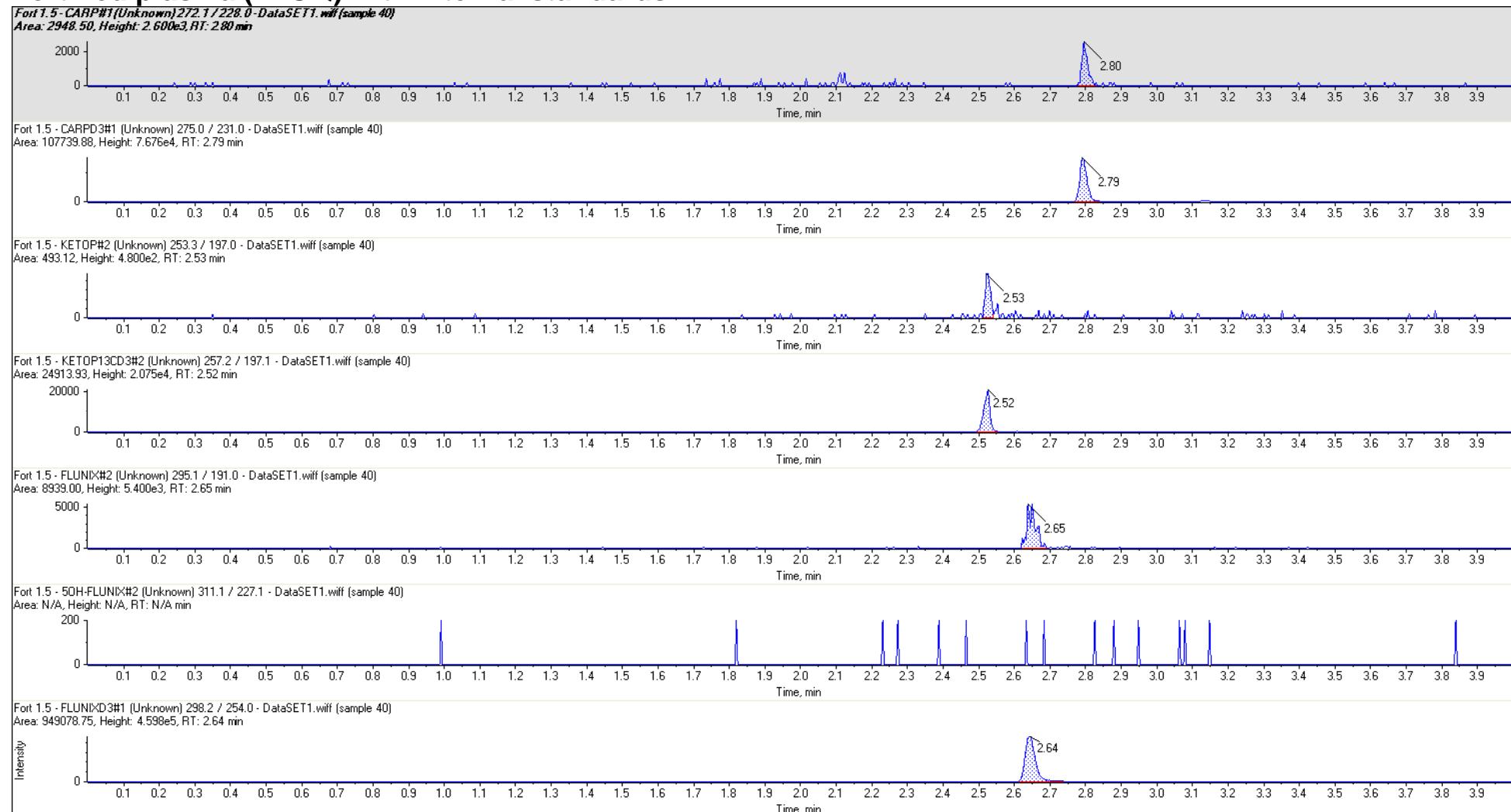
Figure 14: Chromatograms – fortified plasma at LLOQ

Fortified plasma (1000ng/mL) with internal standards
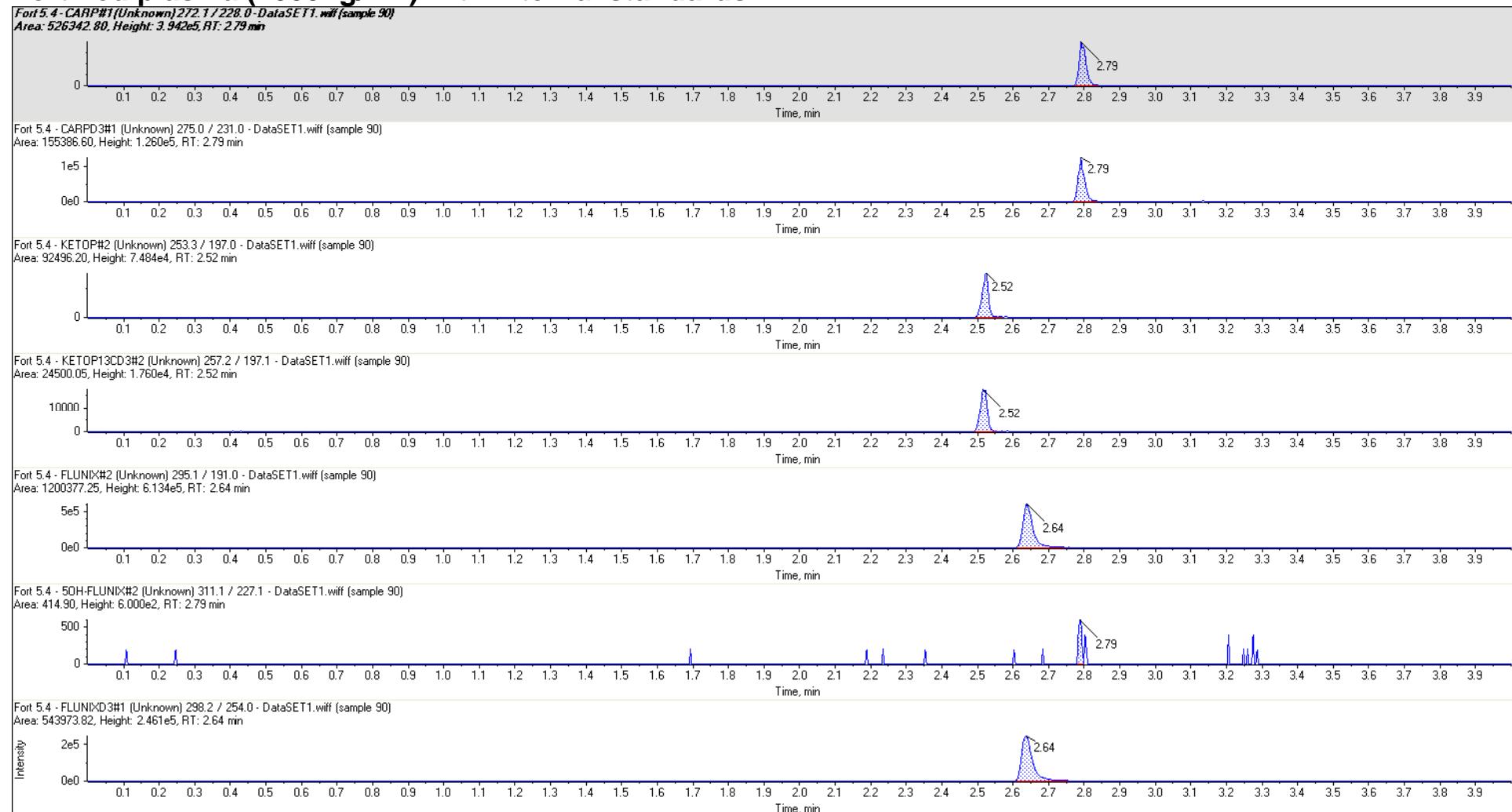
Figure 15: Chromatograms – fortified plasma (1000ng/mL)

Appendix 3 – Calibration curves

5-hydroxyflunixin calibration curve

Analyte Name: 5OH-FLUNIX#2
Internal Standard: FLUNIXD3#1

Data File	DataSET1.wiff	Result Table	131129 Run 4 5 hydroxy flunixin
Acquisition Date	30/11/2013 3:44:36 AM	Algorithm Used	MQ4
Acquisition Method	ATM018 NSAID LCMSMS.dam	Instrument Name	API 3200
Project	1309-066 CSIRO NSAID LCMSMS		

Regression Equation:$y = 5.04925e\text{-}4\ x + 8.86066e\text{-}5$ (r = 0.99981) (weighting: 1 / x)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
2.00	1 of 1	2.00	99.06	N/A	N/A
5.00	1 of 1	5.20	104.43	N/A	N/A
20.00	1 of 1	20.10	100.27	N/A	N/A
80.00	1 of 1	75.80	94.78	N/A	N/A
200.00	1 of 1	202.00	100.98	N/A	N/A
400.00	1 of 1	402.00	100.49	N/A	N/A
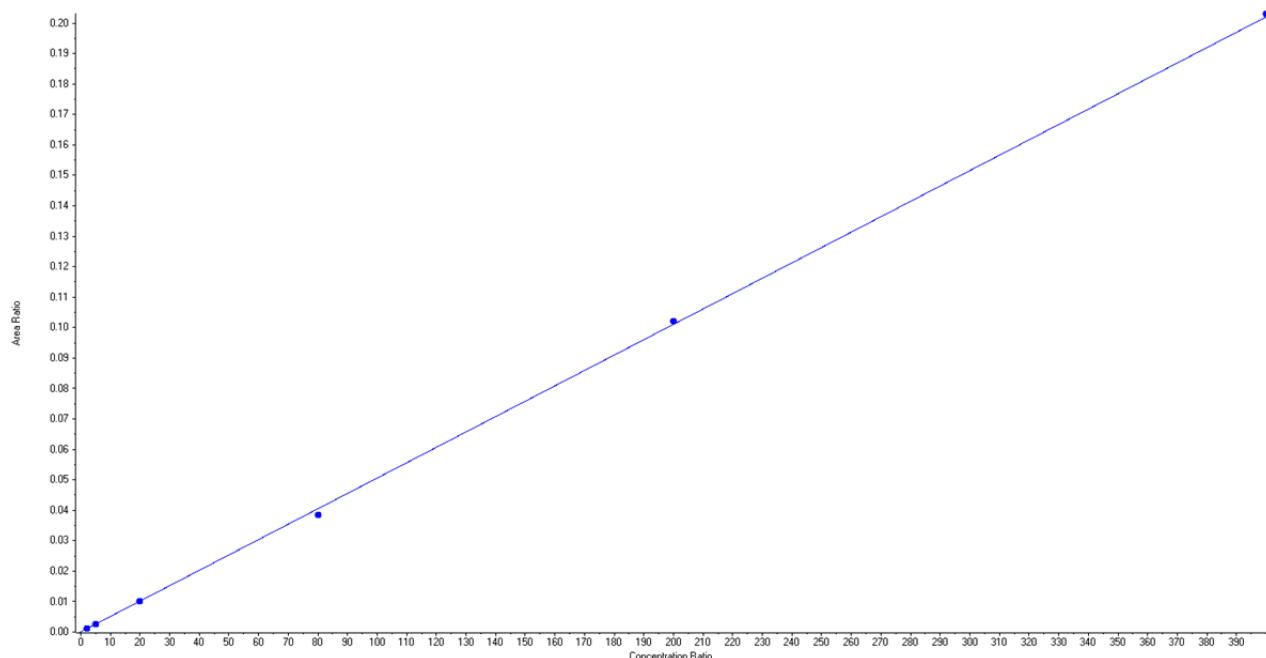
Ketoprofen calibration curve

Analyte Name: KETOP#2
Internal Standard: KETOP13CD3#2

Data File	DataSET1.wiff	Result Table	131122 Accuracy standards
Acquisition Date	23/11/2013 4:30:27 AM	Algorithm Used	MQ4
Acquisition Method	ATM018 NSAID LCMSMS.dam	Instrument Name	API 3200
Project	ATM018 NSAID in plasma LCMSMS		

Regression Equation: $y = 0.00387 x + 0.00382$ (r = 0.99912) (weighting: 1 / x)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
1000.0000	1 of 1	956.00	95.6	N/A	N/A
5.1300	2 of 2	4.52	88.0	0.67	14.9
10.2700	2 of 2	10.01	97.5	1.94	19.4
20.5400	2 of 2	21.50	104.7	1.03	4.8
102.7000	2 of 2	110.02	107.1	2.03	1.8
205.3900	2 of 2	208.76	101.6	4.96	2.4
410.7900	2 of 2	426.63	103.9	8.08	1.9
821.5800	2 of 2	813.31	99.0	33.36	4.1
1026.9700	2 of 2	1008.63	98.2	54.40	5.4
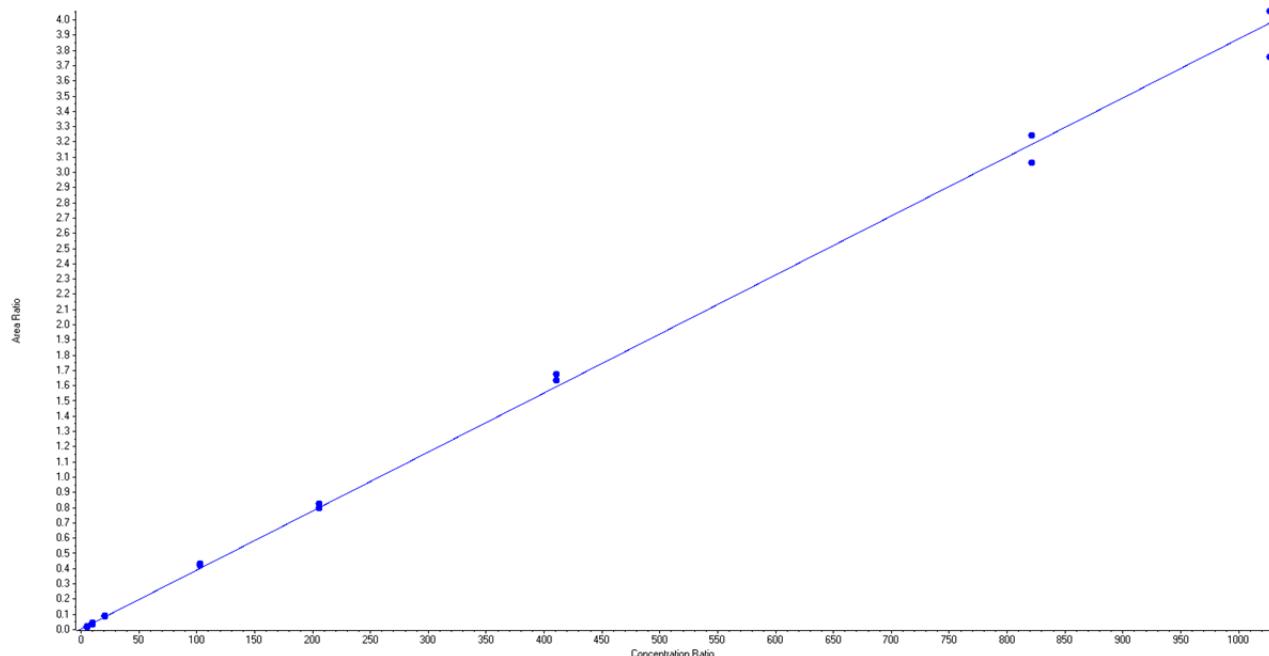
Flunixin calibration curve

Analyte Name: FLUNIX#2
Internal Standard: FLUNIXD3#1

Data File	DataSET1.wiff	Result Table	131122 Accuracy standards
Acquisition Date	23/11/2013 4:30:27 AM	Algorithm Used	MQ4
Acquisition Method	ATM018 NSAID LCMSMS.dam	Instrument Name	API 3200
Project	ATM018 NSAID in plasma LCMSMS		

Regression Equation: $y = 0.00215\,x + -0.00266$ ($r = 0.99951$) (weighting: $1/x$)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
4.9900	2 of 2	5.50	110.4	0.40	7.5
9.9800	2 of 2	9.90	99.5	0.40	3.6
19.9500	2 of 2	19.50	97.5	0.40	1.9
99.7600	2 of 2	96.80	97.0	2.50	2.6
199.5300	2 of 2	183.80	92.1	5.90	3.2
399.0500	2 of 2	410.20	102.8	11.10	2.7
798.1100	2 of 2	794.50	99.6	0.60	0.1
997.6400	2 of 2	1008.80	101.1	18.20	1.8
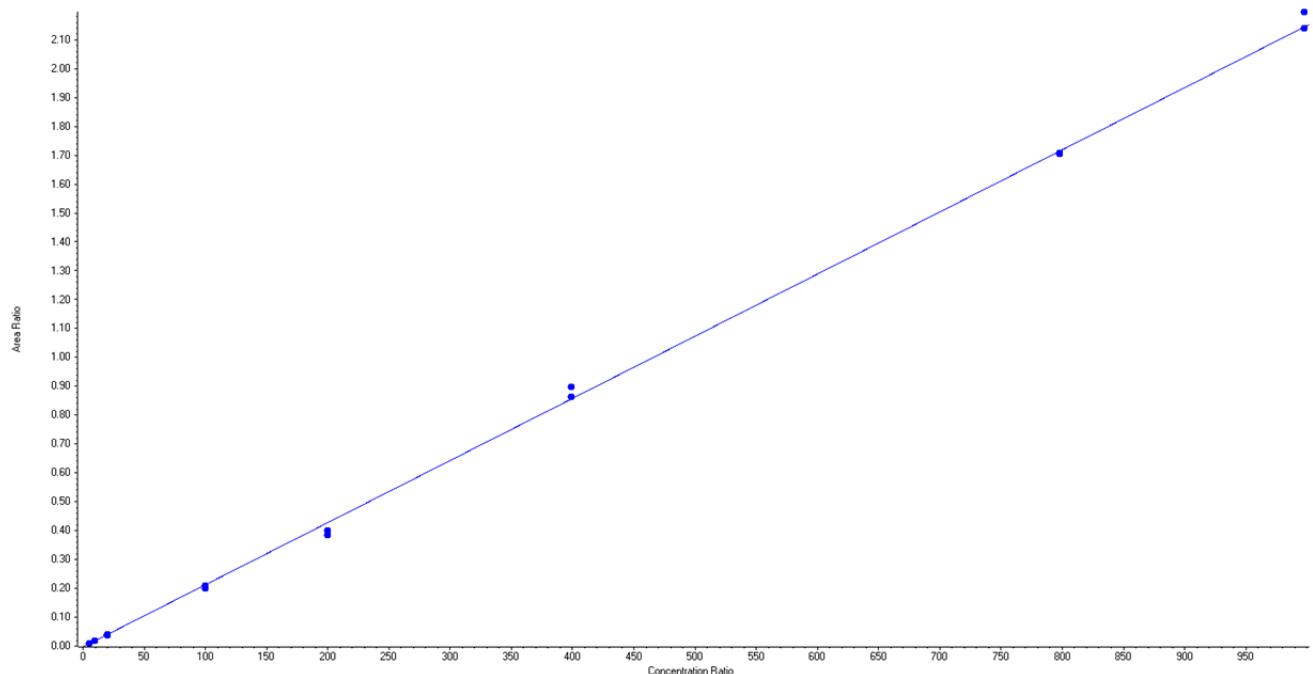
Carprofen Calibration curve (LCMSMS)

Analyte Name: CARP#1
Internal Standard: CARPD3#1

Data File	DataSET1.wiff	Result Table	131122 Accuracy standards
Acquisition Date	23/11/2013 4:30:27 AM	Algorithm Used	MQ4
Acquisition Method	ATM018 NSAID LCMSMS.dam	Instrument Name	API 3200
Project	ATM018 NSAID in plasma LCMSMS		

Regression Equation: $y = 0.00251\,x + 0.00456$ $(r = 0.99873)$ (weighting: 1 / x)

Expected Concentration	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
5.4400	2 of 2	5.13	94.4	0.99	19.4
10.8800	2 of 2	9.28	85.3	0.69	7.4
21.7600	2 of 2	23.36	107.3	1.00	4.3
108.7900	1 of 2	121.27	111.5	N/A	N/A
217.5800	2 of 2	226.59	104.1	11.03	4.9
435.1600	2 of 2	470.13	108.0	9.62	2.1
870.3300	2 of 2	856.00	98.4	21.33	2.5
1087.9000	2 of 2	1052.32	96.7	5.82	0.6
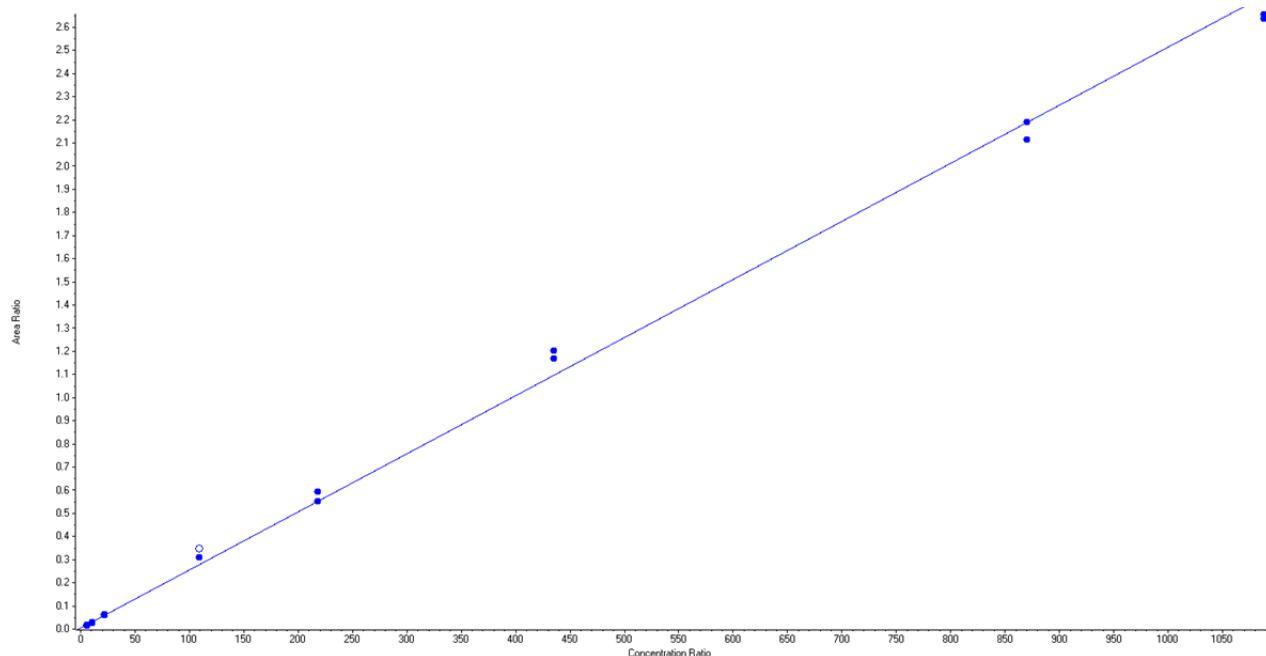
Carprofen (UHPLC-UV)

Calibration Details			
Calibration Type	Lin, WithOffset	Offset (C0)	0.0056
Evaluation Type	Area	Slope (C1)	0.2648
Number of Calibration Points	21	Curve (C2)	0
Number of disabled Calibration Points	0	R-Square	0.9991
		r	0.9996

No.	Injection Name	Calibration Level	X Value	Y Value	Y Value	Area mAU*min	Height mAU
Carprofen	Carprofen	Carprofen	Carprofen UV_VIS_1	Carprofen UV_VIS_1	Carprofen UV_VIS_1	Carprofen UV_VIS_1	Carprofen UV_VIS_1
83	Std 4	1	0.218	0.0699	0.0699	0.07	5.795
84	Std 4	1	0.218	0.0636	0.0636	0.064	5.324
85	Std 5	2	0.544	0.1413	0.1413	0.141	11.843
86	Std 5	2	0.544	0.1482	0.1482	0.148	12.403
87	Std 6	3	1.088	0.2713	0.2713	0.271	22.695
88	Std 6	3	1.088	0.2719	0.2719	0.272	22.617
89	Std 7	4	2.176	0.6052	0.6052	0.605	50.46
90	Std 7	4	2.176	0.6068	0.6068	0.607	50.532
91	Std 8	5	4.352	1.1807	1.1807	1.181	98.629
92	Std 8	5	4.352	1.1927	1.1927	1.193	99.506
93	Std 9	6	10.879	2.8053	2.8053	2.805	233.694
94	Std 9	6	10.879	2.8421	2.8421	2.842	236.991
95	Std 10	7	21.758	5.6055	5.6055	5.606	467.211
96	Std 10	7	21.758	5.8713	5.8713	5.871	489.619
210	Std 4	1	0.218	0.0624	0.0624	0.062	5.288
211	Std 5	2	0.544	0.1473	0.1473	0.147	12.429
212	Std 6	3	1.088	0.2772	0.2772	0.277	23.272
213	Std 7	4	2.176	0.6044	0.6044	0.604	50.637
214	Std 8	5	4.352	1.204	1.204	1.204	100.732
215	Std 9	6	10.879	2.8485	2.8485	2.848	239.09
216	Std 10	7	21.758	5.8852	5.8852	5.885	495.26
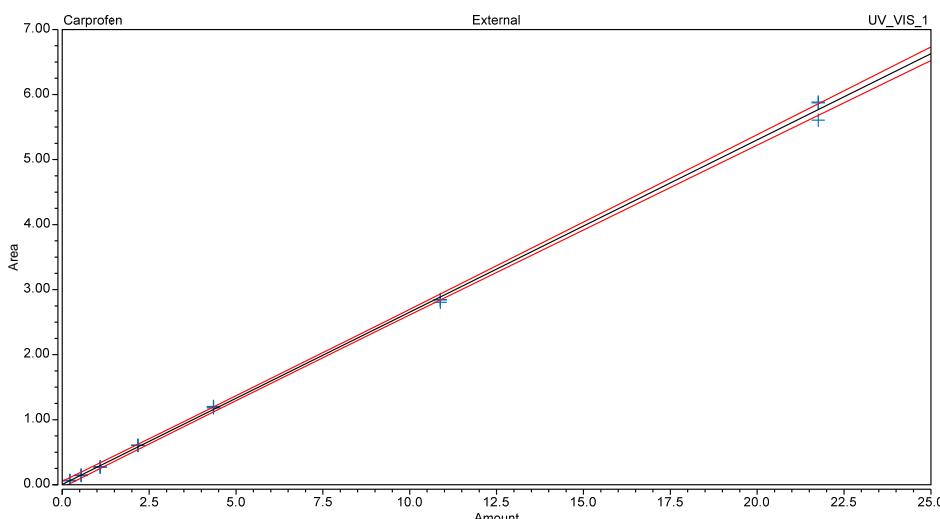
Appendix 4 – Sample Schedule

SEQUENCE # on tube	ANIMAL_ID	TREATMENT	TIME POINT
No sequence just animal number and time point written on label	675	Carprofen	-24
	576	Saline	-24
	716	Saline	-24
	572	Flunixin	-24
	730	Ketoprofen	-24
	437	Ketoprofen	-24
	404	Carprofen	-24
	555	Flunixin	-24
	314	Saline	-24
	760	Ketoprofen	-24
	498	Flunixin	-24
	605	Carprofen	-24
	558	Carprofen	-24
	311	Ketoprofen	-24
	495	Saline	-24
	422	Flunixin	-24
	494	Flunixin	-24
	493	Ketoprofen	-24
	312	Carprofen	-24
	652	Saline	-24
No sequence just animal number and time point written on label	675	Carprofen	-22
	576	Saline	-22
	716	Saline	-22
	572	Flunixin	-22
	730	Ketoprofen	-22
	437	Ketoprofen	-22
	404	Carprofen	-22
	555	Flunixin	-22
	314	Saline	-22
	760	Ketoprofen	-22
	498	Flunixin	-22
	605	Carprofen	-22
	558	Carprofen	-22
	311	Ketoprofen	-22
	495	Saline	-22
	422	Flunixin	-22
	494	Flunixin	-22
	493	Ketoprofen	-22
	312	Carprofen	-22
	652	Saline	-22

41	675	Carprofen	0hrs
42	576	Saline	0hrs
43	716	Saline	0hrs
44	572	Flunixin	0hrs
45	730	Ketoprofen	0hrs
46	437	Ketoprofen	0hrs
47	404	Carprofen	0hrs
48	555	Flunixin	0hrs
49	314	Saline	0hrs
50	760	Ketoprofen	0hrs
51	498	Flunixin	0hrs
52	605	Carprofen	0hrs
53	558	Carprofen	0hrs
54	311	Ketoprofen	0hrs
55	495	Saline	0hrs
56	422	Flunixin	0hrs
57	494	Flunixin	0hrs
58	493	Ketoprofen	0hrs
59	312	Carprofen	0hrs
60	652	Saline	0hrs
61	675	Carprofen	2hrs
62	576	Saline	2hrs
63	716	Saline	2hrs
64	572	Flunixin	2hrs
65	730	Ketoprofen	2hrs
66	437	Ketoprofen	2hrs
67	404	Carprofen	2hrs
68	555	Flunixin	2hrs
69	314	Saline	2hrs
70	760	Ketoprofen	2hrs
71	498	Flunixin	2hrs
72	605	Carprofen	2hrs
73	558	Carprofen	2hrs
74	311	Ketoprofen	2hrs
75	495	Saline	2hrs
76	422	Flunixin	2hrs
77	494	Flunixin	2hrs
78	493	Ketoprofen	2hrs
79	312	Carprofen	2hrs
80	652	Saline	2hrs

81	675	Carprofen	24hrs
82	576	Saline	24hrs
83	716	Saline	24hrs
84	572	Flunixin	24hrs
85	730	Ketoprofen	24hrs
86	437	Ketoprofen	24hrs
87	404	Carprofen	24hrs
88	555	Flunixin	24hrs
89	314	Saline	24hrs
90	760	Ketoprofen	24hrs
91	498	Flunixin	24hrs
92	605	Carprofen	24hrs
93	558	Carprofen	24hrs
94	311	Ketoprofen	24hrs
95	495	Saline	24hrs
96	422	Flunixin	24hrs
97	494	Flunixin	24hrs
98	493	Ketoprofen	24hrs
99	312	Carprofen	24hrs
100	652	Saline	24hrs
101	675	Carprofen	26hrs
102	576	Saline	26hrs
103	716	Saline	26hrs
104	572	Flunixin	26hrs
105	730	Ketoprofen	26hrs
106	437	Ketoprofen	26hrs
107	404	Carprofen	26hrs
108	555	Flunixin	26hrs
109	314	Saline	26hrs
110	760	Ketoprofen	26hrs
111	498	Flunixin	26hrs
112	605	Carprofen	26hrs
113	558	Carprofen	26hrs
114	311	Ketoprofen	26hrs
115	495	Saline	26hrs
116	422	Flunixin	26hrs
117	494	Flunixin	26hrs
118	493	Ketoprofen	26hrs
119	312	Carprofen	26hrs
120	652	Saline	26hrs

121	675	Carprofen	48hrs
122	576	Saline	48hrs
123	716	Saline	48hrs
124	572	3	48hrs
125	730	Ketoprofen	48hrs
126	437	Ketoprofen	48hrs
127	404	Carprofen	48hrs
128	555	Flunixin	48hrs
129	314	Saline	48hrs
130	760	Ketoprofen	48hrs
131	498	Flunixin	48hrs
132	605	Carprofen	48hrs
133	558	Carprofen	48hrs
134	311	Ketoprofen	48hrs
135	495	Saline	48hrs
136	422	Flunixin	48hrs
137	494	Flunixin	48hrs
138	493	Ketoprofen	48hrs
139	312	Carprofen	48hrs
140	652	Saline	48hrs
141	675	Carprofen	50hrs
142	576	Saline	50hrs
143	716	Saline	50hrs
144	572	Flunixin	50hrs
145	730	Ketoprofen	50hrs
146	437	Ketoprofen	50hrs
147	404	Carprofen	50hrs
148	555	Flunixin	50hrs
149	314	Saline	50hrs
150	760	Ketoprofen	50hrs
151	498	Flunixin	50hrs
152	605	Carprofen	50hrs
153	558	Carprofen	50hrs
154	311	Ketoprofen	50hrs
155	495	Saline	50hrs
156	422	Flunixin	50hrs
157	494	Flunixin	50hrs
158	493	Ketoprofen	50hrs
159	312	Carprofen	50hrs
160	652	Saline	50hrs

161	675	Carprofen	72hrs
162	576	Saline	72hrs
163	716	Saline	72hrs
164	572	Flunixin	72hrs
165	730	Ketoprofen	72hrs
166	437	Ketoprofen	72hrs
167	404	Carprofen	72hrs
168	555	Flunixin	72hrs
169	314	Saline	72hrs
170	760	Ketoprofen	72hrs
171	498	Flunixin	72hrs
172	605	Carprofen	72hrs
173	558	Carprofen	72hrs
174	311	Ketoprofen	72hrs
175	495	Saline	72hrs
176	422	Flunixin	72hrs
177	494	Flunixin	72hrs
178	493	Ketoprofen	72hrs
179	312	Carprofen	72hrs
180	652	Saline	72hrs
181	675	Carprofen	74hrs
182	576	Saline	74hrs
183	716	Saline	74hrs
184	572	Flunixin	74hrs
185	730	Ketoprofen	74hrs
186	437	Ketoprofen	74hrs
187	404	Carprofen	74hrs
188	555	Flunixin	74hrs
189	314	Saline	74hrs
190	760	Ketoprofen	74hrs
191	498	Flunixin	74hrs
192	605	Carprofen	74hrs
193	558	Carprofen	74hrs
194	311	Ketoprofen	74hrs
195	495	Saline	74hrs
196	422	Flunixin	74hrs
197	494	Flunixin	74hrs
198	493	Ketoprofen	74hrs
199	312	Carprofen	74hrs
200	652	Saline	74hrs

201	675	Carprofen	96hrs
202	576	Saline	96hrs
203	716	Saline	96hrs
204	572	Flunixin	96hrs
205	730	Ketoprofen	96hrs
206	437	Ketoprofen	96hrs
207	404	Carprofen	96hrs
208	555	Flunixin	96hrs
209	314	Saline	96hrs
210	760	Ketoprofen	96hrs
211	498	Flunixin	96hrs
212	605	Carprofen	96hrs
213	558	Carprofen	96hrs
214	311	Ketoprofen	96hrs
215	495	Saline	96hrs
216	422	Flunixin	96hrs
217	494	Flunixin	96hrs
218	493	Ketoprofen	96hrs
219	312	Carprofen	96hrs
220	652	Saline	96hrs
221	675	Carprofen	98hrs
222	576	Saline	98hrs
223	716	Saline	98hrs
224	572	Flunixin	98hrs
225	730	Ketoprofen	98hrs
226	437	Ketoprofen	98hrs
227	404	Carprofen	98hrs
228	555	Flunixin	98hrs
229	314	Saline	98hrs
230	760	Ketoprofen	98hrs
231	498	Flunixin	98hrs
232	605	Carprofen	98hrs
233	558	Carprofen	98hrs
234	311	Ketoprofen	98hrs
235	495	Saline	98hrs
236	422	Flunixin	98hrs
237	494	Flunixin	98hrs
238	493	Ketoprofen	98hrs
239	312	Carprofen	98hrs
240	652	Saline	98hrs

SEQUENCE # on tube	ANIMAL_ID	TREATMENT	TIME POINT
241	306	Ketoprofen	-24
242	313	Ketoprofen	-24
243	492	Flunixin	-24
244	458	Saline	-24
245	310	Carprofen	-24
246	585	Carprofen	-24
247	698	Saline	-24
248	340	Saline	-24
249	607	Flunixin	-24
250	520	Ketoprofen	-24
251	436	Ketoprofen	-24
252	502	Carprofen	-24
253	302	Flunixin	-24
254	301	Saline	-24
255	727	Carprofen	-24
256	488	Flunixin	-24
257	559	Saline	-24
258	784	Ketoprofen	-24
259	640	Carprofen	-24
260	769	Flunixin	-24
261	306	Ketoprofen	-22
262	313	Ketoprofen	-22
263	492	Flunixin	-22
264	458	Saline	-22
265	310	Carprofen	-22
266	585	Carprofen	-22
267	698	Saline	-22
268	340	Saline	-22
269	607	Flunixin	-22
270	520	Ketoprofen	-22
271	436	Ketoprofen	-22
272	502	Carprofen	-22
273	302	Flunixin	-22
274	301	Saline	-22
275	727	Carprofen	-22
276	488	Flunixin	-22
277	559	Saline	-22
278	784	Ketoprofen	-22
279	640	Carprofen	-22
280	769	Flunixin	-22

281	306	Ketoprofen	0
282	313	Ketoprofen	0
283	492	Flunixin	0
284	458	Saline	0
285	310	Carprofen	0
286	585	Carprofen	0
287	698	Saline	0
288	340	Saline	0
289	607	Flunixin	0
290	520	Ketoprofen	0
291	436	Ketoprofen	0
292	502	Carprofen	0
293	302	Flunixin	0
294	301	Saline	0
295	727	Carprofen	0
296	488	Flunixin	0
297	559	Saline	0
298	784	Ketoprofen	0
299	640	Carprofen	0
300	769	Flunixin	0
301	306	Ketoprofen	2 hrs
302	313	Ketoprofen	2 hrs
303	492	Flunixin	2 hrs
304	458	Saline	2 hrs
305	310	Carprofen	2 hrs
306	585	Carprofen	2 hrs
307	698	Saline	2 hrs
308	340	Saline	2 hrs
309	607	Flunixin	2 hrs
310	520	Ketoprofen	2 hrs
311	436	Ketoprofen	2 hrs
312	502	Carprofen	2 hrs
313	302	Flunixin	2 hrs
314	301	Saline	2 hrs
315	727	Carprofen	2 hrs
316	488	Flunixin	2 hrs
317	559	Saline	2 hrs
318	784	Ketoprofen	2 hrs
319	640	Carprofen	2 hrs
320	769	Flunixin	2 hrs

321	306	Ketoprofen	24hrs
322	313	Ketoprofen	24hrs
323	492	Flunixin	24hrs
324	458	Saline	24hrs
325	310	Carprofen	24hrs
326	585	Carprofen	24hrs
327	698	Saline	24hrs
328	340	Saline	24hrs
329	607	Flunixin	24hrs
330	520	Ketoprofen	24hrs
331	436	Ketoprofen	24hrs
332	502	Carprofen	24hrs
333	302	Flunixin	24hrs
334	301	Saline	24hrs
335	727	Carprofen	24hrs
336	488	Flunixin	24hrs
337	559	Saline	24hrs
338	784	Ketoprofen	24hrs
339	640	Carprofen	24hrs
340	769	Flunixin	24hrs
341	306	Ketoprofen	26hrs
342	313	Ketoprofen	26hrs
343	492	Flunixin	26hrs
344	458	Saline	26hrs
345	310	Carprofen	26hrs
346	585	Carprofen	26hrs
347	698	Saline	26hrs
348	340	Saline	26hrs
349	607	Flunixin	26hrs
350	520	Ketoprofen	26hrs
351	436	Ketoprofen	26hrs
352	502	Carprofen	26hrs
353	302	Flunixin	26hrs
354	301	Saline	26hrs
355	727	Carprofen	26hrs
356	488	Flunixin	26hrs
357	559	Saline	26hrs
358	784	Ketoprofen	26hrs
359	640	Carprofen	26hrs
360	769	Flunixin	26hrs

361	306	Ketoprofen	48hrs
362	313	Ketoprofen	48hrs
363	492	Flunixin	48hrs
364	458	Saline	48hrs
365	310	Carprofen	48hrs
366	585	Carprofen	48hrs
367	698	Saline	48hrs
368	340	Saline	48hrs
369	607	Flunixin	48hrs
370	520	Ketoprofen	48hrs
371	436	Ketoprofen	48hrs
372	502	Carprofen	48hrs
373	302	Flunixin	48hrs
374	301	Saline	48hrs
375	727	Carprofen	48hrs
376	488	Flunixin	48hrs
377	559	Saline	48hrs
378	784	Ketoprofen	48hrs
379	640	Carprofen	48hrs
380	769	Flunixin	48hrs
381	306	Ketoprofen	50hrs
382	313	Ketoprofen	50hrs
383	492	Flunixin	50hrs
384	458	Saline	50hrs
385	310	Carprofen	50hrs
386	585	Carprofen	50hrs
387	698	Saline	50hrs
388	340	Saline	50hrs
389	607	Flunixin	50hrs
390	520	Ketoprofen	50hrs
391	436	Ketoprofen	50hrs
392	502	Carprofen	50hrs
393	302	Flunixin	50hrs
394	301	Saline	50hrs
395	727	Carprofen	50hrs
396	488	Flunixin	50hrs
397	559	Saline	50hrs
398	784	Ketoprofen	50hrs
399	640	Carprofen	50hrs
400	769	Flunixin	50hrs

401	306	Ketoprofen	72hrs
402	313	Ketoprofen	72hrs
403	492	Flunixin	72hrs
404	458	Saline	72hrs
405	310	Carprofen	72hrs
406	585	Carprofen	72hrs
407	698	Saline	72hrs
408	340	Saline	72hrs
409	607	Flunixin	72hrs
410	520	Ketoprofen	72hrs
411	436	Ketoprofen	72hrs
412	502	Carprofen	72hrs
413	302	Flunixin	72hrs
414	301	Saline	72hrs
415	727	Carprofen	72hrs
416	488	Flunixin	72hrs
417	559	Saline	72hrs
418	784	Ketoprofen	72hrs
419	640	Carprofen	72hrs
420	769	Flunixin	72hrs
421	306	Ketoprofen	74hrs
422	313	Ketoprofen	74hrs
423	492	Flunixin	74hrs
424	458	Saline	74hrs
425	310	Carprofen	74hrs
426	585	Carprofen	74hrs
427	698	Saline	74hrs
428	340	Saline	74hrs
429	607	Flunixin	74hrs
430	520	Ketoprofen	74hrs
431	436	Ketoprofen	74hrs
432	502	Carprofen	74hrs
433	302	Flunixin	74hrs
434	301	Saline	74hrs
435	727	Carprofen	74hrs
436	488	Flunixin	74hrs
437	559	Saline	74hrs
438	784	Ketoprofen	74hrs
439	640	Carprofen	74hrs
440	769	Flunixin	74hrs

441	306	Ketoprofen	96hrs
442	313	Ketoprofen	96hrs
443	492	Flunixin	96hrs
444	458	Saline	96hrs
445	310	Carprofen	96hrs
446	585	Carprofen	96hrs
447	698	Saline	96hrs
448	340	Saline	96hrs
449	607	Flunixin	96hrs
450	520	Ketoprofen	96hrs
451	436	Ketoprofen	96hrs
452	502	Carprofen	96hrs
453	302	Flunixin	96hrs
454	301	Saline	96hrs
455	727	Carprofen	96hrs
456	488	Flunixin	96hrs
457	559	Saline	96hrs
458	784	Ketoprofen	96hrs
459	640	Carprofen	96hrs
460	769	Flunixin	96hrs
461	306	Ketoprofen	98hrs
462	313	Ketoprofen	98hrs
463	492	Flunixin	98hrs
464	458	Saline	98hrs
465	310	Carprofen	98hrs
466	585	Carprofen	98hrs
467	698	Saline	98hrs
468	340	Saline	98hrs
469	607	Flunixin	98hrs
470	520	Ketoprofen	98hrs
471	436	Ketoprofen	98hrs
472	502	Carprofen	98hrs
473	302	Flunixin	98hrs
474	301	Saline	98hrs
475	727	Carprofen	98hrs
476	488	Flunixin	98hrs
477	559	Saline	98hrs
478	784	Ketoprofen	98hrs
479	640	Carprofen	98hrs
480	769	Flunixin	98hrs

Appendix 5 – Sample Table

1309-066 Cohort 1

Lab. No.	Animal No.	Active	Time point
1309-066-001	311	Ketoprofen	-24
1309-066-002	311	Ketoprofen	-22
1309-066-003	311	Ketoprofen	0hrs
1309-066-004	311	Ketoprofen	2hrs
1309-066-005	311	Ketoprofen	24hrs
1309-066-006	311	Ketoprofen	26hrs
1309-066-007	311	Ketoprofen	48hrs
1309-066-008	311	Ketoprofen	50hrs
1309-066-009	311	Ketoprofen	72hrs
1309-066-010	311	Ketoprofen	74hrs
1309-066-011	311	Ketoprofen	96hrs
1309-066-012	311	Ketoprofen	98hrs
1309-066-013	437	Ketoprofen	-24
1309-066-014	437	Ketoprofen	-22
1309-066-015	437	Ketoprofen	0hrs
1309-066-016	437	Ketoprofen	2hrs
1309-066-017	437	Ketoprofen	24hrs
1309-066-018	437	Ketoprofen	26hrs
1309-066-019	437	Ketoprofen	48hrs
1309-066-020	437	Ketoprofen	50hrs
1309-066-021	437	Ketoprofen	72hrs
1309-066-022	437	Ketoprofen	74hrs
1309-066-023	437	Ketoprofen	96hrs
1309-066-024	437	Ketoprofen	98hrs
1309-066-025	493	Ketoprofen	-24
1309-066-026	493	Ketoprofen	-22
1309-066-027	493	Ketoprofen	0hrs
1309-066-028	493	Ketoprofen	2hrs
1309-066-029	493	Ketoprofen	24hrs
1309-066-030	493	Ketoprofen	26hrs
1309-066-031	493	Ketoprofen	48hrs
1309-066-032	493	Ketoprofen	50hrs
1309-066-033	493	Ketoprofen	72hrs
1309-066-034	493	Ketoprofen	74hrs
1309-066-035	493	Ketoprofen	96hrs
1309-066-036	493	Ketoprofen	98hrs
1309-066-037	730	Ketoprofen	-24
1309-066-038	730	Ketoprofen	-22
1309-066-039	730	Ketoprofen	0hrs
1309-066-040	730	Ketoprofen	2hrs
1309-066-041	730	Ketoprofen	24hrs
1309-066-042	730	Ketoprofen	26hrs
1309-066-043	730	Ketoprofen	48hrs
1309-066-044	730	Ketoprofen	50hrs
1309-066-045	730	Ketoprofen	72hrs
1309-066-046	730	Ketoprofen	74hrs
1309-066-047	730	Ketoprofen	96hrs
1309-066-048	730	Ketoprofen	98hrs
1309-066-049	760	Ketoprofen	-24

1309-066 Cohort 1

Lab. No.	Animal No.	Active	Time point
1309-066-050	760	Ketoprofen	-22
1309-066-051	760	Ketoprofen	0hrs
1309-066-052	760	Ketoprofen	2hrs
1309-066-053	760	Ketoprofen	24hrs
1309-066-054	760	Ketoprofen	26hrs
1309-066-055	760	Ketoprofen	48hrs
1309-066-056	760	Ketoprofen	50hrs
1309-066-057	760	Ketoprofen	72hrs
1309-066-058	760	Ketoprofen	74hrs
1309-066-059	760	Ketoprofen	96hrs
1309-066-060	760	Ketoprofen	98hrs
1309-066-061	312	Carprofen	-24
1309-066-062	312	Carprofen	-22
1309-066-063	312	Carprofen	0hrs
1309-066-064	312	Carprofen	2hrs
1309-066-065	312	Carprofen	24hrs
1309-066-066	312	Carprofen	26hrs
1309-066-067	312	Carprofen	48hrs
1309-066-068	312	Carprofen	50hrs
1309-066-069	312	Carprofen	72hrs
1309-066-070	312	Carprofen	74hrs
1309-066-071	312	Carprofen	96hrs
1309-066-072	312	Carprofen	98hrs
1309-066-073	404	Carprofen	-24
1309-066-074	404	Carprofen	-22
1309-066-075	404	Carprofen	0hrs
1309-066-076	404	Carprofen	2hrs
1309-066-077	404	Carprofen	24hrs
1309-066-078	404	Carprofen	26hrs
1309-066-079	404	Carprofen	48hrs
1309-066-080	404	Carprofen	50hrs
1309-066-081	404	Carprofen	72hrs
1309-066-082	404	Carprofen	74hrs
1309-066-083	404	Carprofen	96hrs
1309-066-084	404	Carprofen	98hrs
1309-066-085	558	Carprofen	-24
1309-066-086	558	Carprofen	-22
1309-066-087	558	Carprofen	0hrs
1309-066-088	558	Carprofen	2hrs
1309-066-089	558	Carprofen	24hrs
1309-066-090	558	Carprofen	26hrs
1309-066-091	558	Carprofen	48hrs
1309-066-092	558	Carprofen	50hrs
1309-066-093	558	Carprofen	72hrs
1309-066-094	558	Carprofen	74hrs
1309-066-095	558	Carprofen	96hrs
1309-066-096	558	Carprofen	98hrs
1309-066-097	605	Carprofen	-24
1309-066-098	605	Carprofen	-22

1309-066 Cohort 1

Lab. No.	Animal No.	Active	Time point
1309-066-099	605	Carprofen	0hrs
1309-066-100	605	Carprofen	2hrs
1309-066-101	605	Carprofen	24hrs
1309-066-102	605	Carprofen	26hrs
1309-066-103	605	Carprofen	48hrs
1309-066-104	605	Carprofen	50hrs
1309-066-105	605	Carprofen	72hrs
1309-066-106	605	Carprofen	74hrs
1309-066-107	605	Carprofen	96hrs
1309-066-108	605	Carprofen	98hrs
1309-066-109	675	Carprofen	-24
1309-066-110	675	Carprofen	-22
1309-066-111	675	Carprofen	0hrs
1309-066-112	675	Carprofen	2hrs
1309-066-113	675	Carprofen	24hrs
1309-066-114	675	Carprofen	26hrs
1309-066-115	675	Carprofen	48hrs
1309-066-116	675	Carprofen	50hrs
1309-066-117	675	Carprofen	72hrs
1309-066-118	675	Carprofen	74hrs
1309-066-119	675	Carprofen	96hrs
1309-066-120	675	Carprofen	98hrs
1309-066-121	422	Flunixin	-24
1309-066-122	422	Flunixin	-22
1309-066-123	422	Flunixin	0hrs
1309-066-124	422	Flunixin	2hrs
1309-066-125	422	Flunixin	24hrs
1309-066-126	422	Flunixin	26hrs
1309-066-127	422	Flunixin	48hrs
1309-066-128	422	Flunixin	50hrs
1309-066-129	422	Flunixin	72hrs
1309-066-130	422	Flunixin	74hrs
1309-066-131	422	Flunixin	96hrs
1309-066-132	422	Flunixin	98hrs
1309-066-133	494	Flunixin	-24
1309-066-134	494	Flunixin	-22
1309-066-135	494	Flunixin	0hrs
1309-066-136	494	Flunixin	2hrs
1309-066-137	494	Flunixin	24hrs
1309-066-138	494	Flunixin	26hrs
1309-066-139	494	Flunixin	48hrs
1309-066-140	494	Flunixin	50hrs
1309-066-141	494	Flunixin	72hrs
1309-066-142	494	Flunixin	74hrs
1309-066-143	494	Flunixin	96hrs
1309-066-144	494	Flunixin	98hrs
1309-066-145	498	Flunixin	-24
1309-066-146	498	Flunixin	-22
1309-066-147	498	Flunixin	0hrs

1309-066 Cohort 1

Lab. No.	Animal No.	Active	Time point
1309-066-148	498	Flunixin	2hrs
1309-066-149	498	Flunixin	24hrs
1309-066-150	498	Flunixin	26hrs
1309-066-151	498	Flunixin	48hrs
1309-066-152	498	Flunixin	50hrs
1309-066-153	498	Flunixin	72hrs
1309-066-154	498	Flunixin	74hrs
1309-066-155	498	Flunixin	96hrs
1309-066-156	498	Flunixin	98hrs
1309-066-157	555	Flunixin	-24
1309-066-158	555	Flunixin	-22
1309-066-159	555	Flunixin	0hrs
1309-066-160	555	Flunixin	2hrs
1309-066-161	555	Flunixin	24hrs
1309-066-162	555	Flunixin	26hrs
1309-066-163	555	Flunixin	48hrs
1309-066-164	555	Flunixin	50hrs
1309-066-165	555	Flunixin	72hrs
1309-066-166	555	Flunixin	74hrs
1309-066-167	555	Flunixin	96hrs
1309-066-168	555	Flunixin	98hrs
1309-066-169	572	Flunixin	-24
1309-066-170	572	Flunixin	-22
1309-066-171	572	Flunixin	0hrs
1309-066-172	572	Flunixin	2hrs
1309-066-173	572	Flunixin	24hrs
1309-066-174	572	Flunixin	26hrs
1309-066-175	572	Flunixin	48hrs
1309-066-176	572	Flunixin	50hrs
1309-066-177	572	Flunixin	72hrs
1309-066-178	572	Flunixin	74hrs
1309-066-179	572	Flunixin	96hrs
1309-066-180	572	Flunixin	98hrs
1309-066-181	314	Saline	-24
1309-066-182	314	Saline	-22
1309-066-183	314	Saline	0hrs
1309-066-184	314	Saline	2hrs
1309-066-185	314	Saline	24hrs
1309-066-186	314	Saline	26hrs
1309-066-187	314	Saline	48hrs
1309-066-188	314	Saline	50hrs
1309-066-189	314	Saline	72hrs
1309-066-190	314	Saline	74hrs
1309-066-191	314	Saline	96hrs
1309-066-192	314	Saline	98hrs
1309-066-193	495	Saline	-24
1309-066-194	495	Saline	-22
1309-066-195	495	Saline	0hrs
1309-066-196	495	Saline	2hrs

1309-066 Cohort 1

Lab. No.	Animal No.	Active	Time point
1309-066-197	495	Saline	24hrs
1309-066-198	495	Saline	26hrs
1309-066-199	495	Saline	48hrs
1309-066-200	495	Saline	50hrs
1309-066-201	495	Saline	72hrs
1309-066-202	495	Saline	74hrs
1309-066-203	495	Saline	96hrs
1309-066-204	495	Saline	98hrs
1309-066-205	576	Saline	-24
1309-066-206	576	Saline	-22
1309-066-207	576	Saline	0hrs
1309-066-208	576	Saline	2hrs
1309-066-209	576	Saline	24hrs
1309-066-210	576	Saline	26hrs
1309-066-211	576	Saline	48hrs
1309-066-212	576	Saline	50hrs
1309-066-213	576	Saline	72hrs
1309-066-214	576	Saline	74hrs
1309-066-215	576	Saline	96hrs
1309-066-216	576	Saline	98hrs
1309-066-217	652	Saline	-24
1309-066-218	652	Saline	-22
1309-066-219	652	Saline	0hrs
1309-066-220	652	Saline	2hrs
1309-066-221	652	Saline	24hrs
1309-066-222	652	Saline	26hrs
1309-066-223	652	Saline	48hrs
1309-066-224	652	Saline	50hrs
1309-066-225	652	Saline	72hrs
1309-066-226	652	Saline	74hrs
1309-066-227	652	Saline	96hrs
1309-066-228	652	Saline	98hrs
1309-066-229	716	Saline	-24
1309-066-230	716	Saline	-22
1309-066-231	716	Saline	0hrs
1309-066-232	716	Saline	2hrs
1309-066-233	716	Saline	24hrs
1309-066-234	716	Saline	26hrs
1309-066-235	716	Saline	48hrs
1309-066-236	716	Saline	50hrs
1309-066-237	716	Saline	72hrs
1309-066-238	716	Saline	74hrs
1309-066-239	716	Saline	96hrs
1309-066-240	716	Saline	98hrs

Lab ID	ANIMAL_ID	TREATMENT	TIME POINT
1306-066-241	310	Carprofen	-24
1306-066-242	310	Carprofen	-22
1306-066-243	310	Carprofen	0
1306-066-244	310	Carprofen	2 hrs
1306-066-245	310	Carprofen	24hrs
1306-066-246	310	Carprofen	26hrs
1306-066-247	310	Carprofen	48hrs
1306-066-248	310	Carprofen	50hrs
1306-066-249	310	Carprofen	72hrs
1306-066-250	310	Carprofen	74hrs
1306-066-251	310	Carprofen	96hrs
1306-066-252	310	Carprofen	98hrs
1306-066-253	502	Carprofen	-24
1306-066-254	502	Carprofen	-22
1306-066-255	502	Carprofen	0
1306-066-256	502	Carprofen	2 hrs
1306-066-257	502	Carprofen	24hrs
1306-066-258	502	Carprofen	26hrs
1306-066-259	502	Carprofen	48hrs
1306-066-260	502	Carprofen	50hrs
1306-066-261	502	Carprofen	72hrs
1306-066-262	502	Carprofen	74hrs
1306-066-263	502	Carprofen	96hrs
1306-066-264	502	Carprofen	98hrs
1306-066-265	585	Carprofen	-24
1306-066-266	585	Carprofen	-22
1306-066-267	585	Carprofen	0
1306-066-268	585	Carprofen	2 hrs
1306-066-269	585	Carprofen	24hrs
1306-066-270	585	Carprofen	26hrs
1306-066-271	585	Carprofen	48hrs
1306-066-272	585	Carprofen	50hrs
1306-066-273	585	Carprofen	72hrs
1306-066-274	585	Carprofen	74hrs
1306-066-275	585	Carprofen	96hrs
1306-066-276	585	Carprofen	98hrs
1306-066-277	640	Carprofen	-24
1306-066-278	640	Carprofen	-22
1306-066-279	640	Carprofen	0
1306-066-280	640	Carprofen	2 hrs

Lab ID	ANIMAL_ID	TREATMENT	TIME POINT
1306-066-281	640	Carprofen	24hrs
1306-066-282	640	Carprofen	26hrs
1306-066-283	640	Carprofen	48hrs
1306-066-284	640	Carprofen	50hrs
1306-066-285	640	Carprofen	72hrs
1306-066-286	640	Carprofen	74hrs
1306-066-287	640	Carprofen	96hrs
1306-066-288	640	Carprofen	98hrs
1306-066-289	727	Carprofen	-24
1306-066-290	727	Carprofen	-22
1306-066-291	727	Carprofen	0
1306-066-292	727	Carprofen	2 hrs
1306-066-293	727	Carprofen	24hrs
1306-066-294	727	Carprofen	26hrs
1306-066-295	727	Carprofen	48hrs
1306-066-296	727	Carprofen	50hrs
1306-066-297	727	Carprofen	72hrs
1306-066-298	727	Carprofen	74hrs
1306-066-299	727	Carprofen	96hrs
1306-066-300	727	Carprofen	98hrs
1306-066-301	302	Flunixin	-24
1306-066-302	302	Flunixin	-22
1306-066-303	302	Flunixin	0
1306-066-304	302	Flunixin	2 hrs
1306-066-305	302	Flunixin	24hrs
1306-066-306	302	Flunixin	26hrs
1306-066-307	302	Flunixin	48hrs
1306-066-308	302	Flunixin	50hrs
1306-066-309	302	Flunixin	72hrs
1306-066-310	302	Flunixin	74hrs
1306-066-311	302	Flunixin	96hrs
1306-066-312	302	Flunixin	98hrs
1306-066-313	488	Flunixin	-24
1306-066-314	488	Flunixin	-22
1306-066-315	488	Flunixin	0
1306-066-316	488	Flunixin	2 hrs
1306-066-317	488	Flunixin	24hrs
1306-066-318	488	Flunixin	26hrs
1306-066-319	488	Flunixin	48hrs
1306-066-320	488	Flunixin	50hrs

Lab ID	ANIMAL_ID	TREATMENT	TIME POINT
1306-066-321	488	Flunixin	72hrs
1306-066-322	488	Flunixin	74hrs
1306-066-323	488	Flunixin	96hrs
1306-066-324	488	Flunixin	98hrs
1306-066-325	492	Flunixin	-24
1306-066-326	492	Flunixin	-22
1306-066-327	492	Flunixin	0
1306-066-328	492	Flunixin	2 hrs
1306-066-329	492	Flunixin	24hrs
1306-066-330	492	Flunixin	26hrs
1306-066-331	492	Flunixin	48hrs
1306-066-332	492	Flunixin	50hrs
1306-066-333	492	Flunixin	72hrs
1306-066-334	492	Flunixin	74hrs
1306-066-335	492	Flunixin	96hrs
1306-066-336	492	Flunixin	98hrs
1306-066-337	607	Flunixin	-24
1306-066-338	607	Flunixin	-22
1306-066-339	607	Flunixin	0
1306-066-340	607	Flunixin	2 hrs
1306-066-341	607	Flunixin	24hrs
1306-066-342	607	Flunixin	26hrs
1306-066-343	607	Flunixin	48hrs
1306-066-344	607	Flunixin	50hrs
1306-066-345	607	Flunixin	72hrs
1306-066-346	607	Flunixin	74hrs
1306-066-347	607	Flunixin	96hrs
1306-066-348	607	Flunixin	98hrs
1306-066-349	769	Flunixin	-24
1306-066-350	769	Flunixin	-22
1306-066-351	769	Flunixin	0
1306-066-352	769	Flunixin	2 hrs
1306-066-353	769	Flunixin	24hrs
1306-066-354	769	Flunixin	26hrs
1306-066-355	769	Flunixin	48hrs
1306-066-356	769	Flunixin	50hrs
1306-066-357	769	Flunixin	72hrs
1306-066-358	769	Flunixin	74hrs
1306-066-359	769	Flunixin	96hrs
1306-066-360	769	Flunixin	98hrs

Lab ID	ANIMAL_ID	TREATMENT	TIME POINT
1306-066-361	306	Ketoprofen	-24
1306-066-362	306	Ketoprofen	-22
1306-066-363	306	Ketoprofen	0
1306-066-364	306	Ketoprofen	2 hrs
1306-066-365	306	Ketoprofen	24hrs
1306-066-366	306	Ketoprofen	26hrs
1306-066-367	306	Ketoprofen	48hrs
1306-066-368	306	Ketoprofen	50hrs
1306-066-369	306	Ketoprofen	72hrs
1306-066-370	306	Ketoprofen	74hrs
1306-066-371	306	Ketoprofen	96hrs
1306-066-372	306	Ketoprofen	98hrs
1306-066-373	313	Ketoprofen	-24
1306-066-374	313	Ketoprofen	-22
1306-066-375	313	Ketoprofen	0
1306-066-376	313	Ketoprofen	2 hrs
1306-066-377	313	Ketoprofen	24hrs
1306-066-378	313	Ketoprofen	26hrs
1306-066-379	313	Ketoprofen	48hrs
1306-066-380	313	Ketoprofen	50hrs
1306-066-381	313	Ketoprofen	72hrs
1306-066-382	313	Ketoprofen	74hrs
1306-066-383	313	Ketoprofen	96hrs
1306-066-384	313	Ketoprofen	98hrs
1306-066-385	436	Ketoprofen	-24
1306-066-386	436	Ketoprofen	-22
1306-066-387	436	Ketoprofen	0
1306-066-388	436	Ketoprofen	2 hrs
1306-066-389	436	Ketoprofen	24hrs
1306-066-390	436	Ketoprofen	26hrs
1306-066-391	436	Ketoprofen	48hrs
1306-066-392	436	Ketoprofen	50hrs
1306-066-393	436	Ketoprofen	72hrs
1306-066-394	436	Ketoprofen	74hrs
1306-066-395	436	Ketoprofen	96hrs
1306-066-396	436	Ketoprofen	98hrs
1306-066-397	520	Ketoprofen	-24
1306-066-398	520	Ketoprofen	-22
1306-066-399	520	Ketoprofen	0
1306-066-400	520	Ketoprofen	2 hrs

Lab ID	ANIMAL_ID	TREATMENT	TIME POINT
1306-066-401	520	Ketoprofen	24hrs
1306-066-402	520	Ketoprofen	26hrs
1306-066-403	520	Ketoprofen	48hrs
1306-066-404	520	Ketoprofen	50hrs
1306-066-405	520	Ketoprofen	72hrs
1306-066-406	520	Ketoprofen	74hrs
1306-066-407	520	Ketoprofen	96hrs
1306-066-408	520	Ketoprofen	98hrs
1306-066-409	784	Ketoprofen	-24
1306-066-410	784	Ketoprofen	-22
1306-066-411	784	Ketoprofen	0
1306-066-412	784	Ketoprofen	2 hrs
1306-066-413	784	Ketoprofen	24hrs
1306-066-414	784	Ketoprofen	26hrs
1306-066-415	784	Ketoprofen	48hrs
1306-066-416	784	Ketoprofen	50hrs
1306-066-417	784	Ketoprofen	72hrs
1306-066-418	784	Ketoprofen	74hrs
1306-066-419	784	Ketoprofen	96hrs
1306-066-420	784	Ketoprofen	98hrs
1306-066-421	301	Saline	-24
1306-066-422	301	Saline	-22
1306-066-423	301	Saline	0
1306-066-424	301	Saline	2 hrs
1306-066-425	301	Saline	24hrs
1306-066-426	301	Saline	26hrs
1306-066-427	301	Saline	48hrs
1306-066-428	301	Saline	50hrs
1306-066-429	301	Saline	72hrs
1306-066-430	301	Saline	74hrs
1306-066-431	301	Saline	96hrs
1306-066-432	301	Saline	98hrs
1306-066-433	340	Saline	-24
1306-066-434	340	Saline	-22
1306-066-435	340	Saline	0
1306-066-436	340	Saline	2 hrs
1306-066-437	340	Saline	24hrs
1306-066-438	340	Saline	26hrs
1306-066-439	340	Saline	48hrs
1306-066-440	340	Saline	50hrs

Lab ID	ANIMAL_ID	TREATMENT	TIME POINT
1306-066-441	340	Saline	72hrs
1306-066-442	340	Saline	74hrs
1306-066-443	340	Saline	96hrs
1306-066-444	340	Saline	98hrs
1306-066-445	458	Saline	-24
1306-066-446	458	Saline	-22
1306-066-447	458	Saline	0
1306-066-448	458	Saline	2 hrs
1306-066-449	458	Saline	24hrs
1306-066-450	458	Saline	26hrs
1306-066-451	458	Saline	48hrs
1306-066-452	458	Saline	50hrs
1306-066-453	458	Saline	72hrs
1306-066-454	458	Saline	74hrs
1306-066-455	458	Saline	96hrs
1306-066-456	458	Saline	98hrs
1306-066-457	559	Saline	-24
1306-066-458	559	Saline	-22
1306-066-459	559	Saline	0
1306-066-460	559	Saline	2 hrs
1306-066-461	559	Saline	24hrs
1306-066-462	559	Saline	26hrs
1306-066-463	559	Saline	48hrs
1306-066-464	559	Saline	50hrs
1306-066-465	559	Saline	72hrs
1306-066-466	559	Saline	74hrs
1306-066-467	559	Saline	96hrs
1306-066-468	559	Saline	98hrs
1306-066-469	698	Saline	-24
1306-066-470	698	Saline	-22
1306-066-471	698	Saline	0
1306-066-472	698	Saline	2 hrs
1306-066-473	698	Saline	24hrs
1306-066-474	698	Saline	26hrs
1306-066-475	698	Saline	48hrs
1306-066-476	698	Saline	50hrs
1306-066-477	698	Saline	72hrs
1306-066-478	698	Saline	74hrs
1306-066-479	698	Saline	96hrs
1306-066-480	698	Saline	98hrs

Appendix 6– Example Chromatograms

Ketoprofen run Chromatograms

Sample Name	Reagent blank		Injection Vial	39
Sample ID			Injection Volume	10.00
Sample Type	Unknown		Algorithm Used	MQ4
Acquisition Date	26/11/2013 5:21:19 AM		Dilution Factor	1.00
Acquisition Method	ATM018 NSAID LCMSMS.dam		Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS		Instrument Name	API 3200
Data File	DataSET1.wiff			
Result Table	131125 Run 1 Ketoprofen			
Sample Comment				

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP13CD3#2	N/A	N/A	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP#1	9.213e+01	2.56	N/A	N/A
KETOP#2	2.764e+01	2.48	N/A	N/A

KETOP13CD3#2 (Internal Standard)

RT (Exp. RT):   N/A(2.55) min

Concentration: 1.0000

Sample Type: (Unknown)

KETOP#1 (253.3 / 209.0)

RT (Exp. RT):   2.56 (2.55) min

Calculated conc: N/A

Area Ratio: N/A

Sample Type: (Unknown)

KETOP#2 (253.3 / 197.0)

RT (Exp. RT):   2.48 (2.55) min

Calculated conc: N/A

Area Ratio: N/A

Sample Type: (Unknown)
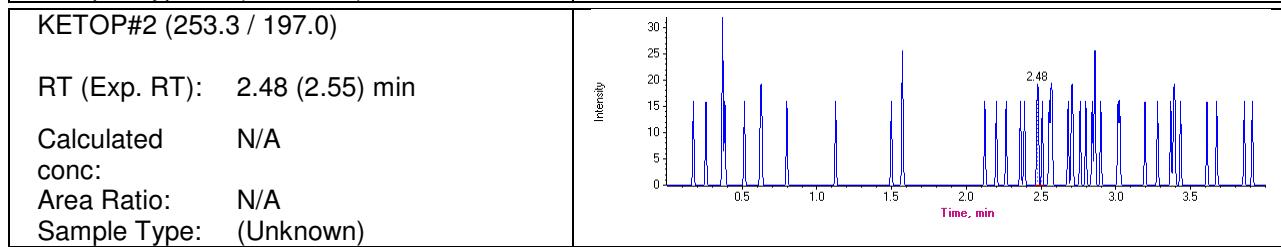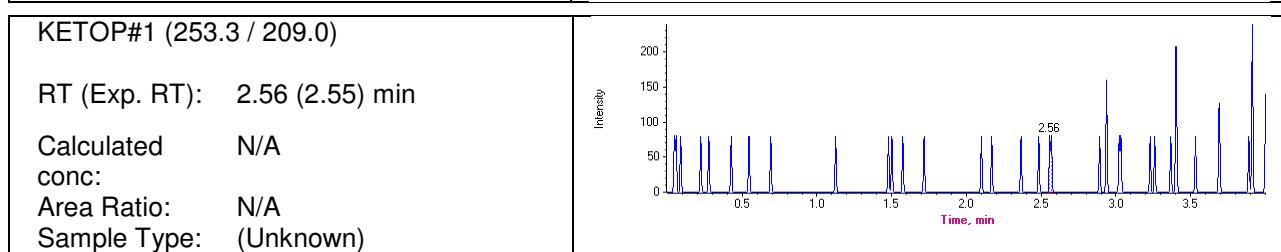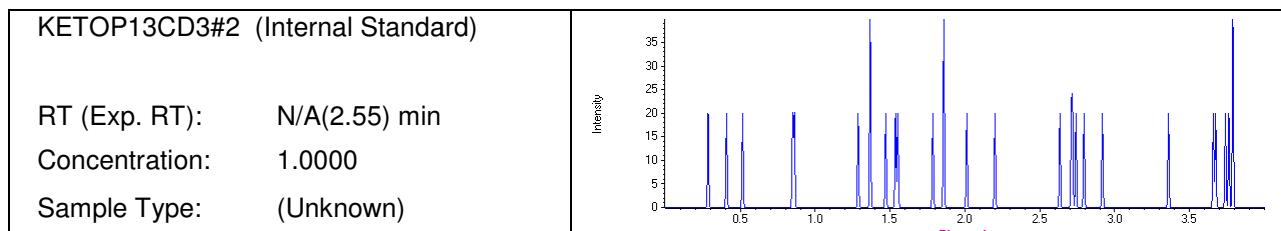
Sample Name	Blank plasma extract	Injection Vial	55
Sample ID		Injection Volume	10.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	26/11/2013 3:58:35 AM	Dilution Factor	1.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131125 Run 1 Ketoprofen		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP13CD3#2	11.52	2.58	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP#1	5.524e+02	2.53	N/A	143663.62
KETOP#2	1.180e+02	2.53	N/A	1973.51

KETOP13CD3#2 (Internal Standard)

RT (Exp. RT):   2.58(2.55) min

Concentration: 1.0000

Sample Type: (Unknown)

KETOP#1 (253.3 / 209.0)

RT (Exp. RT):   2.53 (2.55) min

Calculated conc: 143663.62

Area Ratio: 4.794e1

Sample Type: (Unknown)

KETOP#2 (253.3 / 197.0)

RT (Exp. RT):   2.53 (2.55) min

Calculated conc: 1973.51

Area Ratio: 1.024e1

Sample Type: (Unknown)
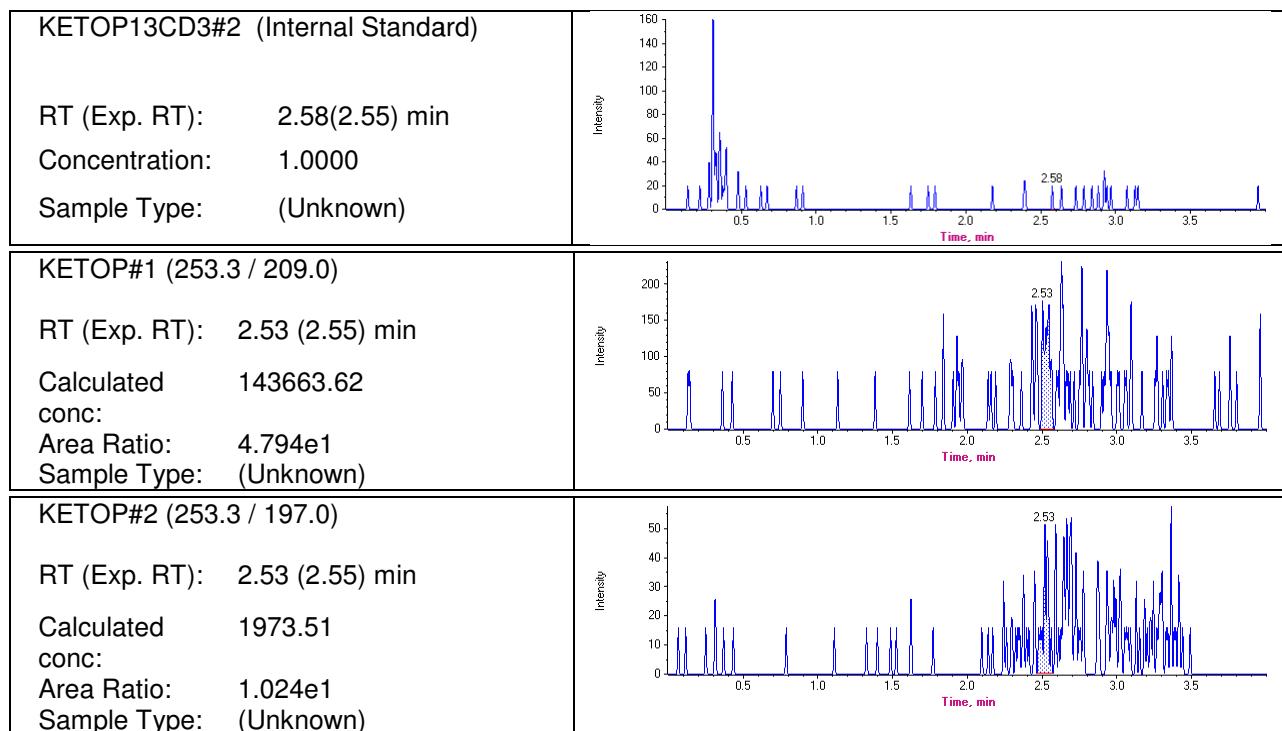
Sample Name	Blank plasma with internal stds	Injection Vial	56
Sample ID		Injection Volume	10.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	26/11/2013 4:03:04 AM	Dilution Factor	1.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131125 Run 1 Ketoprofen		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP13CD3#2	10101.50	2.55	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP#1	1.843e+02	2.49	N/A	40.76
KETOP#2	4.604e+01	2.58	N/A	< 0

KETOP13CD3#2 (Internal Standard)

RT (Exp. RT):   2.55(2.55) min

Concentration: 1.0000

Sample Type: (Unknown)

KETOP#1 (253.3 / 209.0)

RT (Exp. RT):   2.49 (2.55) min

Calculated conc: 40.76

Area Ratio: 1.824e-2

Sample Type: (Unknown)

KETOP#2 (253.3 / 197.0)

RT (Exp. RT):   2.58 (2.55) min

Calculated conc: < 0

Area Ratio: 4.558e-3

Sample Type: (Unknown)
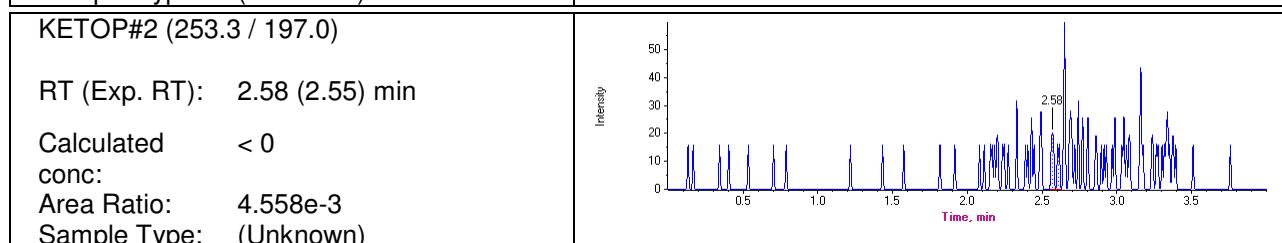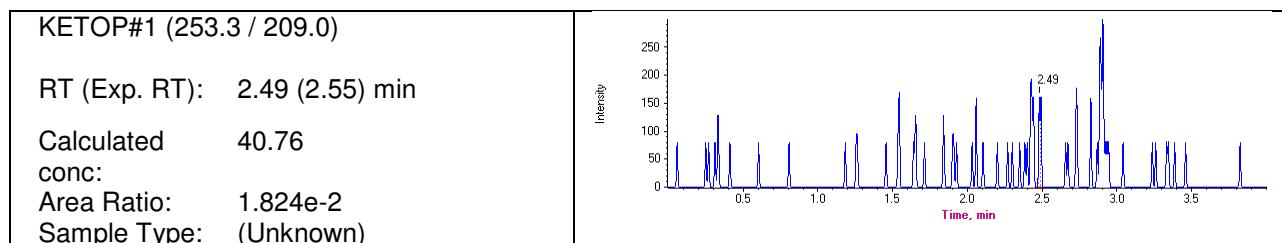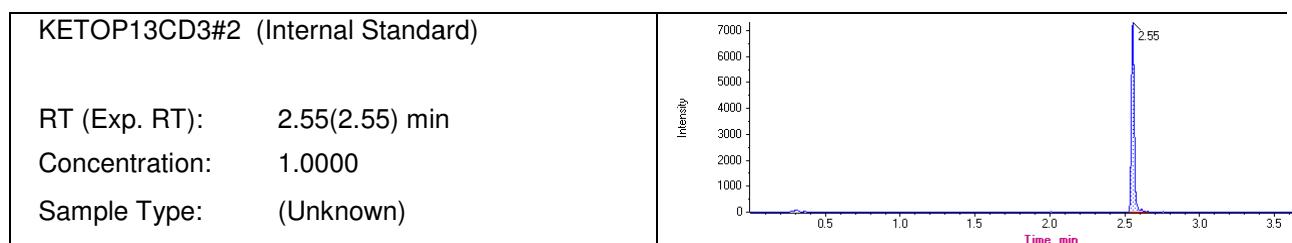
Sample Name	Std 6 (410.79ng/mL ketoprofen)	Injection Vial	46
Sample ID		Injection Volume	10.00
Sample Type	Standard	Algorithm Used	MQ4
Acquisition Date	26/11/2013 6:10:44 AM	Dilution Factor	1.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131125 Run 1 Ketoprofen		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP13CD3#2	14478.46	2.53	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP#1	2.488e+03	2.54	410.7900	501.06
KETOP#2	3.373e+04	2.53	410.7900	447.63

KETOP13CD3#2 (Internal Standard)

RT (Exp. RT):   2.53(2.55) min

Concentration: 1.0000

Sample Type: (Standard)

KETOP#1 (253.3 / 209.0)

RT (Exp. RT):   2.54 (2.55) min

Calculated conc: 501.06

Area Ratio: 1.718e-1

Sample Type: (Standard)

KETOP#2 (253.3 / 197.0)

RT (Exp. RT):   2.53 (2.55) min

Calculated conc: 447.63

Area Ratio: 2.330e0

Sample Type: (Standard)
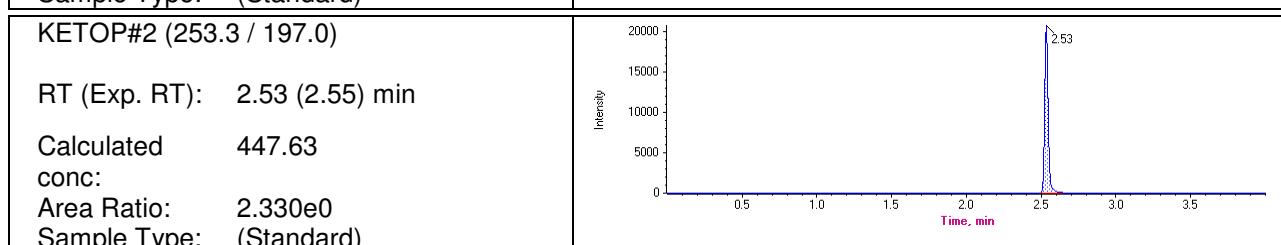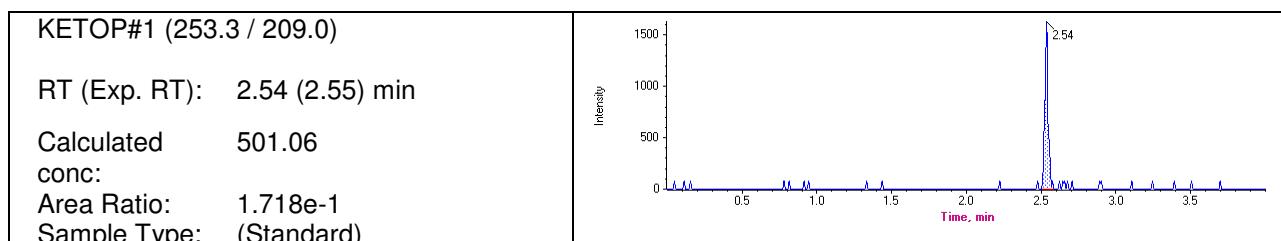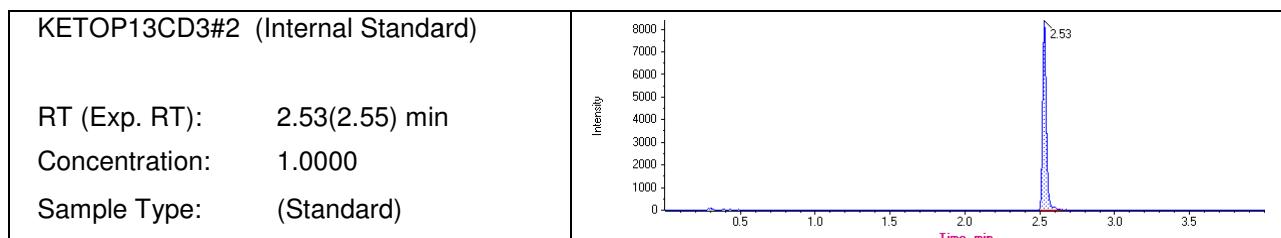
Sample Name	Spike 1A (10.27ng/mL ketoprofen)	Injection Vial	1
Sample ID		Injection Volume	10.00
Sample Type	Quality Control	Algorithm Used	MQ4
Acquisition Date	26/11/2013 6:46:41 AM	Dilution Factor	2.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131125 Run 1 Ketoprofen		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP13CD3#2	11667.99	2.53	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP#1	3.685e+02	2.61	0.0000	161.49
KETOP#2	4.331e+02	2.53	10.2700	10.78

KETOP13CD3#2 (Internal Standard)

RT (Exp. RT):   2.53(2.55) min

Concentration: 1.0000

Sample Type: (Quality Control)

KETOP#1 (253.3 / 209.0)

RT (Exp. RT):   2.61 (2.55) min

Calculated conc: 161.49

Area Ratio: 3.158e-2

Sample Type: (Quality Control)

KETOP#2 (253.3 / 197.0)

RT (Exp. RT):   2.53 (2.55) min

Calculated conc: 10.78

Area Ratio: 3.712e-2

Sample Type: (Quality Control)
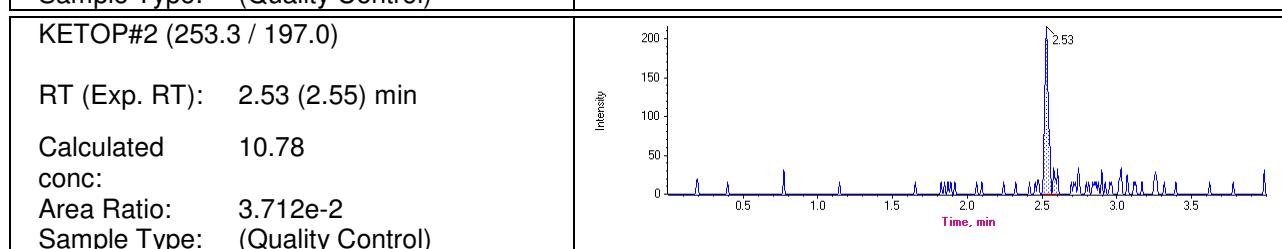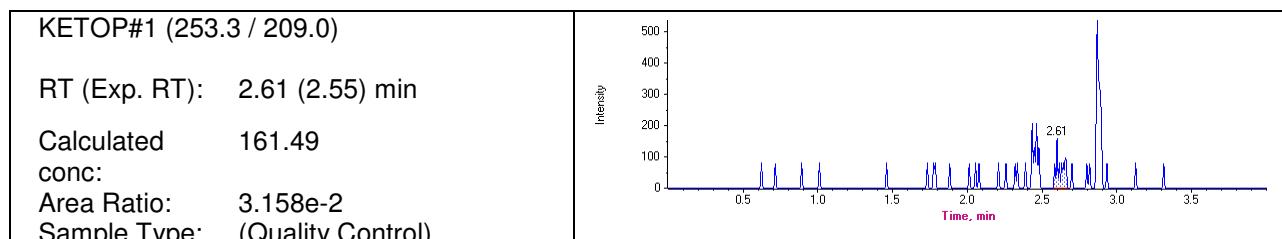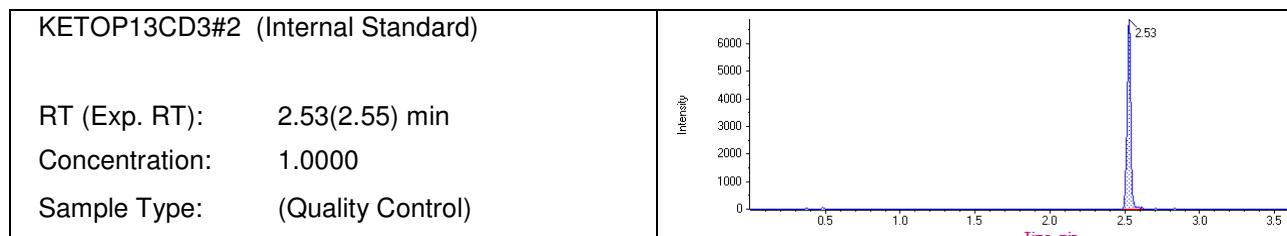
Sample Name	Sample 1309-066-001	Injection Vial	2
Sample ID	Reported <10ng/mL	Injection Volume	10.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	26/11/2013 8:43:33 AM	Dilution Factor	2.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131125 Run 1 Ketoprofen		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP13CD3#2	12224.17	2.53	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP#1	1.841e+02	2.52	N/A	62.47
KETOP#2	4.606e+01	2.53	N/A	< 0

KETOP13CD3#2 (Internal Standard)

RT (Exp. RT):　　2.53(2.55) min

Concentration:　　1.0000

Sample Type:　　(Unknown)

KETOP#1 (253.3 / 209.0)

RT (Exp. RT):　2.52 (2.55) min

Calculated conc:　62.47

Area Ratio:　1.506e-2

Sample Type:　(Unknown)

KETOP#2 (253.3 / 197.0)

RT (Exp. RT):　2.53 (2.55) min

Calculated conc:　< 0

Area Ratio:　3.768e-3

Sample Type:　(Unknown)
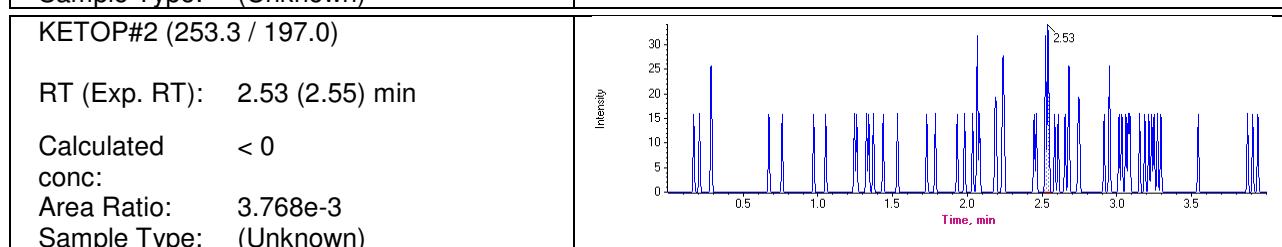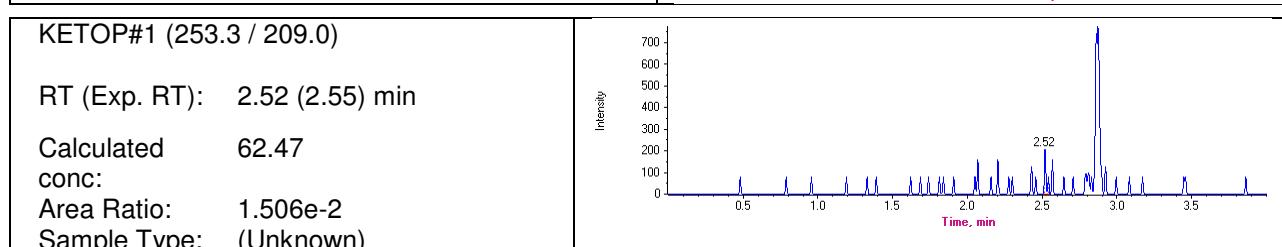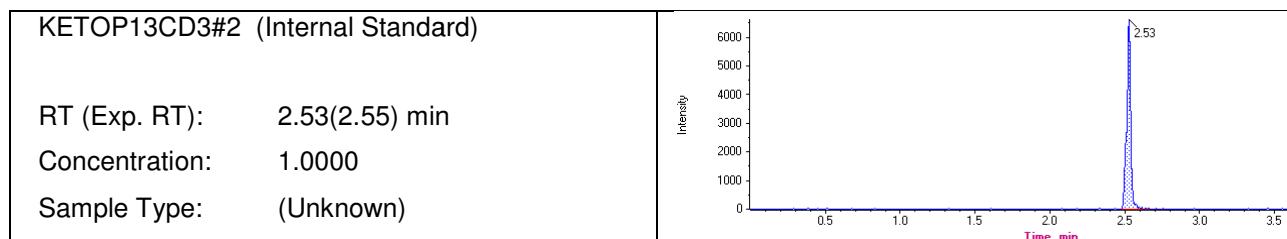
Sample Name	Sample 1309-066-002	Injection Vial	3
Sample ID	Reported 7409.1ng/mL	Injection Volume	10.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	26/11/2013 8:48:02 AM	Dilution Factor	2.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131125 Run 1 Ketoprofen		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP13CD3#2	9722.92	2.53	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP#1	1.326e+04	2.54	N/A	8148.05
KETOP#2	1.868e+05	2.54	N/A	7409.06

KETOP13CD3#2 (Internal Standard)

RT (Exp. RT):   2.53(2.55) min

Concentration: 1.0000

Sample Type: (Unknown)

KETOP#1 (253.3 / 209.0)

RT (Exp. RT):   2.54 (2.55) min

Calculated conc: 8148.05

Area Ratio: 1.364e0

Sample Type: (Unknown)

KETOP#2 (253.3 / 197.0)

RT (Exp. RT):   2.54 (2.55) min

Calculated conc: 7409.06

Area Ratio: 1.921e1

Sample Type: (Unknown)
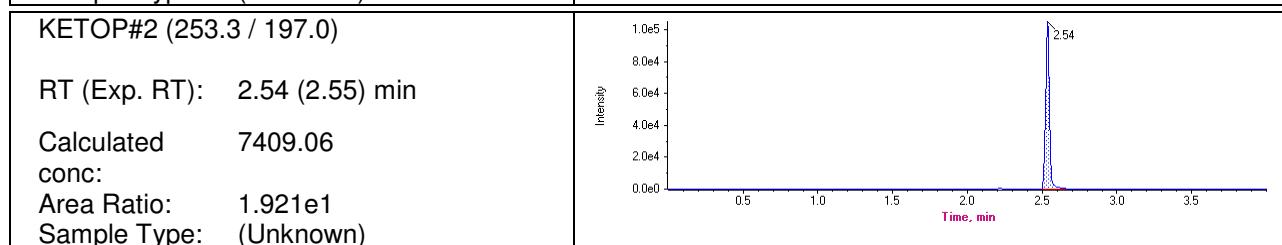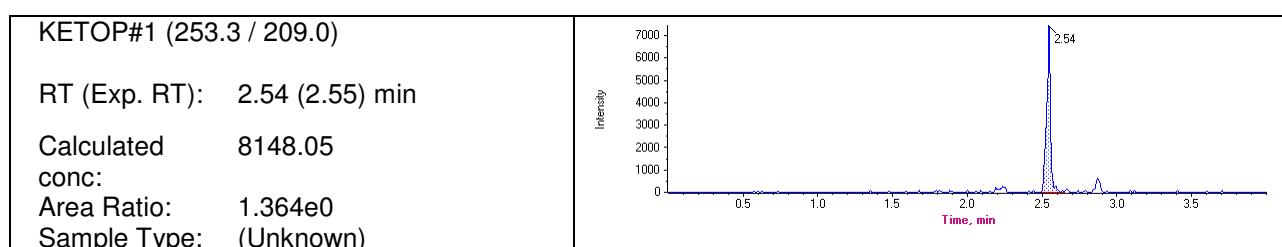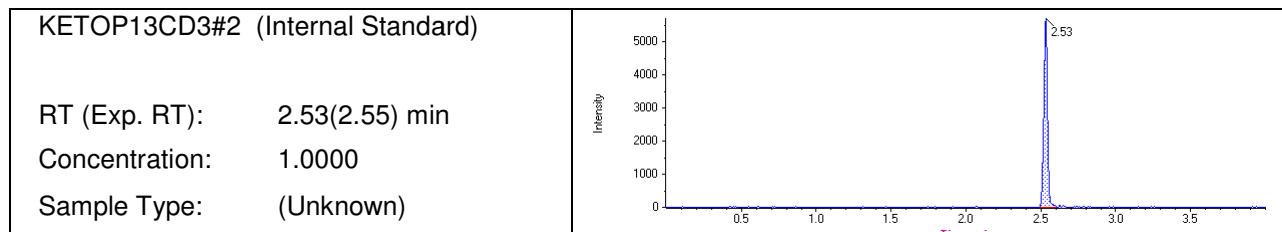
Sample Name	Sample 1309-066-047	Injection Vial	56
Sample ID	Reported <10ng/mL	Injection Volume	10.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	26/11/2013 12:46:25 PM	Dilution Factor	2.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131125 Run 1 Ketoprofen		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP13CD3#2	12430.06	2.52	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP#1	2.763e+02	2.63	N/A	105.43
KETOP#2	3.687e+02	2.53	N/A	7.90

KETOP13CD3#2 (Internal Standard)

RT (Exp. RT):   2.52(2.55) min

Concentration: 1.0000

Sample Type: (Unknown)


KETOP#1 (253.3 / 209.0)

RT (Exp. RT):   2.63 (2.55) min

Calculated conc: 105.43

Area Ratio: 2.223e-2

Sample Type: (Unknown)

KETOP#2 (253.3 / 197.0)

RT (Exp. RT):   2.53 (2.55) min

Calculated conc: 7.90

Area Ratio: 2.966e-2

Sample Type: (Unknown)
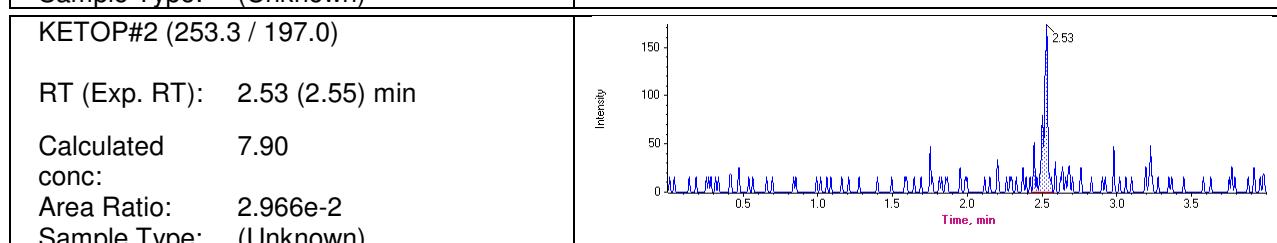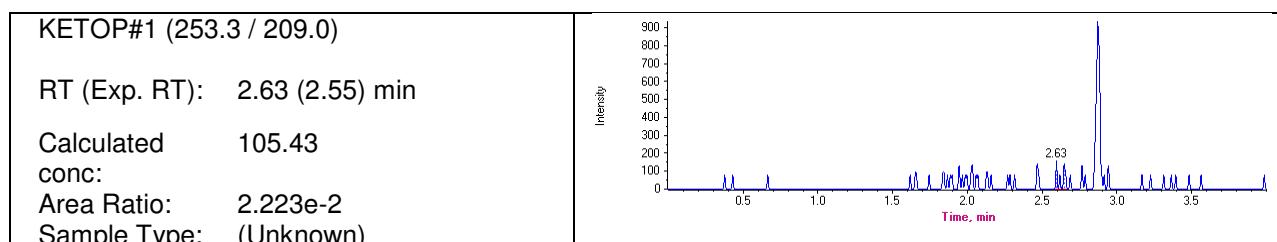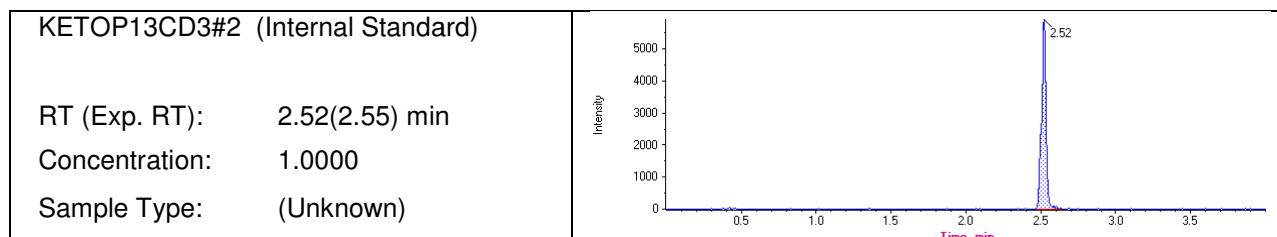
Sample Name	Sample 1309-066-048	Injection Vial	57
Sample ID	Reported 4737.6ng/mL	Injection Volume	10.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	26/11/2013 12:50:55 PM	Dilution Factor	2.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131125 Run 1 Ketoprofen		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP13CD3#2	10586.79	2.52	1.0000	-
FLUNIXD3#1	498684.42	2.64	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
KETOP#1	8.524e+03	2.52	N/A	4798.74
KETOP#2	1.301e+05	2.52	N/A	4737.64

KETOP13CD3#2 (Internal Standard)

RT (Exp. RT):   2.52(2.55) min

Concentration: 1.0000

Sample Type: (Unknown)

KETOP#1 (253.3 / 209.0)

RT (Exp. RT):   2.52 (2.55) min

Calculated conc: 4798.74

Area Ratio: 8.052e-1

Sample Type: (Unknown)

KETOP#2 (253.3 / 197.0)

RT (Exp. RT):   2.52 (2.55) min

Calculated conc: 4737.64

Area Ratio: 1.229e1

Sample Type: (Unknown)
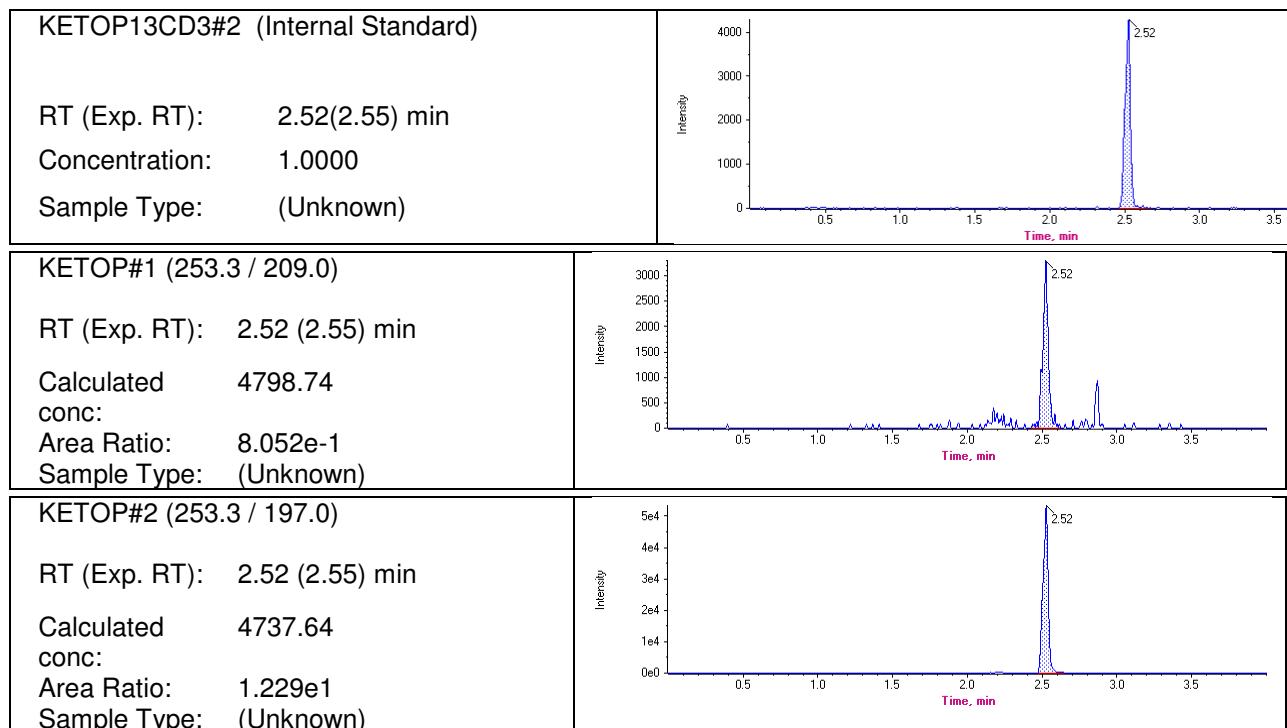
Flunixin/ 5-hydroxyflunixin run Chromatograms

Sample Name	Reagent blank		Injection Vial	47
Sample ID			Injection Volume	5.00
Sample Type	Unknown		Algorithm Used	MQ4
Acquisition Date	30/11/2013 3:49:48 AM		Dilution Factor	1.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	562.57	2.74	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	5.890e+02	2.76	N/A	degenerate
FLUNIX#2	N/A	N/A	N/A	N/A
5OH-FLUNIX#2	9.211e+01	2.50	N/A	degenerate

FLUNIXD3#1 (Internal Standard)
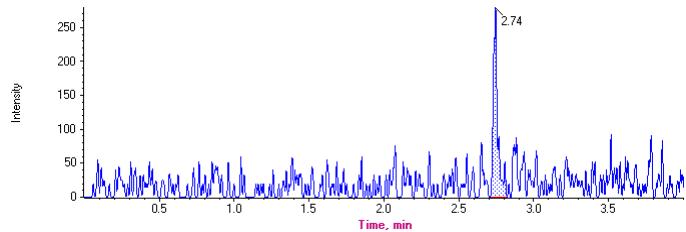
RT (Exp. RT): 2.74(2.68) min

Concentration: 1.0000

Sample Type: (Unknown)

FLUNIX#1 (295.1 / 251.1)
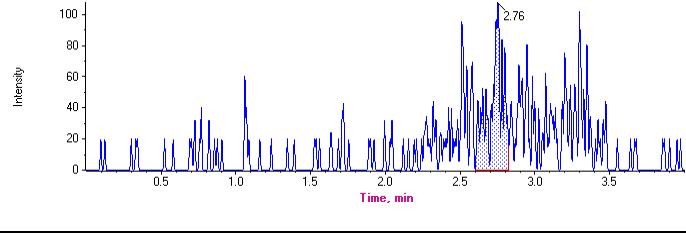
RT (Exp. RT): 2.76 (2.68) min

Calculated conc: degenerate

Area Ratio: 1.047e0

Sample Type: (Unknown)
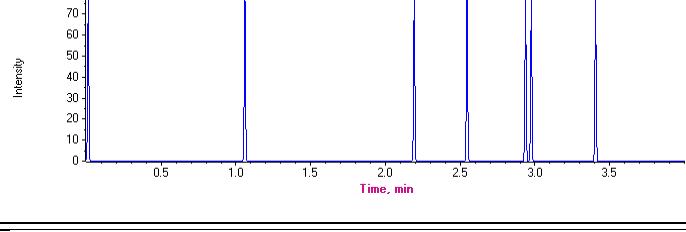
FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT): N/A (2.68) min

Calculated conc: N/A

Area Ratio: N/A

Sample Type: (Unknown)

5OH-FLUNIX#2 (311.1 / 227.1)
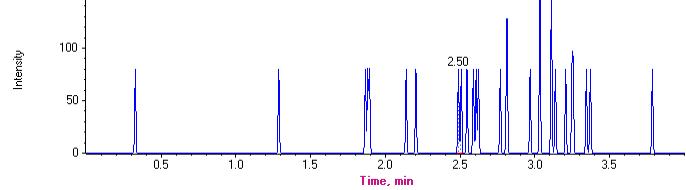
RT (Exp. RT): 2.50 (2.47) min

Calculated conc: degenerate

Area Ratio: 1.637e-1

Sample Type: (Unknown)

Sample Name	Blank plasma		Injection Vial	11
Sample ID			Injection Volume	5.00
Sample Type	Unknown		Algorithm Used	MQ4
Acquisition Date	30/11/2013 3:58:46 AM		Dilution Factor	1.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	229.63	2.74	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	5.199e+02	2.71	N/A	degenerate
FLUNIX#2	4.603e+01	2.71	N/A	103.5
5OH-FLUNIX#2	4.607e+01	2.40	N/A	degenerate

FLUNIXD3#1 (Internal Standard)

RT (Exp. RT):   2.74(2.68) min

Concentration: 1.0000

Sample Type: (Unknown)

FLUNIX#1 (295.1 / 251.1)

RT (Exp. RT):   2.71 (2.68) min

Calculated conc: degenerate

Area Ratio: 2.264e0

Sample Type: (Unknown)

FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT):   2.71 (2.68) min

Calculated conc: 103.5

Area Ratio: 2.005e-1

Sample Type: (Unknown)

5OH-FLUNIX#2 (311.1 / 227.1)

RT (Exp. RT):   2.40 (2.47) min

Calculated conc: degenerate

Area Ratio: 2.006e-1

Sample Type: (Unknown)

Sample Name	Blank plasma with internal standard	Injection Vial	12
Sample ID		Injection Volume	5.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	30/11/2013 4:07:48 AM	Dilution Factor	1.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	250494.46	2.72	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	3.456e+02	2.68	N/A	degenerate
FLUNIX#2	4.601e+01	2.71	N/A	< 0
5OH-FLUNIX#2	9.219e+01	2.55	N/A	degenerate

FLUNIXD3#1 (Internal Standard)
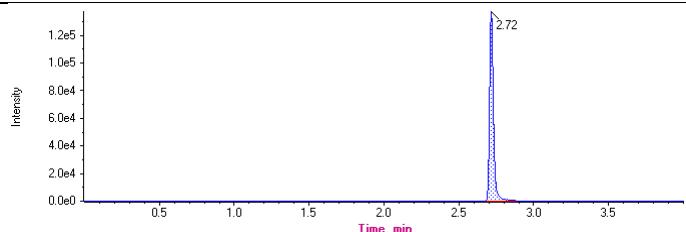
RT (Exp. RT):   2.72(2.68) min

Concentration: 1.0000

Sample Type: (Unknown)

FLUNIX#1 (295.1 / 251.1)

RT (Exp. RT):   2.68 (2.68) min

Calculated conc: degenerate

Area Ratio: 1.380e-3
Sample Type: (Unknown)

FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT):   2.71 (2.68) min

Calculated conc: < 0

Area Ratio: 1.837e-4
Sample Type: (Unknown)

5OH-FLUNIX#2 (311.1 / 227.1)

RT (Exp. RT):   2.55 (2.47) min

Calculated conc: degenerate

Area Ratio: 3.680e-4
Sample Type: (Unknown)
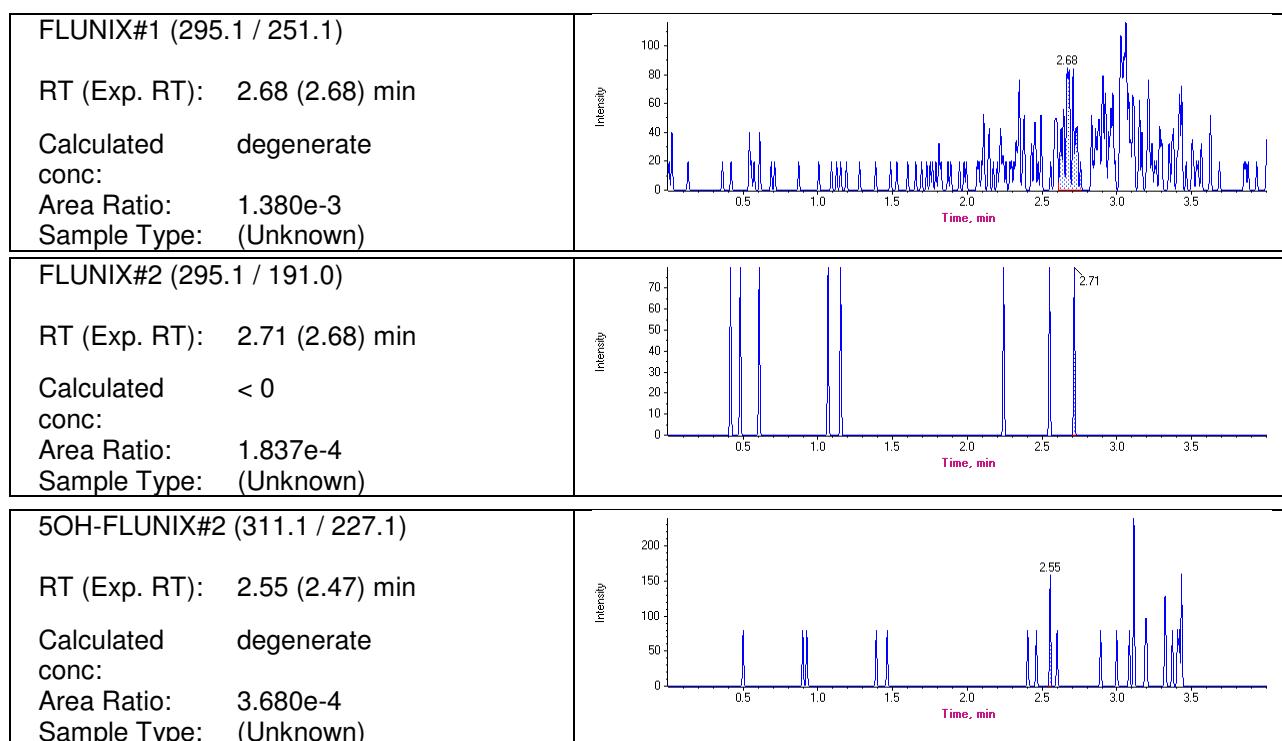
Sample Name	Std 7 (798.11ng/mL flunixin)	Injection Vial	7
Sample ID		Injection Volume	5.00
Sample Type	Standard	Algorithm Used	MQ4
Acquisition Date	30/11/2013 5:55:43 AM	Dilution Factor	1.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	222760.53	2.71	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	9.964e+05	2.72	0.0000	degenerate
FLUNIX#2	3.350e+05	2.72	798.1100	781.0
5OH-FLUNIX#2	1.382e+02	2.45	0.0000	degenerate

FLUNIXD3#1 (Internal Standard)

RT (Exp. RT):   2.71(2.68) min

Concentration: 1.0000

Sample Type: (Standard)

FLUNIX#1 (295.1 / 251.1)

RT (Exp. RT):   2.72 (2.68) min

Calculated conc: degenerate

Area Ratio: 4.473e0

Sample Type: (Standard)

FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT):   2.72 (2.68) min

Calculated conc: 781.0

Area Ratio: 1.504e0

Sample Type: (Standard)

5OH-FLUNIX#2 (311.1 / 227.1)

RT (Exp. RT):   2.45 (2.47) min

Calculated conc: degenerate

Area Ratio: 6.205e-4

Sample Type: (Standard)

Sample Name	Fort 4.1 (9976.35ng/mL flunixin)	Injection Vial	85
Sample ID		Injection Volume	5.00
Sample Type	Quality Control	Algorithm Used	MQ4
Acquisition Date	30/11/2013 9:54:08 AM	Dilution Factor	10.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	186005.33	2.70	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	1.054e+06	2.70	9976.3500	degenerate
FLUNIX#2	3.516e+05	2.70	9976.3500	9817.5
5OH-FLUNIX#1	3.096e+02	2.42	0.0000	degenerate
5OH-FLUNIX#2	4.607e+01	2.43	0.0000	degenerate

FLUNIXD3#1 (Internal Standard)

RT (Exp. RT):   2.70(2.68) min

Concentration: 1.0000

Sample Type: (Quality Control)
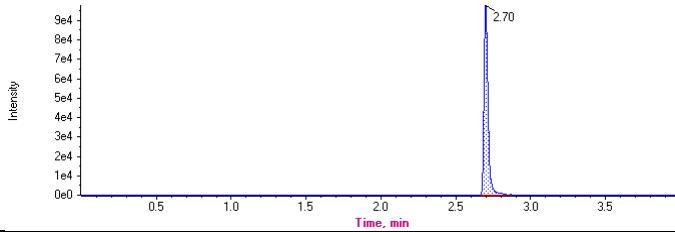
FLUNIX#1 (295.1 / 251.1)

RT (Exp. RT):   2.70 (2.68) min

Calculated   degenerate
conc:
Area Ratio: 5.666e0
Sample Type: (Quality Control)
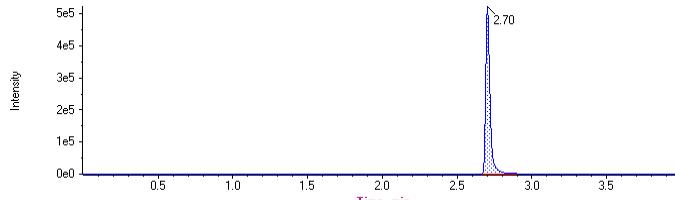
FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT):   2.70 (2.68) min

Calculated   9817.5
conc:
Area Ratio: 1.890e0
Sample Type: (Quality Control)
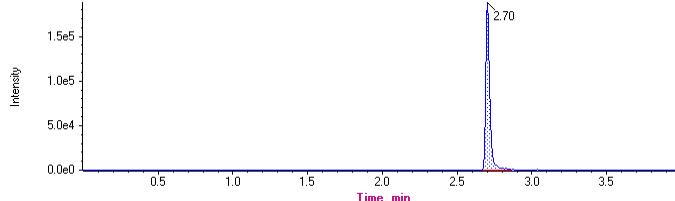
5OH-FLUNIX#2 (311.1 / 227.1)

RT (Exp. RT):   2.43 (2.47) min

Calculated   degenerate
conc:
Area Ratio: 2.477e-4
Sample Type: (Quality Control)
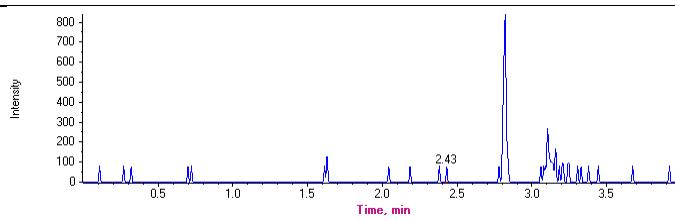
Sample Name	Sample 1309-066-154	Injection Vial	89
Sample ID	Reported 3258.6ng/mL flunixin	Injection Volume	5.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	30/11/2013 2:05:54 PM	Dilution Factor	10.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	186374.81	2.69	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	2.865e+05	2.70	N/A	degenerate
FLUNIX#2	1.171e+05	2.70	N/A	3258.6
5OH-FLUNIX#2	1.704e+03	2.55	N/A	degenerate

FLUNIXD3#1 (Internal Standard)

RT (Exp. RT):   2.69(2.68) min

Concentration: 1.0000

Sample Type: (Unknown)

FLUNIX#1 (295.1 / 251.1)

RT (Exp. RT):   2.70 (2.68) min

Calculated   degenerate
conc:
Area Ratio: 1.537e0
Sample Type: (Unknown)

FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT):   2.70 (2.68) min

Calculated   3258.6
conc:
Area Ratio: 6.283e-1
Sample Type: (Unknown)

5OH-FLUNIX#2 (311.1 / 227.1)

RT (Exp. RT):   2.55 (2.47) min

Calculated   degenerate
conc:
Area Ratio: 9.144e-3
Sample Type: (Unknown)

Sample Name	Sample 1309-066-155	Injection Vial	90
Sample ID	Reported 41.0ng/mL flunixin	Injection Volume	5.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	30/11/2013 2:10:23 PM	Dilution Factor	10.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	197541.16	2.71	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	7.331e+03	2.71	N/A	degenerate
FLUNIX#2	1.797e+03	2.71	N/A	41.0
5OH-FLUNIX#2	9.202e+01	2.40	N/A	degenerate

FLUNIXD3#1 (Internal Standard)

RT (Exp. RT):   2.71(2.68) min

Concentration: 1.0000

Sample Type: (Unknown)

FLUNIX#1 (295.1 / 251.1)

RT (Exp. RT):   2.71 (2.68) min

Calculated   degenerate
conc:
Area Ratio: 3.711e-2
Sample Type: (Unknown)

FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT):   2.71 (2.68) min

Calculated   41.0
conc:
Area Ratio: 9.098e-3
Sample Type: (Unknown)

5OH-FLUNIX#2 (311.1 / 227.1)

RT (Exp. RT):   2.40 (2.47) min

Calculated   degenerate
conc:
Area Ratio: 4.658e-4
Sample Type: (Unknown)

Sample Name	5OH STD 13 (20.0ng/mL 5-OH Flunixin)	Injection Vial	15
Sample ID		Injection Volume	5.00
Sample Type	Standard	Algorithm Used	MQ4
Acquisition Date	30/11/2013 6:49:39 AM	Dilution Factor	1.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	256974.57	2.71	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	8.715e+02	2.72	0.0000	degenerate
FLUNIX#2	2.765e+02	2.72	0.0000	degenerate
5OH-FLUNIX#2	2.625e+03	2.57	20.0000	20.1

FLUNIXD3#1 (Internal Standard)

RT (Exp. RT):   2.71(2.68) min

Concentration: 1.0000

Sample Type: (Standard)

FLUNIX#1 (295.1 / 251.1)

RT (Exp. RT):   2.72 (2.68) min

Calculated   degenerate
conc:
Area Ratio: 3.391e-3
Sample Type: (Standard)

FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT):   2.72 (2.68) min

Calculated   degenerate
conc:
Area Ratio: 1.076e-3
Sample Type: (Standard)

5OH-FLUNIX#2 (311.1 / 227.1)

RT (Exp. RT):   2.57 (2.57) min

Calculated   20.1
conc:
Area Ratio: 1.021e-2
Sample Type: (Standard)

Sample Name	Fort 5.3 (20ng/mL5-OH Flunixin)	Injection Vial	31
Sample ID		Injection Volume	5.00
Sample Type	Quality Control	Algorithm Used	MQ4
Acquisition Date	30/11/2013 10:30:08 AM	Dilution Factor	10.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	221928.04	2.69	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	4.707e+02	2.70	0.0000	degenerate
FLUNIX#2	N/A	N/A	0.0000	N/A
5OH-FLUNIX#2	2.303e+02	2.55	20.0000	18.8

FLUNIXD3#1 (Internal Standard)	
RT (Exp. RT): 2.69(2.68) min	
Concentration: 1.0000	
Sample Type: (Quality Control)	

FLUNIX#1 (295.1 / 251.1)	
RT (Exp. RT): 2.70 (2.68) min	
Calculated conc: degenerate	
Area Ratio: 2.121e-3	
Sample Type: (Quality Control)	

FLUNIX#2 (295.1 / 191.0)	
RT (Exp. RT): N/A (2.68) min	
Calculated conc: N/A	
Area Ratio: N/A	
Sample Type: (Quality Control)	

5OH-FLUNIX#2 (311.1 / 227.1)	
RT (Exp. RT): 2.55 (2.57) min	
Calculated conc: 18.8	
Area Ratio: 1.038e-3	
Sample Type: (Quality Control)	

Sample Name	Sample 1309-066-139	Injection Vial	70
Sample ID	Reported <20ng/mL	Injection Volume	5.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	30/11/2013 12:31:32 PM	Dilution Factor	10.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	200366.61	2.71	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	7.011e+03	2.71	N/A	degenerate
FLUNIX#2	1.797e+03	2.71	N/A	degenerate
5OH-FLUNIX#2	9.213e+01	2.65	N/A	7.4

FLUNIXD3#1 (Internal Standard)

RT (Exp. RT):   2.71(2.68) min

Concentration: 1.0000

Sample Type: (Unknown)

FLUNIX#1 (295.1 / 251.1)

RT (Exp. RT):   2.71 (2.68) min

Calculated   degenerate
conc:
Area Ratio: 3.499e-2
Sample Type: (Unknown)

FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT):   2.71 (2.68) min

Calculated   degenerate
conc:
Area Ratio: 8.967e-3
Sample Type: (Unknown)

5OH-FLUNIX#2 (311.1 / 227.1)

RT (Exp. RT):   2.65 (2.57) min

Calculated   7.4
conc:
Area Ratio: 4.598e-4
Sample Type: (Unknown)

Sample Name	Sample 1309-066-140	Injection Vial	71
Sample ID	Reported 128.6ng/mL 5-OH Flunixin	Injection Volume	5.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	30/11/2013 12:36:02 PM	Dilution Factor	10.00

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIXD3#1	174901.16	2.71	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
FLUNIX#1	3.049e+05	2.71	N/A	degenerate
FLUNIX#2	9.682e+04	2.71	N/A	degenerate
5OH-FLUNIX#2	1.151e+03	2.57	N/A	128.6

FLUNIXD3#1 (Internal Standard)

RT (Exp. RT):   2.71(2.68) min

Concentration: 1.0000

Sample Type: (Unknown)

FLUNIX#1 (295.1 / 251.1)

RT (Exp. RT):   2.71 (2.68) min

Calculated conc: degenerate

Area Ratio: 1.743e0

Sample Type: (Unknown)

FLUNIX#2 (295.1 / 191.0)

RT (Exp. RT):   2.71 (2.68) min

Calculated conc: degenerate

Area Ratio: 5.535e-1

Sample Type: (Unknown)

5OH-FLUNIX#2 (311.1 / 227.1)

RT (Exp. RT):   2.57 (2.57) min

Calculated conc: 128.6

Area Ratio: 6.582e-3
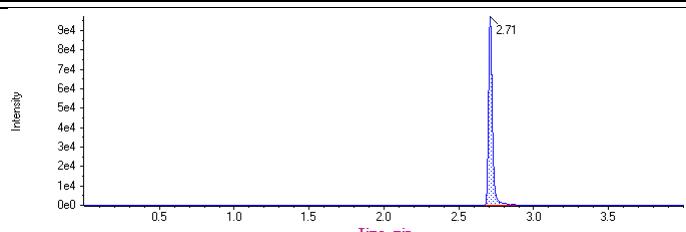
Sample Type: (Unknown)

Carprofen run Chromatograms (LCMSMS)

Sample Name	Blank plasma	**Injection Vial**	59
Sample ID		**Injection Volume**	10.00
Sample Type	Unknown	**Algorithm Used**	MQ4
Acquisition Date	28/11/2013 4:17:33 AM	**Dilution Factor**	1.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	**Weight to Volume**	N/A
Project	1309-066 CSIRO NSAID LCMSMS	**Instrument Name**	API 3200
Data File	DataSET1.wiff		
Result Table	131127 Run 2 Saline group		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARPD3#1	103.65	2.81	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARP#1	4.603e+01	2.75	N/A	133.0
CARP#2	7.398e+01	2.87	N/A	299.1

CARPD3#1 (Internal Standard)

RT (Exp. RT):   2.81(2.80) min

Concentration: 1.0000

Sample Type: (Unknown)

CARP#1 (272.1 / 228.0)

RT (Exp. RT):   2.75 (2.80) min

Calculated conc: 133.0

Area Ratio: 4.441e-1

Sample Type: (Unknown)

CARP#2 (272.1 / 226.0)

RT (Exp. RT):   2.87 (2.80) min

Calculated conc: 299.1

Area Ratio: 7.137e-1

Sample Type: (Unknown)
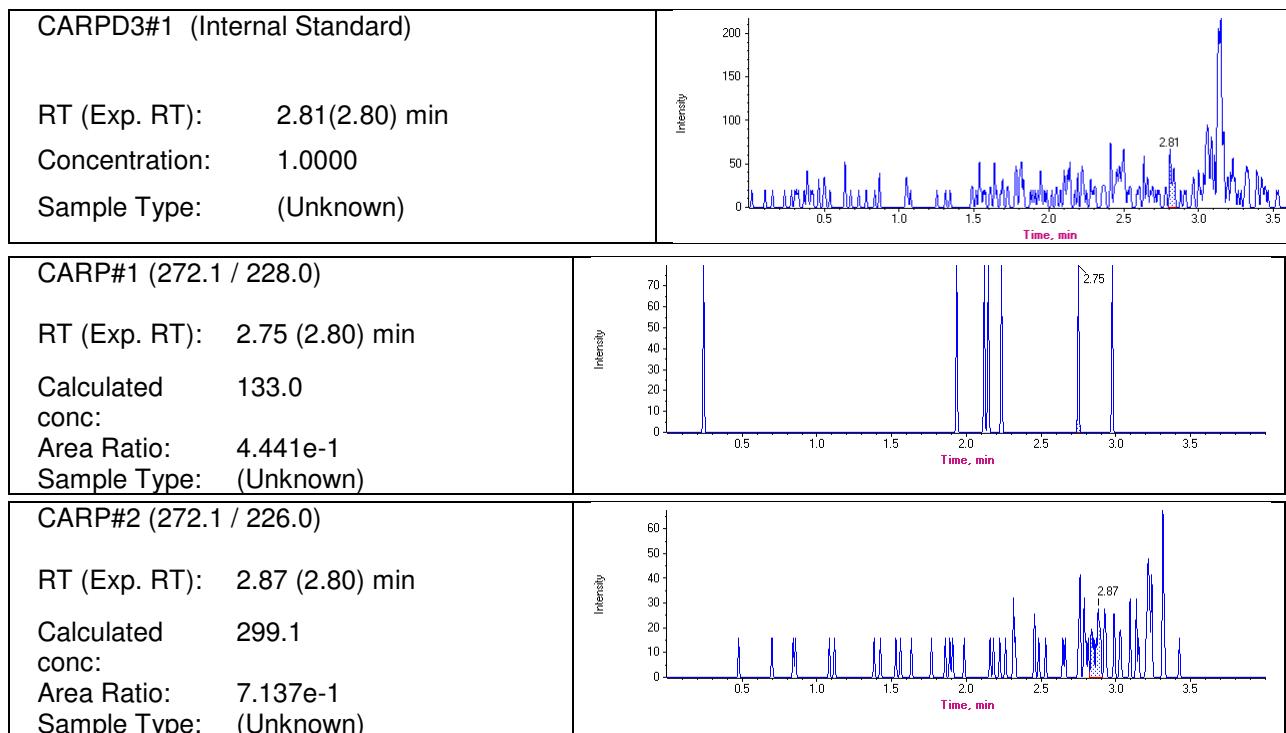
Sample Name	Blank plasma with internal std	Injection Vial	60
Sample ID		Injection Volume	10.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	28/11/2013 4:22:03 AM	Dilution Factor	1.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131127 Run 2 Saline group		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARPD3#1	16825.61	2.81	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARP#1	9.216e+01	2.74	N/A	1.5
CARP#2	1.293e+02	2.88	N/A	2.4

CARPD3#1 (Internal Standard)

RT (Exp. RT):   2.81(2.80) min

Concentration: 1.0000

Sample Type: (Unknown)

CARP#1 (272.1 / 228.0)

RT (Exp. RT):   2.74 (2.80) min

Calculated conc: 1.5

Area Ratio: 5.477e-3

Sample Type: (Unknown)

CARP#2 (272.1 / 226.0)

RT (Exp. RT):   2.88 (2.80) min

Calculated conc: 2.4

Area Ratio: 7.681e-3

Sample Type: (Unknown)
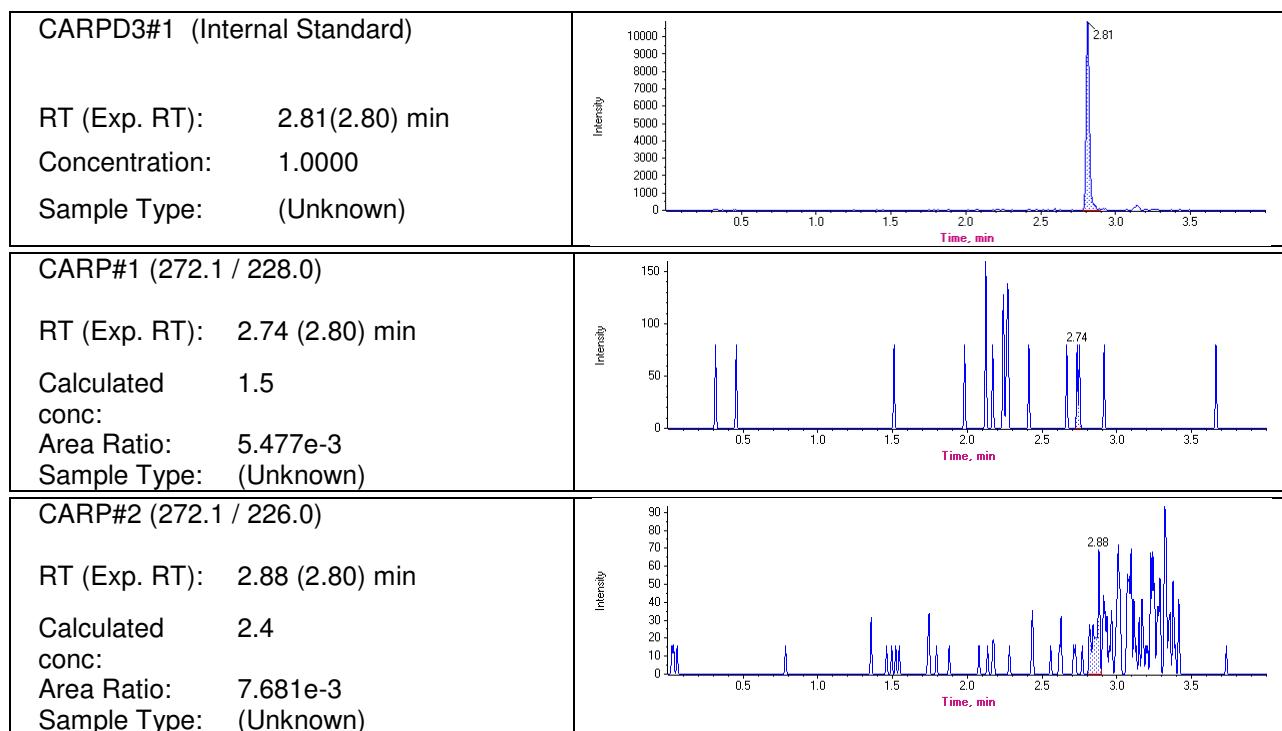
Sample Name	Std 7 (870.33ng/mL carprofen)	Injection Vial	55
Sample ID		Injection Volume	10.00
Sample Type	Standard	Algorithm Used	MQ4
Acquisition Date	28/11/2013 6:05:24 AM	Dilution Factor	1.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131127 Run 2 Saline group		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARPD3#1	36580.85	2.81	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARP#1	9.760e+04	2.81	870.3200	799.9
CARP#2	6.703e+04	2.81	870.3300	769.2

CARPD3#1 (Internal Standard)

RT (Exp. RT):   2.81(2.80) min

Concentration: 1.0000

Sample Type: (Standard)

CARP#1 (272.1 / 228.0)

RT (Exp. RT):   2.81 (2.80) min

Calculated conc: 799.9

Area Ratio: 2.668e0

Sample Type: (Standard)

CARP#2 (272.1 / 226.0)

RT (Exp. RT):   2.81 (2.80) min

Calculated conc: 769.2

Area Ratio: 1.832e0

Sample Type: (Standard)
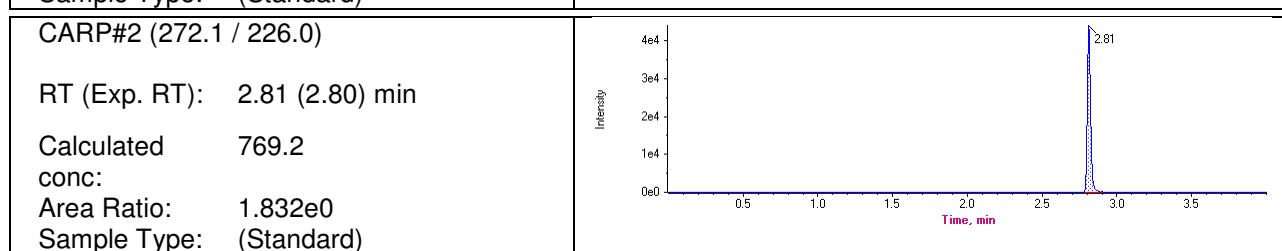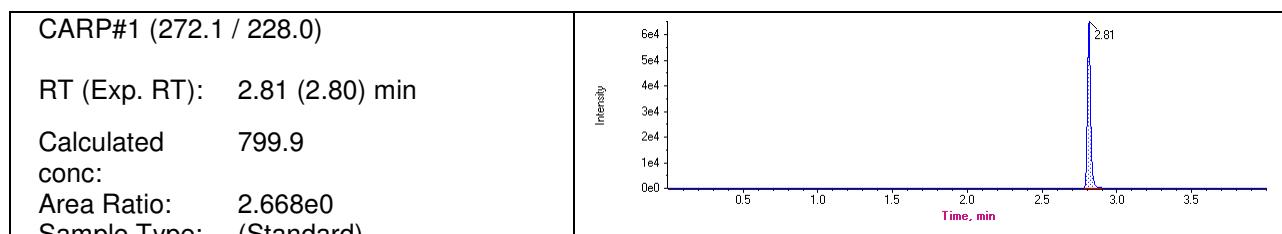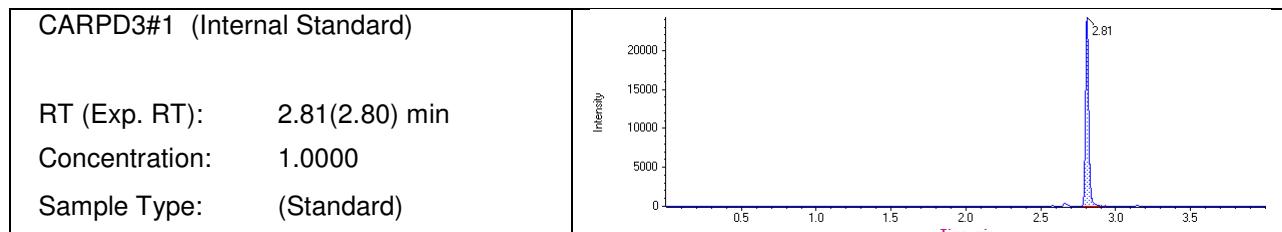
Sample Name	Spike 3C (435.2ng/mL carprofen)	Injection Vial	25
Sample ID		Injection Volume	10.00
Sample Type	Quality Control	Algorithm Used	MQ4
Acquisition Date	28/11/2013 8:02:16 AM	Dilution Factor	2.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131127 Run 2 Saline group		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARPD3#1	18973.14	2.81	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARP#1	1.248e+04	2.81	435.1600	394.2
CARP#2	1.020e+04	2.81	435.2000	450.1

CARPD3#1 (Internal Standard)

RT (Exp. RT):   2.81(2.80) min

Concentration: 1.0000

Sample Type: (Quality Control)

CARP#1 (272.1 / 228.0)

RT (Exp. RT):   2.81 (2.80) min

Calculated conc: 394.2

Area Ratio: 6.579e-1

Sample Type: (Quality Control)

CARP#2 (272.1 / 226.0)

RT (Exp. RT):   2.81 (2.80) min

Calculated conc: 450.1

Area Ratio: 5.375e-1

Sample Type: (Quality Control)
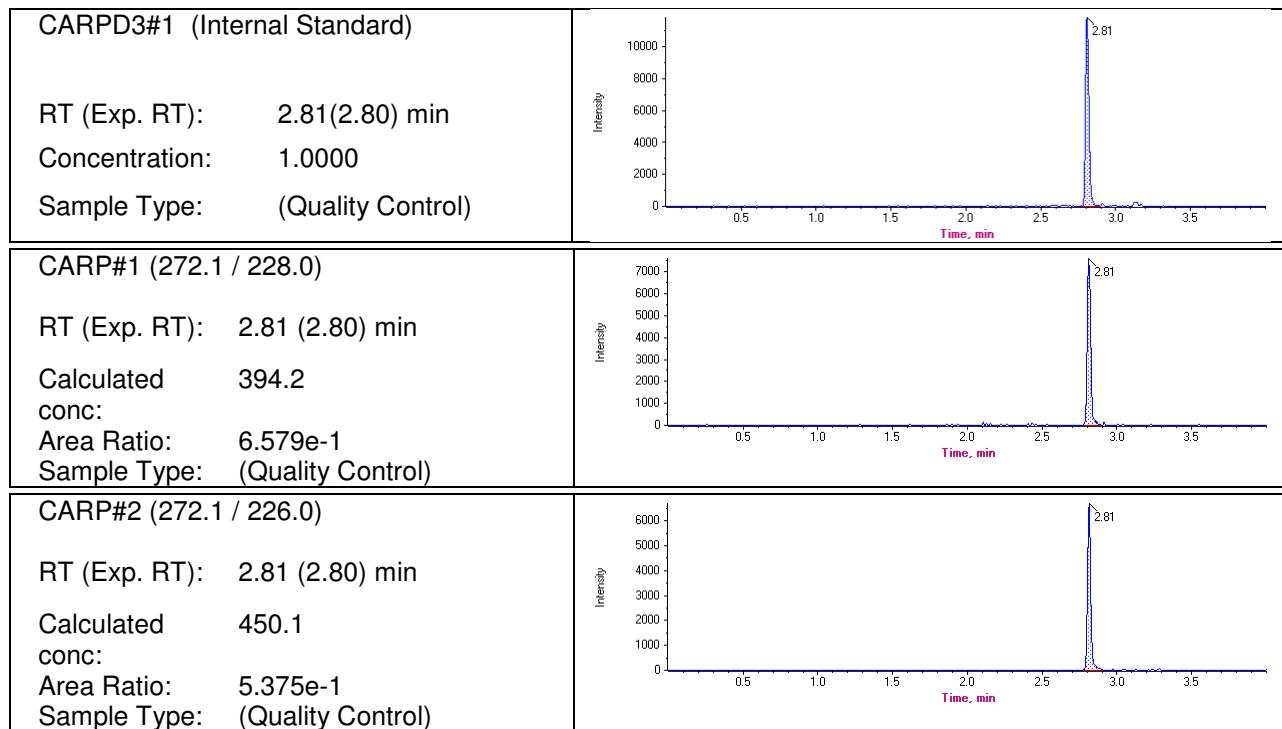
Sample Name	Sample1309-066-198	Injection Vial	21
Sample ID	Reported <20ng/mL	Injection Volume	10.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	28/11/2013 10:12:39 AM	Dilution Factor	2.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131127 Run 2 Saline group		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARPD3#1	6564.33	2.79	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARP#1	4.604e+01	2.85	N/A	3.8
CARP#2	9.238e+01	2.82	N/A	10.1

CARPD3#1 (Internal Standard)

RT (Exp. RT):   2.79(2.80) min

Concentration: 1.0000

Sample Type: (Unknown)

CARP#1 (272.1 / 228.0)

RT (Exp. RT):   2.85 (2.80) min

Calculated conc: 3.8

Area Ratio: 7.013e-3

Sample Type: (Unknown)

CARP#2 (272.1 / 226.0)

RT (Exp. RT):   2.82 (2.80) min

Calculated conc: 10.1

Area Ratio: 1.407e-2

Sample Type: (Unknown)
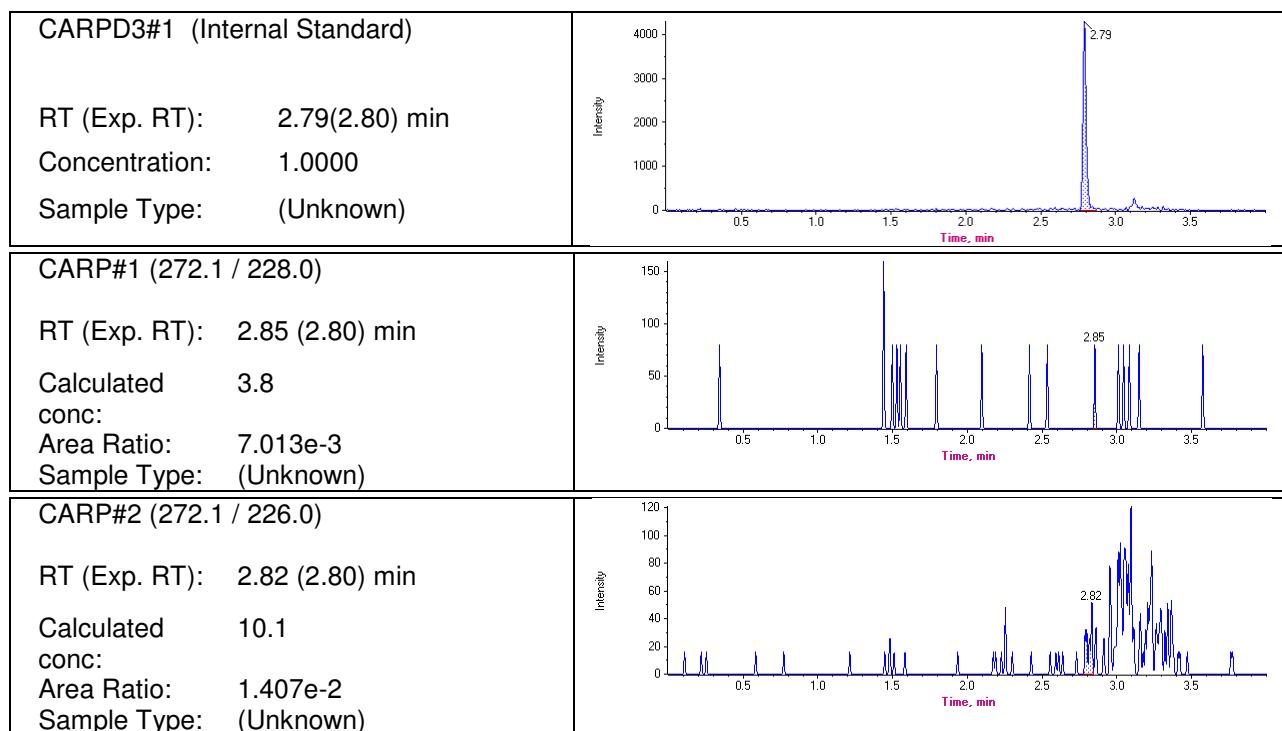
Sample Name	Sample 1309-066-199	Injection Vial	22
Sample ID	Reported <20ng/mL	Injection Volume	10.00
Sample Type	Unknown	Algorithm Used	MQ4
Acquisition Date	28/11/2013 10:17:08 AM	Dilution Factor	2.00
Acquisition Method	ATM018 NSAID LCMSMS.dam	Weight to Volume	N/A
Project	1309-066 CSIRO NSAID LCMSMS	Instrument Name	API 3200
Data File	DataSET1.wiff		
Result Table	131127 Run 2 Saline group		
Sample Comment			

Internal Standard	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARPD3#1	12954.90	2.80	1.0000	-

Target Analyte	Area (cps)	RT (min)	Target conc. ()	Calc. Conc. ()
CARP#1	4.601e+01	2.86	N/A	1.8
CARP#2	8.235e+01	2.85	N/A	3.6

CARPD3#1 (Internal Standard)

RT (Exp. RT):   2.80(2.80) min

Concentration: 1.0000

Sample Type: (Unknown)

CARP#1 (272.1 / 228.0)

RT (Exp. RT):   2.86 (2.80) min

Calculated conc: 1.8

Area Ratio: 3.552e-3

Sample Type: (Unknown)

CARP#2 (272.1 / 226.0)

RT (Exp. RT):   2.85 (2.80) min

Calculated conc: 3.6

Area Ratio: 6.357e-3

Sample Type: (Unknown)
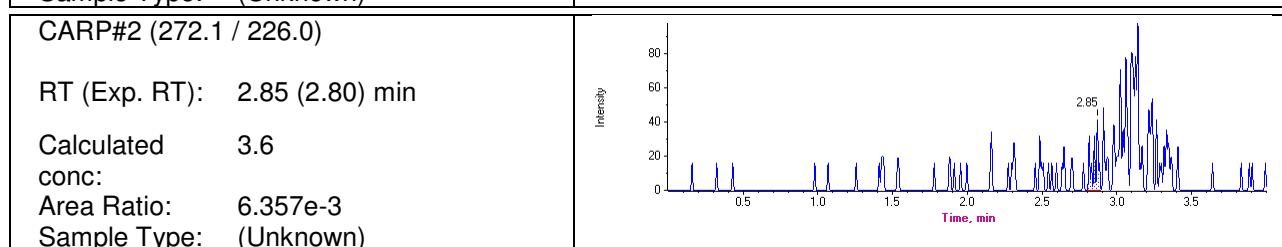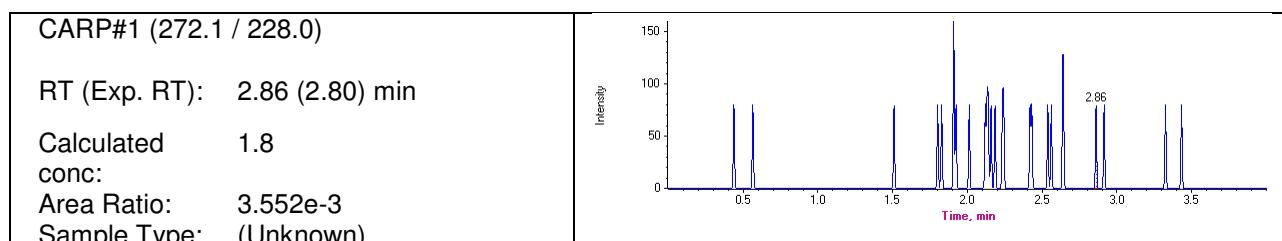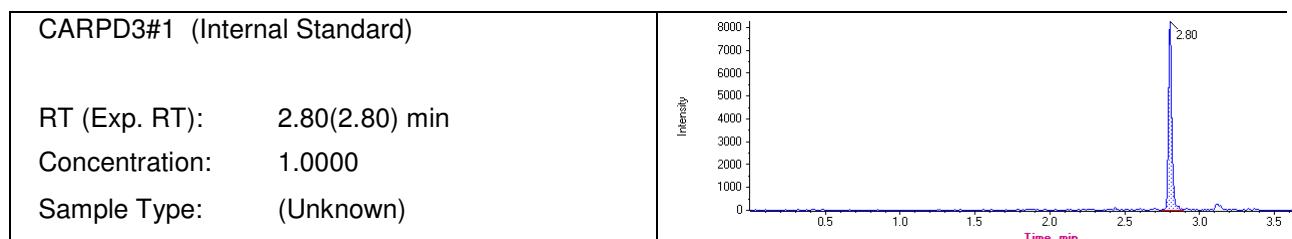
Chromatogram and Results

Injection Details

Injection Name:	**1309-066-0241 start plate 10**	*Run Time (min):*	**4.00**
Vial Number:	**GA1**	*Injection Volume:*	**2.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**240.0**
Instrument Method:	**ATM019 Carprofen instrument method**	*Bandwidth:*	**1**
Processing Method:	**ATM019 Carprofen processing method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**09/Dec/13 11:31**	*Sample Weight:*	**0.2500**
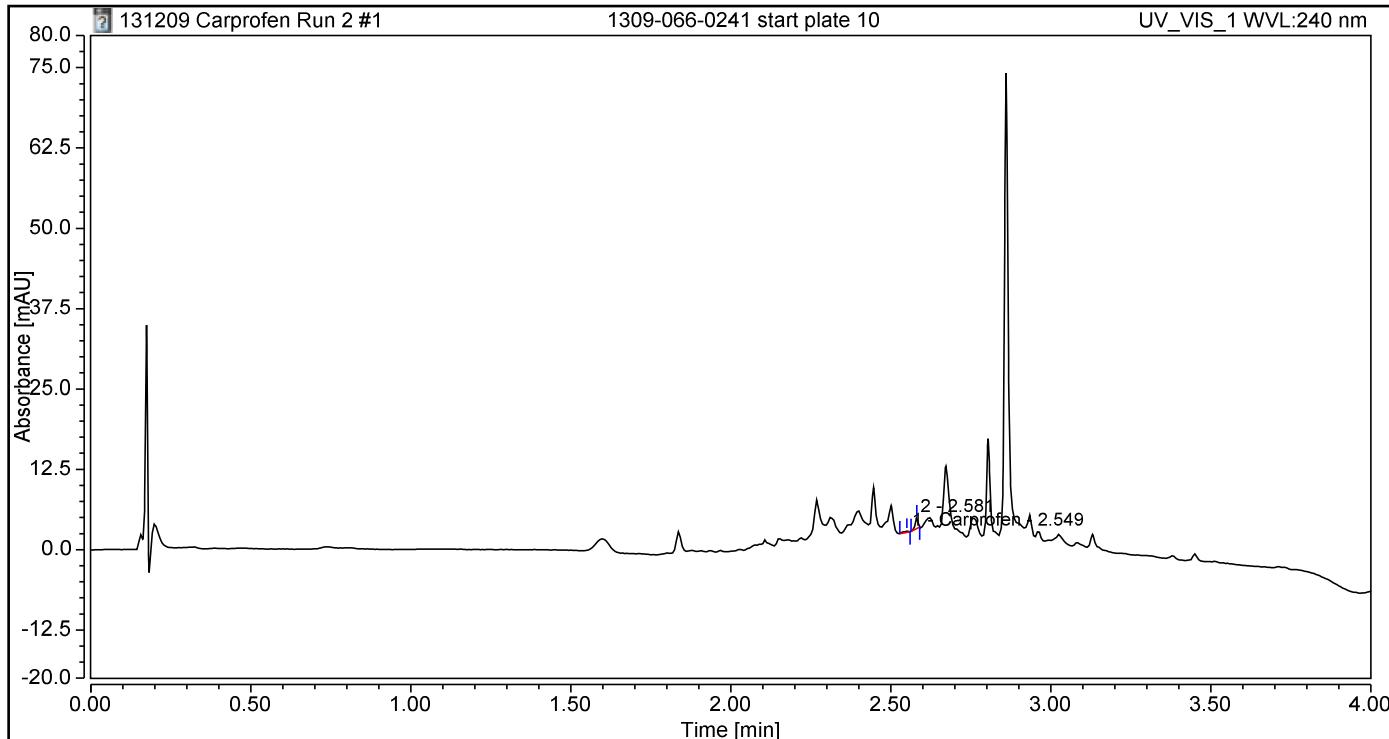
Chromatogram

131209 Carprofen Run 2 #1   1309-066-0241 start plate 10   UV_VIS_1 WVL:240 nm

Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	Carprofen	2.549	0.004	0.197	19.33	10.59	n.a.
2		2.581	0.017	1.664	80.67	89.41	n.a.
Total:			**0.021**	**1.860**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**Fort 6.4**	*Run Time (min):*	**4.00**
Vial Number:	**BH6**	*Injection Volume:*	**2.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**240.0**
Instrument Method:	**ATM019 Carprofen instrument method**	*Bandwidth:*	**1**
Processing Method:	**ATM019 Carprofen processing method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**10/Dec/13 01:27**	*Sample Weight:*	**0.2500**
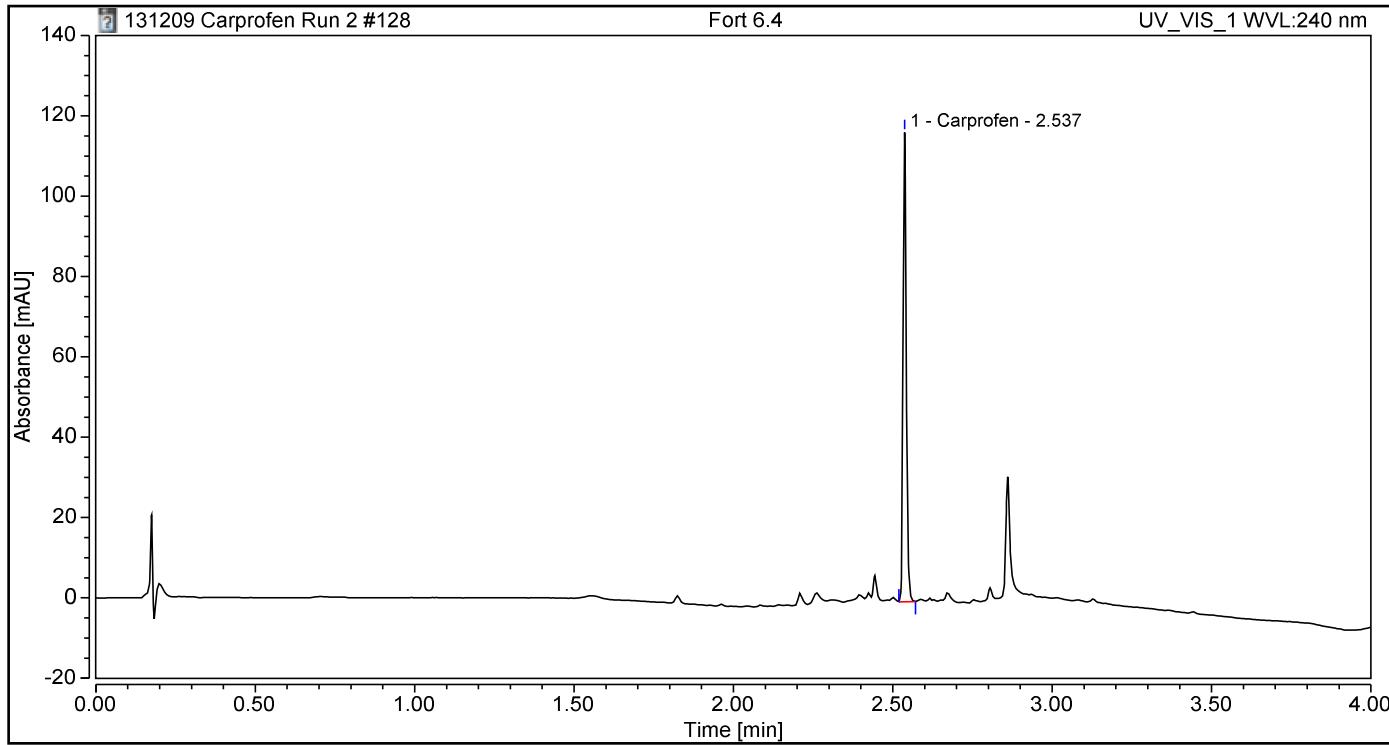
Chromatogram

131209 Carprofen Run 2 #128   Fort 6.4   UV_VIS_1 WVL:240 nm

Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	Carprofen	2.537	1.390	116.841	100.00	100.00	20.9076
Total:			**1.390**	**116.841**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**Fort 3.4**	*Run Time (min):*	**4.00**
Vial Number:	**BG9**	*Injection Volume:*	**2.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**240.0**
Instrument Method:	**ATM019 Carprofen instrument method**	*Bandwidth:*	**1**
Processing Method:	**ATM019 Carprofen processing method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**10/Dec/13 00:08**	*Sample Weight:*	**0.2500**
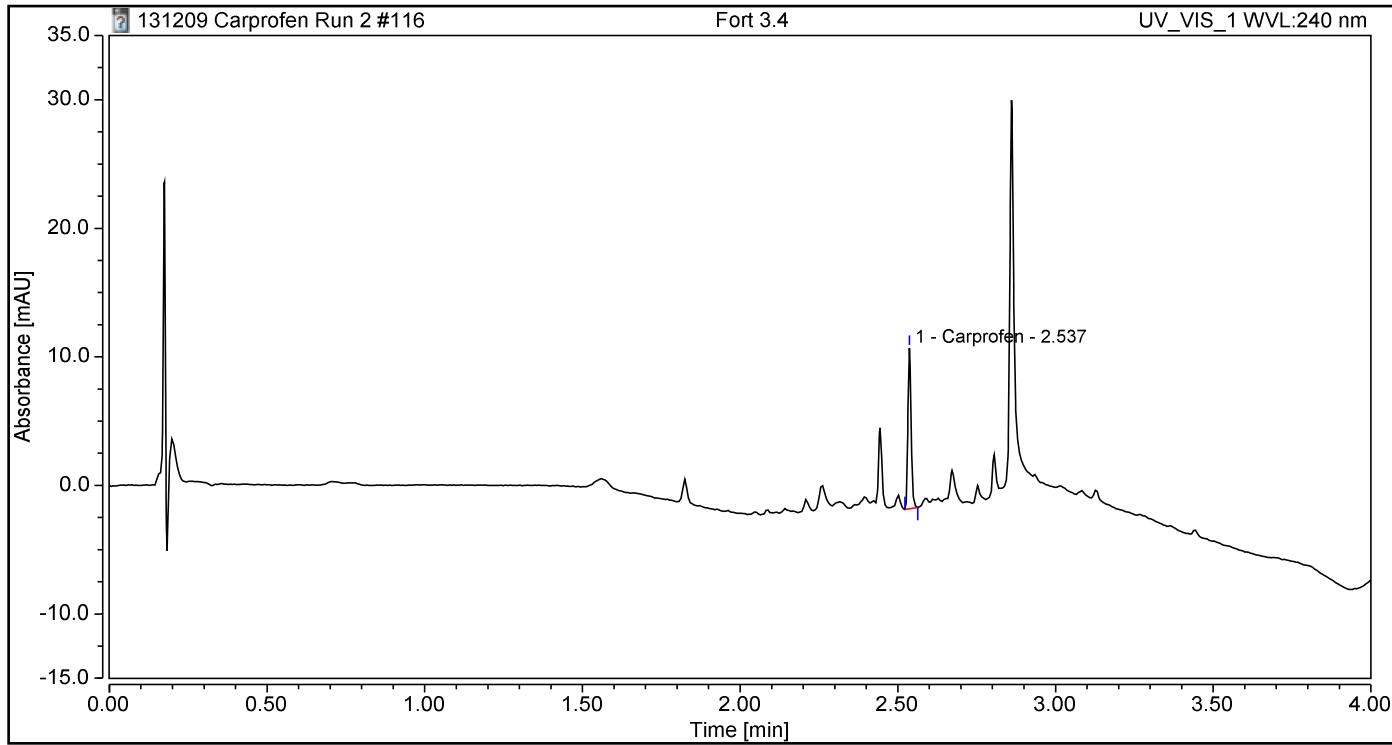
Chromatogram

Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	Carprofen	2.537	0.148	12.496	100.00	100.00	2.1551
Total:			**0.148**	**12.496**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**Std 10**	*Run Time (min):*	**4.00**
Vial Number:	**RA7**	*Injection Volume:*	**2.00**
Injection Type:	**Calibration Standard**	*Channel:*	**UV_VIS_1**
Calibration Level:	**07**	*Wavelength:*	**240.0**
Instrument Method:	**ATM019 Carprofen instrument method**	*Bandwidth:*	**1**
Processing Method:	**ATM019 Carprofen processing method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**09/Dec/13 21:50**	*Sample Weight:*	**1.0000**

Chromatogram
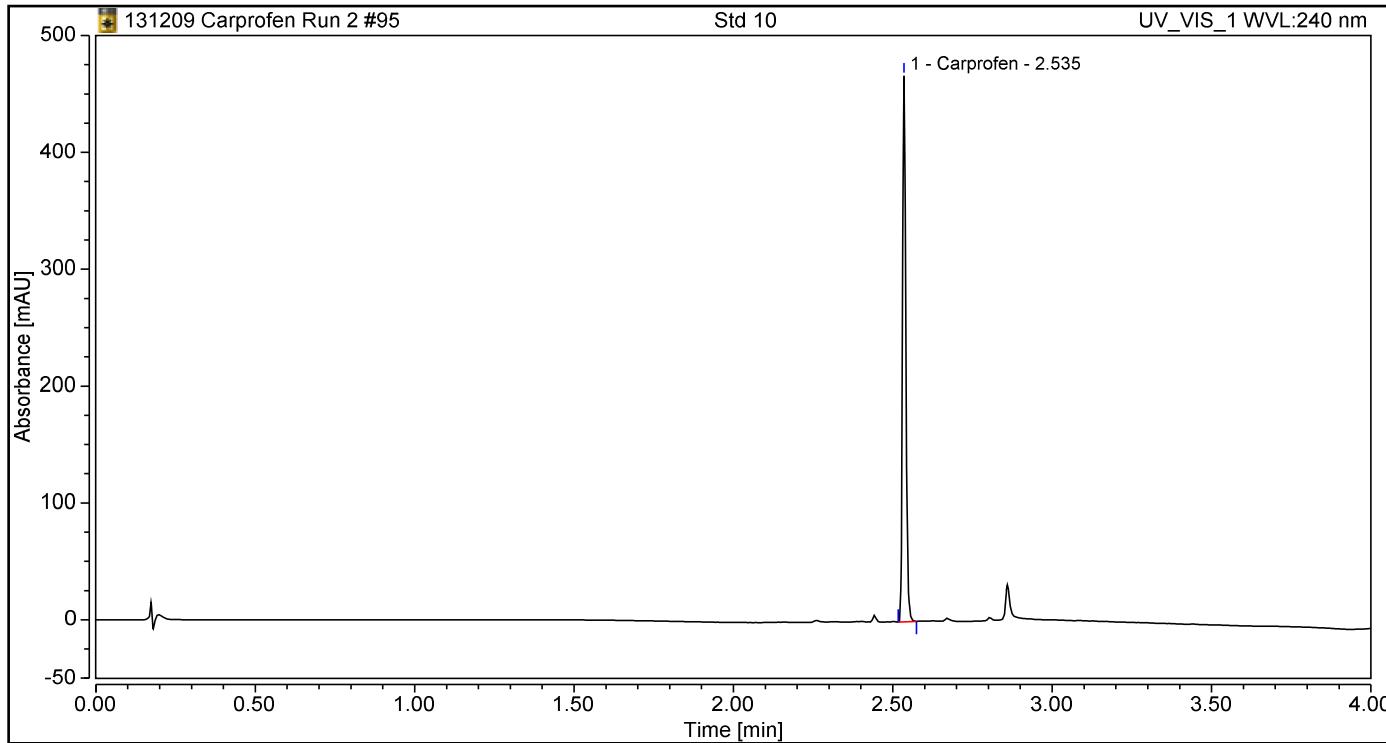
131209 Carprofen Run 2 #95   Std 10   UV_VIS_1 WVL:240 nm

1 - Carprofen - 2.535

Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	Carprofen	2.535	5.606	467.211	100.00	100.00	21.1444
Total:			**5.606**	**467.211**	**100.00**	**100.00**	

31/12/13

Chromatogram and Results

Injection Details

Injection Name:	**1309-066-245**	*Run Time (min):*	**4.00**
Vial Number:	**GA5**	*Injection Volume:*	**2.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**240.0**
Instrument Method:	**ATM019 Carprofen instrument method**	*Bandwidth:*	**1**
Processing Method:	**ATM019 Carprofen processing method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**09/Dec/13 11:57**	*Sample Weight:*	**0.2500**
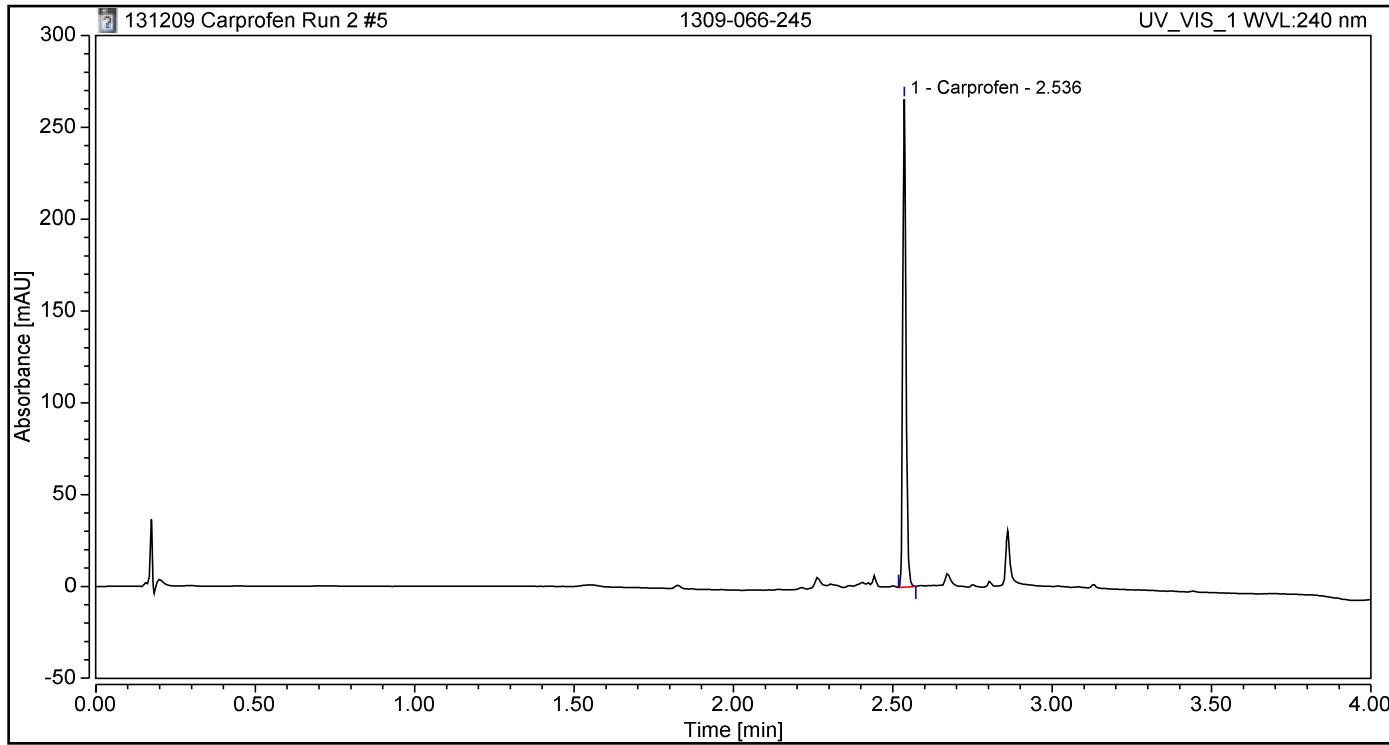
Chromatogram

131209 Carprofen Run 2 #5 — 1309-066-245 — UV_VIS_1 WVL:240 nm — 1 - Carprofen - 2.536

Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	Carprofen	2.536	3.110	265.594	100.00	100.00	46.8902
Total:			**3.110**	**265.594**	**100.00**	**100.00**	

Chromatogram and Results

Injection Details

Injection Name:	**1309-066-243**	*Run Time (min):*	**4.00**
Vial Number:	**GA3**	*Injection Volume:*	**2.00**
Injection Type:	**Unknown**	*Channel:*	**UV_VIS_1**
Calibration Level:		*Wavelength:*	**240.0**
Instrument Method:	**ATM019 Carprofen instrument method**	*Bandwidth:*	**1**
Processing Method:	**ATM019 Carprofen processing method**	*Dilution Factor:*	**1.0000**
Injection Date/Time:	**09/Dec/13 11:44**	*Sample Weight:*	**0.2500**
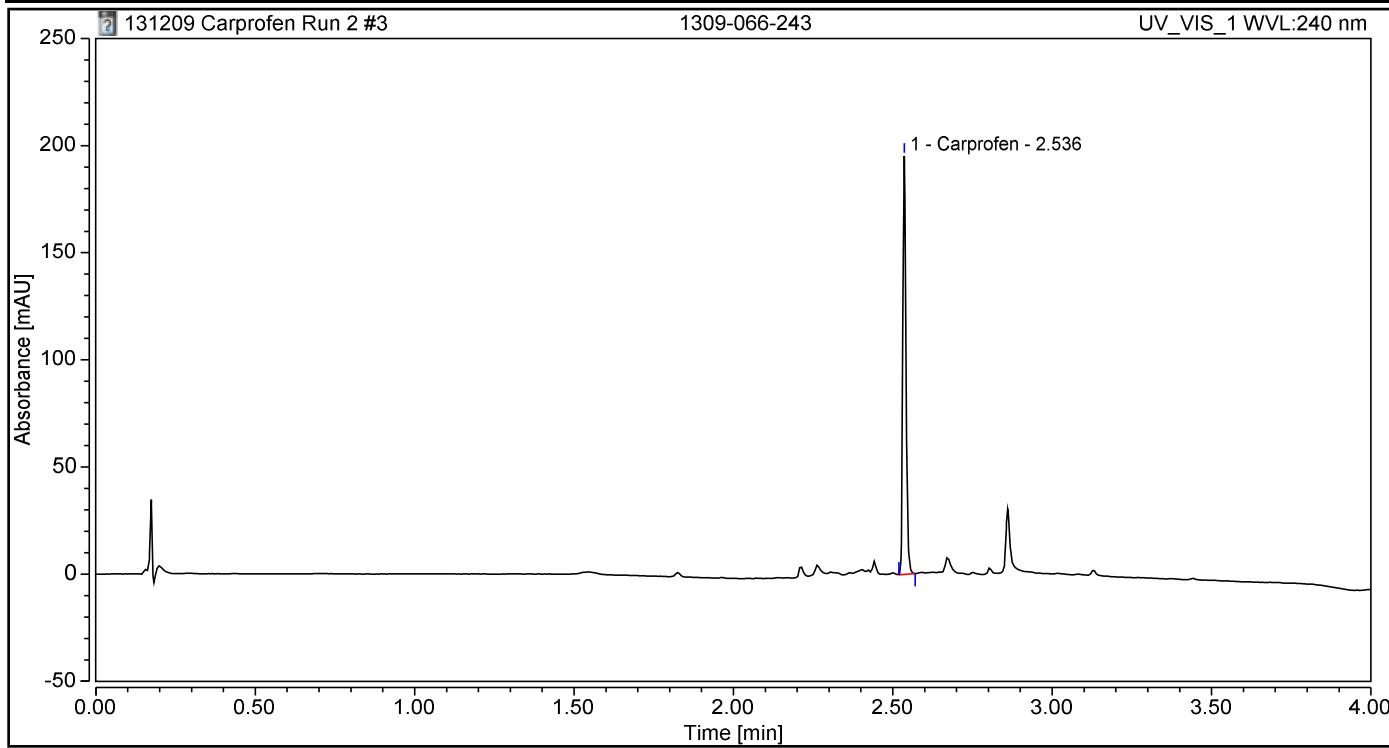
Chromatogram

131209 Carprofen Run 2 #3   1309-066-243   UV_VIS_1 WVL:240 nm

1 - Carprofen - 2.536

Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount
1	Carprofen	2.536	2.287	195.312	100.00	100.00	34.4548
Total:			**2.287**	**195.312**	**100.00**	**100.00**	