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Automated liquid chromatography–tandem mass spectrometry method for the analysis of firocoxib in urine and plasma from horse and dog

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Abstract

A rugged, sensitive and efficient liquid chromatography–tandem mass spectrometry method was developed and validated for the quantitative analysis of firocoxib in urine from 5 to 3000 ng/mL and in plasma from 1 to 3000 ng/mL. The method requires 200 μL of either plasma or urine and includes sample preparation in 96-well solid phase extraction (SPE) plates using a BIOMEK® 2000 Laboratory Automated Workstation. Chromatographic separation of firocoxib from matrix interferences was achieved using isocratic reversed phase chromatography on a PHENOMENEX LUNA® Phenyl-Hexyl column. The mobile phase was 45% acetonitrile and 55% of a 2 mM ammonium formate buffer. The method was accurate (88–107%) and precise (CV < 12.2%) within and between sets. Extraction efficiencies (recovery) >93% were achieved and ionization efficiencies (due to matrix effects) were >72%. Extensive stability and ruggedness testing was also performed; therefore, the method can be used for pharmacokinetic studies as well as drug monitoring and screening. The data presented here is the first LC–MS/MS method for the quantitation of firocoxib in plasma (LLOQ of 1 ng/mL), a 25-fold improvement in sensitivity over the HPLC-UV method and the first quantitative method for firocoxib in urine (LLOQ of 5 ng/mL). Additionally the sample preparation process has been automated to improve efficiency.

Keywords: LC–MS/MS; NSAID; Urine; Plasma; Automation; Firocoxib; Horse; Dog

1. Introduction

Firocoxib, a non-steroidal anti-inflammatory drug (NSAID), provides therapeutic efficacy by inhibition of prostaglandin synthesis via selective binding to the type 2 cyclooxygenase (COX-2) isoenzyme [1,2]. Traditional NSAIDs may exhibit undesirable side effects due to the simultaneous inhibition of the type 1 cyclooxygenase (COX-1) isoenzyme responsible for normal physiologic functioning, including protection of the gastric mucosal lining, regulation of renal blood flow, and platelet aggregation [3,4]. Firocoxib is a member of a class of compounds known as coxibs which are highly selective for COX-2 and have little effect on COX-1 isoenzymes even when administered at relatively high concentrations. Firocoxib has been approved as a veterinary pharmaceutical for pain management associated with osteoarthritis in dogs and horses.

Despite their routine use for pain and inflammation during training, NSAIDs are prohibited for use in horses and dogs prior to racing [5–7]. Illicit drug control programs therefore include urine or plasma screens for the presence of pharmacologically active NSAID concentrations. In order for a simple urine screen to be predictive of relevant systemic drug concentrations, the relationship between plasma and urine concentrations must be known. This relationship can be used to assist racing commissions in recommending withdrawal periods and determining the range of the analytical method used in drug control laboratories. A pharmacokinetic study determining urine and plasma concentrations of meloxicam in horses was published for this purpose [5]. It is anticipated that firocoxib will be prohibited from use in race animals as well and included in the screening process. Experiments to determine plasma-to-urine ratios of firocoxib in horse are in progress in our laboratory. Fast and reliable analytical methods for the determination of low levels of firocoxib in urine and plasma are therefore needed and reported in this paper.

Numerous methods for the quantitation of COX-2 inhibitors in biological matrices utilizing LC separation with UV [8–10],

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fluorescence [11,12], and MS [5,13–19] detection have been published, a small number of which are referenced here. Many of these methods were summarized in a review paper by Nageswara Rao et al. [20]. The LC–MS/MS methods generally had a lower limit of quantitation (LLOQ) in the 0.25–10 ng/mL range. Negative and positive ionization were performed using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) MS ionization techniques. Previously, there were no published methods for the quantitation of firocoxib in plasma. A method to determine the concentrations of firocoxib in plasma, developed and validated in our laboratory, has been submitted for publication [21]. This method uses solid phase extraction followed by LC-UV analysis and has a LLOQ of 25 ng/mL in horse and dog plasma. To date, no publications report the quantitation of firocoxib in urine.

The objective of this study was to develop and validate a rugged, sensitive, selective, and efficient method for the analysis of firocoxib in urine and plasma of dog and horse.

2. Experimental

2.1. Biological samples

Control urine and plasma from horse and dog were obtained from Lampire Biological Laboratories (Piperville, PA, USA) and Merial's Missouri Research Center (MO, USA). Biological control samples were collected from animals over 1 year of age which were fasted, except for samples of horse urine for which the animals were not fasted. Dog and horse control samples were from mixed breed, beagle, and mongrel dogs and Quarter Horse Cross, Standard, and Appaloosa Breed horses of both sexes.

2.2. Chemicals

Firocoxib was provided by Archemis, Cedex, France, and was characterized under GMP quality control. Methanol, acetonitrile, and water were purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA). Formic acid (88%), ammonium formate, and ammonium hydroxide (reagent grade) were obtained from J.T. Baker (Mallinckrodt Baker Inc., Phillipsburg, NJ, USA). Acetic acid was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). All chemicals were HPLC grade unless otherwise noted. The following compounds were purchased from Sigma–Aldrich (St. Louis, MO, USA) to show non-interference of common veterinary drugs with the quantitation of firocoxib: acepromazine maleate, phenylbutazone, ketoprofen, carprofen, and ketamine. Praziquantel was purchased from USP (Rockville, MD, USA). Ivermectin and rofecoxib were kindly provided by Merck & Co., Inc. (Rahway, NJ, USA). The following compounds were tested as potential internal standards and were purchased from Cayman Chemical (Ann Arbor, MI, USA): CAY10404, DuP-697, sulindac, and nimesulide.

2.3. Equipment

LC–MS/MS analysis was performed using a WATERS® (Milford, MA, USA) MICROMASS® QUATTRO ULTIMA® or QUATTRO MICRO™ tandem mass spectrometer coupled to a WATERS 2795 HPLC quaternary pump system with integral temperature controlled autosampler and column heater compartment. A RHEODYNE®, PR750-100 switching valve (Rheodyne, L.P., Rohnert Park, CA, USA) was used to divert early eluants away from the MS. Data were acquired using MASSLYNX™ software version 4.0 (Waters Corporation) and processed using QUANLYNX™ software version 4.0 (Waters Corporation). Solid phase extraction was performed using a BECKMAN COULTER (Brea, CA, USA) BIOMEK 2000 Laboratory Automated Workstation equipped with a vacuum manifold and running BIOWORKS™ software version 3.2. A GLAS-COL® (Terre Haute, IN, USA) Pulse Vortex Mixer/Heater was used to mix 96-well plates.

2.4. LC–MS/MS Conditions

Chromatographic separation was achieved using a PHENOMENEX LUNA 100 mm × 2 mm, 3 μm Phenyl-Hexyl column with a phenyl-hexyl guard (Torrance, CA, USA) under isocratic conditions with (45:55, v/v) acetonitrile:aqueous buffer at a flow rate of 0.25 mL/min. The buffer consisted of an aqueous solution of 2 mM ammonium formate combined with 0.1% formic acid and with the pH adjusted to 4.0 using 30% ammonium hydroxide. For each sample, a 20 μL partial loop injection was directed onto the column which was maintained at 40 °C. The autosampler temperature was kept at 10 °C to avoid evaporation of the samples which were reconstituted in mobile phase. For the first 3.5 min of the run, the auxiliary pump supplied

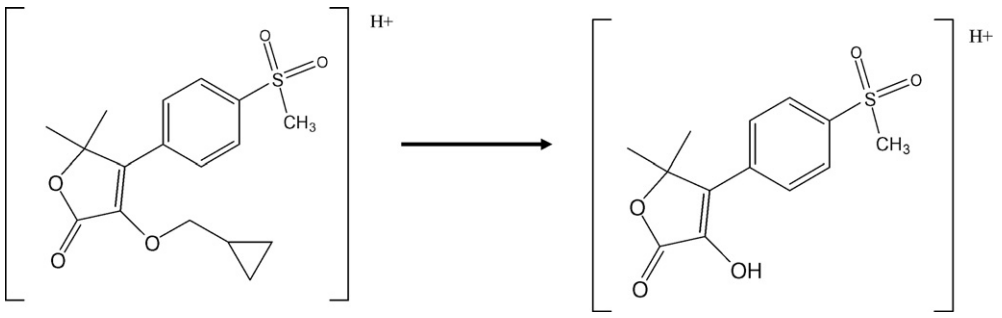

Fig. 1. Monitored ions for MS detection of firocoxib via ESI positive ionization. The transition corresponds to 337.0 → 283.0 *m/z*.

mobile phase to the MS while the column eluate was directed to waste via the switching valve. This step was added to divert any unpurified polar endogenous compounds away from the MS, thereby keeping the instrument clean and maintaining optimal instrument performance over the course of an analytical run. From 3.5 to 5.4 min, the eluate was diverted to the MS, and the auxiliary pump flow went to waste. Firocoxib was ionized using positive ESI and detected via the 337.0 → 283.0 *m/z* MRM transition involving loss of the cyclopropylmethyl group. The transition is shown in Fig. 1.

The following MS parameters were optimized for the detection of firocoxib: cone voltage, 25 V; collision energy, 11 eV; capillary voltage, 2.64 kV; cone gas flow, ~170 L/h; desolvation gas flow, ~690 L/h; source block temperature, 110 °C; desolvation temperature, 500 °C.

2.5. Sample preparation

Frozen urine and plasma standards and samples were thawed at room temperature, sonicated for 10 min, and then kept on ice until use. Rigorously vortexing urine samples immediately prior to taking aliquots greatly reduced analytical variability. A 200 μL aliquot of each urine or plasma standard, quality control (QC) sample, or control sample was transferred into one of the 2 mL cells of a 96-well collection plate and diluted with 400 μL of an aqueous solution of 5% acetic acid. The collection plate was vigorously mixed for 5 min at room temperature using the 96-well plate Pulse Vortex Mixer/Heater. A 30 mg WATERS OASIS® HLB 96-well solid phase extraction plate was conditioned with 1 mL per cell of methanol and equilibrated with 1 mL per cell of water. The entire diluted sample was then added to the extraction plate and pulled through with a vacuum of approximately 127 mm of Hg. The 96-well beds were washed with 1 mL of 5% acetic acid, followed by a 1 mL solution of 25% methanol:75% water. Firocoxib was eluted into a fresh 96-well collection plate with 2 × 0.5 mL of acetonitrile. The samples were evaporated to dryness at 55 °C under a stream of nitrogen, and the residue was reconstituted with 200 μL of mobile phase and mixed in the plate for 10 min. The sealed 96-well collection plate was then put into the autosampler for injection on the LC–MS/MS system. The solid phase extraction procedure and reconstitution steps were automated using the BIOMEK 2000.

2.6. Standard and QC sample preparation

Working stock solutions of firocoxib (0.1–500 μg/mL) were prepared volumetrically in 45:55 acetonitrile:water from a 1.0 mg/mL stock solution and stored at −20 °C and used for the preparation of QC samples and matrix standards. For each standard or QC sample, plasma or urine was fortified with the appropriate neat working stock solution (100-fold dilution). The organic content in each standard/sample was therefore 0.45%. Standard concentrations of 1 (plasma only), 5, 10, 25, 50, 250, 1000, and 3000 ng/mL were used to generate a standard curve in each biological matrix for each species. The 500 μg/mL working stock solution was used to fortify plasma or urine for the dilution QC. The QC samples were prepared at 10, 25, 250, and 2500 ng/mL. For the higher QC samples, preparation was from a separate working stock solution, however, this was not the case for lower QC samples prepared for the data presented here. Subsequent studies in our laboratory have been performed with all QC samples prepared from a separate weighing of firocoxib and results were comparable to those presented here. The plasma and urine standards and QC samples were stored in 2 mL aliquots at −20 °C.

2.7. Internal standard

No suitable internal standard was found for this assay. Other COX-2 inhibitors were investigated but had either very different column retention times or poor SPE recovery. Dueterated firocoxib was not available. Nonetheless, the method met or exceeded all acceptance criteria, including rigorous selectivity testing, without the use of an internal standard.

2.8. Validation procedure

The validation guidelines were adapted from the Bioanalytical Method Validation Guidance for Industry published by the FDA, May 2001 [22].

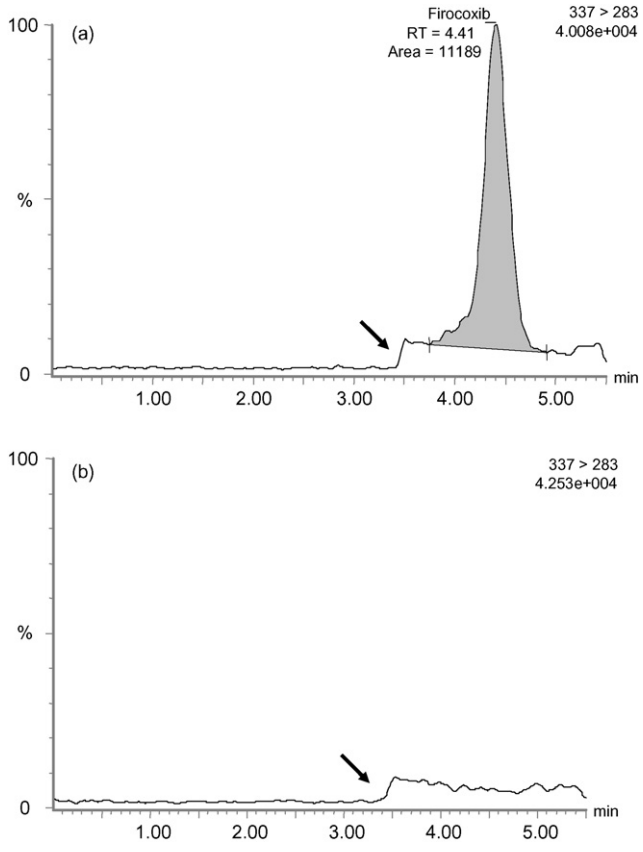

Fig. 2. Extracted dog urine with (a) 5 ng/mL of firocoxib and (b) control dog urine. Vertical axes have been normalized to the height of the 5 ng/mL firocoxib peak. The arrow marks the time when the switching valve begins to divert the column eluant to the MS.

2.8.1. Set acceptance criteria

Each set contained standards prepared in urine or plasma at a minimum of six non-zero fortification levels and run in duplicate, along with two control samples extracted without the addition of firocoxib and a minimum of six quality control samples, two each at low, mid, and high fortification levels. The acceptability of each set was determined by the accuracy of the standards and QC samples, the coefficient of determination (>0.99), and the absence of interference peaks in blank control samples (peak area <20% of the LLOQ). For the run to be accepted at least two-third of all standards and QC samples had to have accuracy within 15% of nominal (20% for the LLOQ). A weighted ($1/x$) regression analysis of the firocoxib standard curve peak area versus nominal concentration was used to generate a quadratic equation for calculating the concentration of firocoxib in each sample. Five injections of a neat firocoxib solution were made at the beginning and end of each night's run to assess suitability of the LC–MS system and the instrument performance over the duration of the run.

2.8.2. Accuracy and precision

Six replicates at each QC level (1, 10, 250, and 2500 ng/mL in plasma and 5, 10, 250, and 2500 ng/mL in urine) were assessed in one set (intraday) and each of three sets (interday) to determine precision and accuracy. The accuracy of the method describes the closeness of the mean results to the true value. For this study, the true value is the nominal concentration, and the accuracy is reported as the ratio of measured concentration to the nominal concentration, expressed as a percent. The mean intraday accuracy is the average accuracy of 6 replicates, and the mean interday accuracy is the average of 18 replicates run in 3 distinct sets on at least 2 days. To assess the intraday precision of the method, the coefficient of variation (CV) of six replicates at each QC level was determined from a single set. Interday precision was determined in the same way using six replicates at each level from each of three sets.

2.8.3. Selectivity

Three replicates of each of six lots of control urine or seven lots of control plasma were evaluated in each species for interfering peaks. Accuracy and precision of three replicates of a 25 ng/mL fortified urine or plasma sample in each lot were also measured. Non-interference from common veterinary drugs was determined by fortifying control plasma with a pool of 500 ng/mL of each of the following compounds: acepromazine maleate, phenylbutazone, ketoprofen, carprofen, ivermectin, ketamine, and praziquantel. Three replicate aliquots of the spiked control sample were analyzed for peaks occurring at the same retention time as firocoxib. QC samples at 25 ng/mL of firocoxib were also spiked with 500 ng/mL of the pooled vet-

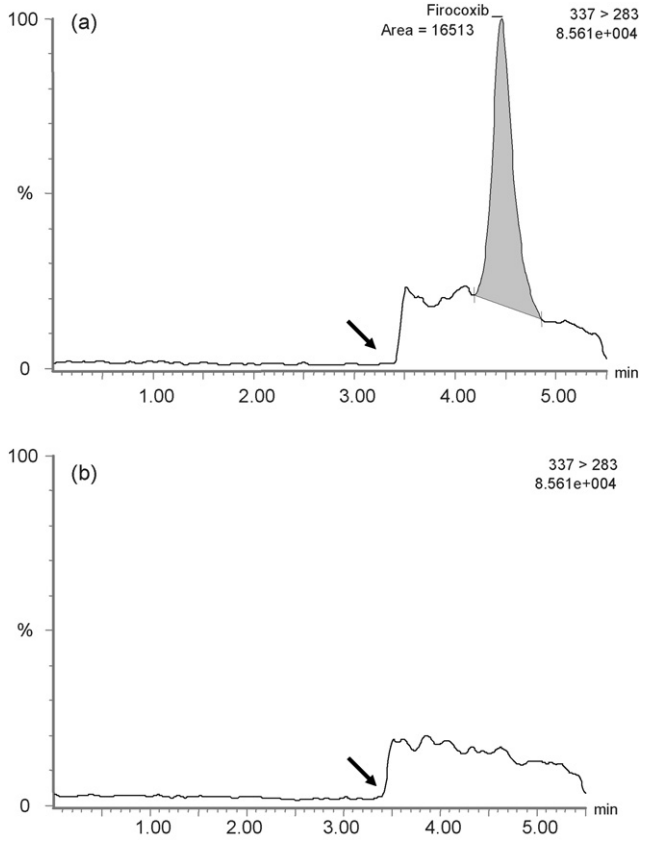

Fig. 3. Extracted horse urine with (a) 5 ng/mL of firocoxib and (b) control horse urine. Vertical axes have been normalized to the height of the 5 ng/mL firocoxib peak. The arrow marks the time when the switching valve begins to divert the column eluant to the MS.

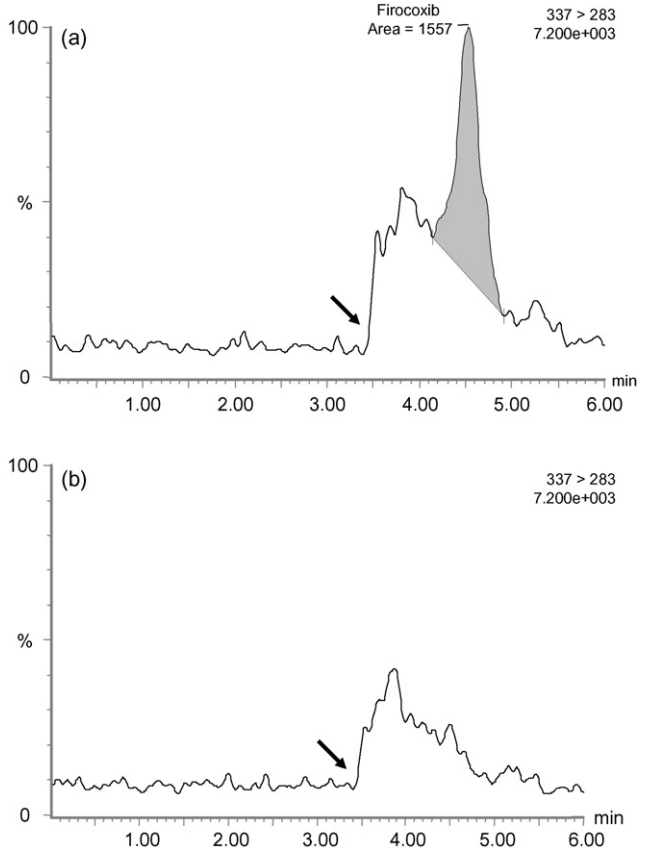

Fig. 4. Extracted dog plasma with (a) 1 ng/mL of firocoxib and (b) control dog plasma. Vertical axes have been normalized to the height of the 1 ng/mL firocoxib peak. The arrow marks the time when the switching valve begins to divert the column eluant to the MS.

erinary drug solution and the accuracy and precision of firocoxib determined.

2.8.4. Sensitivity

The LLOQ was the concentration that consistently gave intra- and interday precision of <15%, intra- and interday mean accuracy within 15% of nominal, and had a signal-to-noise ratio (S/N) of greater than 10. The limit of detection (LOD) was defined as the concentration having a S/N ≥ 3 and precision of ≤33%.

2.8.5. Dilution accuracy

A dilution QC was fortified at a nominal level of 5000 ng/mL and diluted 10-fold in urine or plasma prior to extraction. The accuracy and precision of three replicates of the dilution QC were determined.

2.8.6. Ionization and extraction efficiency (recovery)

Two aspects of overall recovery were investigated in this study: the loss of firocoxib during the sample preparation procedure (extraction efficiency) and the loss or gain of MS signal due to co-eluting residual matrix components (ionization efficiency). The ionization efficiency was measured by performing the extraction process using control urine or plasma samples. The extracted controls were reconstituted with a neat standard (in mobile phase) and identified as spiked extract. The MS peak areas of the spiked extract were compared to the peak areas of the fortified neat reconstitution solution. The difference in the MS response was due to the effect of the residual matrix on the ionization process. To determine the extraction efficiency of firocoxib from urine or plasma, control (untreated) urine or plasma was first spiked with firocoxib to create a QC sample. The firocoxib in the QC sample was then extracted from the urine or plasma and analyzed. The peak area from the extracted sample was compared to the peak area of the spiked extract to isolate only the loss of firocoxib due to the extraction procedure (i.e. both the spiked extract and the extracted QC sample are assumed to have the same LC–MS response to residual matrix components). Overall recovery (matrix QC samples/neat QC samples) was also reported.

2.8.7. Stability

Stability of firocoxib in urine was assessed following three freeze–thaw (F/T) cycles at two QC levels, each run in triplicate. Each F/T cycle consisted of a minimum of 24 h frozen at approximately −20 °C followed by a complete thaw at room temperature. Samples were analyzed prior to being frozen and after the third freeze–thaw cycle against freshly prepared standards. Short-term (benchtop) stability of firocoxib in urine was assessed for fortified samples maintained at room temperature for at least 4 h following one F/T cycle. Stability of firocoxib in plasma was assessed previously in our laboratory [21]. Firocoxib was stable in plasma for at least eight F/T cycles. Long-term stability of frozen (−20 °C) plasma samples was also established for up to 2 years.

Post-preparative stability was determined for a set of extracted and reconstituted plasma QC samples left at approximately 5 °C and at room temperature for >72 h. Stability of the dried eluate from the extraction was also determined under the same conditions. Similarly, post-preparative stability was determined for a set of extracted and reconstituted urine QC samples left at approximately −20 °C (frozen) and at room temperature for >72 h. Stability of the dried eluate from the extraction was also determined under the same conditions for urine QC samples. Short-term, post-preparative, and dried eluate stability were assessed at two (urine) or three (plasma) fortification levels. Post-preparative and dried eluate stability was assessed by determining concentrations based on the original standard curves. Although this method may not be a true assessment of stability, it does replicate the expected actual treatment of the study samples, i.e. the entire set would be stored dry or reconstituted until it could be injected on the LC–MS. A set of extracted, reconstituted samples for each matrix and each species was also injected, left in the autosampler for 72 h at 10 °C, and reinjected to show the ability to reinject samples in case of a failed run.

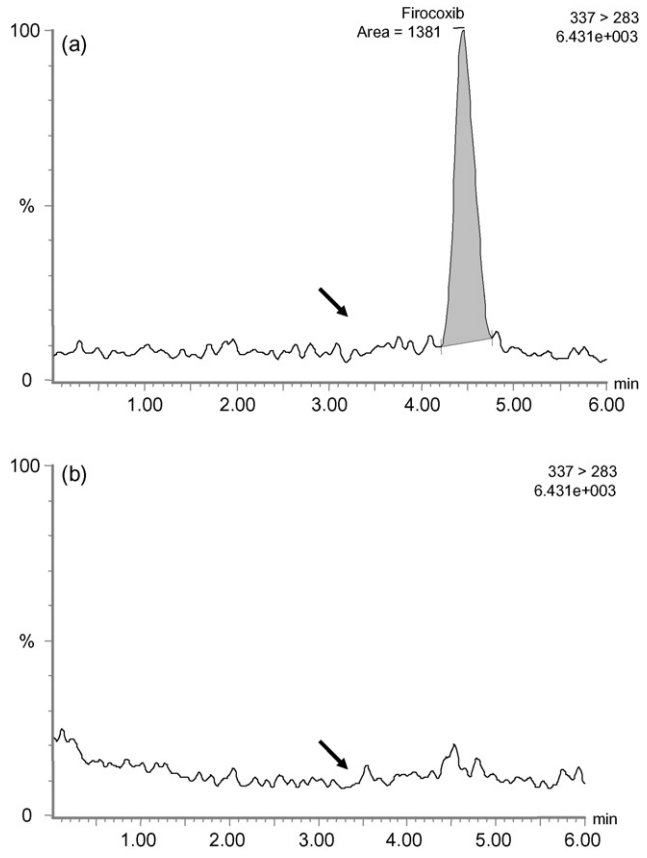

Fig. 5. Extracted horse plasma with (a) 1 ng/mL of firocoxib and (b) control horse plasma. Vertical axes have been normalized to the height of the 1 ng/mL firocoxib peak. The arrow marks the time when the switching valve begins to divert the column eluant to the MS.

2.8.8. Ruggedness

Ruggedness was tested by using two lots of columns, two lots of SPE plates, two LC–MS systems, making small changes in the sample preparation procedure, and experimenting with small changes in the pH of the mobile phase.

Table 1
Selectivity of the method for firocoxib in different lots of urine and plasma from horse and dog

Lot	Mean control concentration ($n = 3$ per lot)	25 ng/mL QC sample ($n = 3$ per lot)		
		Mean measured concentration (ng/mL)	Mean accuracy (%)	Precision (CV, %)
Dog urine				
113058794	NDP	24.6	98.3	1.2
122059030	NDP	22.4	89.4	5.5
122059031	NDP	22.9	91.8	1.9
122059032	NDP	23.0	92.0	2.1
122059033	NDP	21.9	87.6	3.8
AEW 313	NDP	22.1	88.3	3.7
Spiked	NDP	27.2	109	4
Horse urine				
601095572	NDP	22.5	89.9	4.4
601095573	NDP	23.4	93.7	3.0
601095574	NDP	23.3	93.3	3.5
601095575	BLOD	22.4	89.5	3.4
601095576	NDP	22.7	90.6	3.4
AA488	NDP	21.4	85.5	2.4
Spiked	NDP	26.2	105	7
Dog plasma				
122059039	NDP	23.3	93.1	2.0
122059040	NDP	24.4	97.7	0.5
122059041	NDP	24.9	99.5	2.5
122059042	NDP	25.0	99.8	2.7
122059043	NDP	24.8	99.4	3.6
122059044	NDP	25.0	99.9	3.5
670 A	NDP	23.8	95.2	3.2
Spiked	NDP	26.3	105	7
Horse plasma				
121959016	NDP	24.9	99.7	2.3
121959017	NDP	23.0	91.8	2.9
121959018	NDP	25.0	100	2
121959019	NDP	22.8	91.0	3.8
121959020	NDP	24.0	96.0	2.6
121959021	NDP	22.9	91.8	1.5
AA548	NDP	25.3	101	5
Spiked	NDP	24.6	98.3	2.7

The mean control concentration was determined by averaging the calculated concentration of any peak occurring at the retention time of firocoxib. In most cases there was no peak, and in all cases the peak was less than 20% of the LLOQ. Measured concentration, mean accuracy, and precision of three replicate QC samples prepared in each lot are also reported. The lot labeled as 'spiked' was additionally fortified prior to extraction with 500 ng/mL of each of seven common veterinary drugs: acepromazine, carprofen, ivermectin, ketamine, ketoprofen, phenylbutazone, and praziquantel. NDP: no detectable peak; BLOD: below the limit of detection, <1 ng/mL. Accuracies were determined on measured concentrations before rounding.

3. Results and discussion

The method described below fulfills the requirements for a rugged and efficient method for the quantification of firocoxib in dog and horse, urine and plasma, and meets or exceeds FDA guidelines for bioanalytical method validation.

3.1. Method development

The LC–MS method first developed in our laboratory for the assay of firocoxib in urine and plasma had similar sample preparation to the method presented in this paper with a shorter run time (3.5 min versus 5.5 min). The column was a PHENOMENEX LUNA C8(2) with a corresponding guard column, and the mobile phase was 60:40 acetonitrile:water with 0.1% formic acid. The retention time of firocoxib was 2.2 min. Unfortunately, the method was not rugged and did not pass validation criteria. Firocoxib was not separated well from matrix components, resulting in a loss of signal, a higher LOQ, and severe lot-to-lot variability in urine. Decreasing the percent of acetonitrile in the mobile phase gave a longer retention time but did not separate firocoxib from interfering matrix components, including a small peak found in some horse urine lots. Several improvements to the sample preparation were attempted but failed including adding a protein precipitation step, diluting the samples, and changing the SPE chemistry (weak and strong ion exchange). These changes led to unacceptably high variability in the assay. Using a Phenyl-Hexyl column separated firocoxib from matrix components and led to low variability and consistent results for various lots of

Table 2
Factors determining the selection of the curve fitting procedure given for a typical set of standards and QC samples

	Dog urine				Horse urine			
Fitting	Linear	Linear	Quadratic	Quadratic	Linear	Linear	Quadratic	Quadratic
Weighting	None	1/x	None	1/x	None	1/x	None	1/x
Σ Relative error	999	279	1047	222	1365	430	188	131
Percent of std that pass	71.4	71.4	71.4	92.9	42.9	78.6	92.9	100
Mean accuracy (%) ($n=6$)								
5 ng/mL	0	90.8	0	95.5	0	67.8	114	100
25 ng/mL	98.4	117	92.8	115				
250 ng/mL	112	113	113	110	114	120	104	104
2500 ng/mL	99.2	98.7	99.4	98.4	110	106	109	109
Typical regression equation	$y=-0.14495x^2+3477.27x-4628.68$				$y=-0.187369x^2+3171.31x-827.278$			
	Dog plasma				Horse plasma			
Fitting	Linear	Linear	Quadratic	Quadratic	Linear	Linear	Quadratic	Quadratic
Weighting	None	1/x	None	1/x	None	1/x	None	1/x
Σ Relative error	2063	704	516	202	2271	914	1142	281
Percent of std that pass	43.8	68.8	81.3	100	46.7	80.0	80.0	100
Mean accuracy (%) ($n=6$)								
1 ng/mL	0	43	151	96.1	0	17.3	217	100
10 ng/mL	0	103	100	95.3	0	108	113	102
250 ng/mL	106	110	97.4	97.6	108	113	99.6	100
2500 ng/mL	103	100	101	101	109	106	108	108
Typical regression equation	$y=-0.100092x^2+1449.2x-222.434$				$y=-0.078526x^2+1353.6x-52.4208$			

matrix. A number of mobile phase solutions were examined, including using ammonium acetate pH 4.5 and 5.0 as the aqueous buffer and using methanol/acetonitrile combinations for the organic mobile phase. The ammonium formate buffer was chosen because it provided the best LC–MS response and the shortest retention time while still separating firocoxib from the interferences.

Sample preparation was optimized to minimize residual matrix while maximizing firocoxib recovery. The SPE wash steps were included to minimize the matrix components without diminishing the recovery. Increasing the methanol content in the SPE wash to 35% increased the variability of the assay somewhat; therefore, a maximum of 25% methanol is recommended. SPE recovery in plasma was improved by ∼20% when a dilute

Table 3
A summary of intra- and interday accuracy and precision for each QC level in each matrix

	Dog urine				Dog plasma			
	5.00[a]	10.0[a]	250[a]	2500[a]	1.00[a]	10.0[a]	250[a]	2500[a]
Intraday, $n=6$								
Mean concentration (ng/mL)	4.39	9.79	253	2510	1.02	8.94	232	2450
Mean accuracy (%)	87.8	98.0	101	101	102	89.4	92.7	97.7
Precision, CV (%)	4.7	2.9	3	3	6	4.5	2.2	5.5
Interday, three sets, $n=6$ per set								
Mean concentration (ng/mL)	4.74	9.49	242	2400	0.99	9.29	243	2590
Mean accuracy (%)	94.7	94.9	96.8	96.2	98.7	92.8	97.4	104
Precision, CV (%)	7.5	8.1	6.3	6.2	10.0	8.2	4.4	6
	Horse urine				Horse plasma			
	5.00[a]	10.0[a]	250[a]	2500[a]	1.00[a]	10.0[a]	250[a]	2500[a]
Intraday, $n=6$								
Mean concentration (ng/mL)	5.05	8.89	248	2590	0.99	9.88	250	2660
Mean accuracy (%)	101	88.9	99.4	104	99.3	98.8	99.9	106
Precision, CV (%)	8	4.7	2.4	3	9.3	3.0	1.6	2
Interday, three sets, $n=6$ per set								
Mean concentration (ng/mL)	4.81	9.3	253	2610	0.97	9.92	249	2680
Mean accuracy (%)	96.2	93.0	101	105	97.4	99.2	99.5	107
Precision, CV (%)	10.0	8.1	3	3	12.2	8.0	6.3	3

[a] Nominal concentration (ng/mL).

Table 4
Recovery of firocoxib due to the effects of matrix on the analysis (ionization efficiency), from the sample preparation procedure (extraction efficiency) and overall (comparison of QC sample prepared in matrix to neat QC samples)

	10 ng/mL	250 ng/mL	2500 ng/mL
Dog urine			
Ionization efficiency (%)	90.9	103	102
Extraction efficiency (%)	94.5	95.8	92.7
Overall recovery (%)	85.8	98.4	94.2
Horse urine			
Ionization efficiency (%)	82.9	86.4	88.1
Extraction efficiency (%)	98.6	99.3	97.6
Overall recovery (%)	81.7	85.8	86.0
Dog plasma			
Ionization efficiency (%)	72.2	76.3	97.6
Extraction efficiency (%)	94.8	94.2	96.3
Overall recovery (%)	68.5	71.9	94.0
Horse plasma			
Ionization efficiency (%)	83.3	82.7	83.5
Extraction efficiency (%)	101	102	98.3
Overall recovery (%)	83.9	84.3	82.1

acidic solution was added to the samples prior to extraction, presumably disrupting protein binding. Rofecoxib, CAY10404, DuP-697, sulindac, and nimesulide were investigated as potential internal standards. Each compound either had poor SPE recovery, instability under the optimal firocoxib LC–MS conditions, or very early or late elution on the analytical column. Rofecoxib was the most promising compound but because the retention time was shorter than firocoxib, matrix effects were a greater influence on the signal, and the method was less rugged and less precise with the use of this internal standard.

The method was also adapted to be automated using the BIOMEK 2000 Laboratory Automation Workstation. The BIOMEK 2000 has an eight-channel pipette tool which was used for the addition of the dilution solvent and reconstitution of the samples. The eight-channel bulk dispense tool was used for equilibration, conditioning, washing, and elution of the SPE plate. Transfer of the diluted samples from the sample preparation plate to the SPE plate was performed by hand because using the eight-channel pipette tool increased the variability of the assay (although still within acceptable bioanalytical limits). For ruggedness testing, the assay was also performed by hand with similar results to those obtained from the automated procedure.

All MS parameters were optimized including gas temperatures, flow rates, and optic voltages. Positive and negative APCI and ESI ionization techniques were investigated. Although negative ionization has been used for other COX-2 inhibitors including rofecoxib, firocoxib had very poor ionization in negative ion mode. APCI, positive ionization had good signal although the sensitivity was slightly less than that of ESI.

3.2. Selectivity

The results of selectivity testing are given in Table 1. No peaks were present that interfered with the quantitation of firocoxib in any lot of urine or plasma. For most sets, the control chromatograms contained no measurable peaks. Typical chromatograms of the LLOQ and control matrix samples are shown in Figs. 2–5. The broad underlying background seen after diverting the column eluant to the MS (and was most evident in dog plasma) did not interfere with quantitation of firocoxib at the LLOQ. For all plasma and urine lots, the mean accuracy and precision of 25 ng/mL QC samples passed the acceptance criteria. Firocoxib QC samples (25 ng/mL) spiked with 500 ng/mL each of acepromazine, carprofen, ivermectin, ketamine, ketoprofen, phenylbutazone, and praziquantel had acceptable accuracy and precision. The control matrix spiked with the same pool of compounds contained no detectable peaks.

Carryover following the high standard or QC samples was also evaluated in each set and in most cases found to be undetectable except in cases where instrument problems occurred or contamination during sample preparation was suspected.

To confirm the identity of the chromatographic peak monitored for the validation, a typical MS scan and product ion scan of parent mass 337 *m/z* are shown in Fig. 6.

3.3. Goodness of fit of the standard curves

A least squares regression with a quadratic equation and a $1/x$ weighting factor was used to achieve the best accuracy for all standards. A linear equation or unweighted fitting resulted in poor accuracy, especially for low concentration standards, and higher relative error [23] in a set, as shown in Table 2. The $1/x$ weighted fit consistently gave more accurate results for standards and QC samples in each run. The coefficient of determination (R^2) was greater than 0.99 for all sets.

3.4. Accuracy and precision

Intra- and interday accuracy and precision as well as mean calculated concentrations for QCs at four levels for each species and biological matrix are given in Table 3. Intraday accuracies range from 87.8 to 106% with precision of less than 9.3% in all cases. Interday accuracies range from 92.9 to 107% with precision of less than or equal to 12.2% in all cases. The method is precise and accurate and exceeds the acceptance criteria suggested in the FDA bioanalytical validation guidance [22]. The upper limit of quantitation is therefore set at 3000 ng/mL. The LLOQ is discussed in Section 3.5.

3.5. Sensitivity

Intraday accuracy and precision for the 1 ng/mL QC in plasma was $102 \pm 6\%$ (dog) and $99.3 \pm 9.3\%$ (horse). Signal-to-noise for the 1 ng/mL QC was always greater than 10. The LLOQ in plasma for both species is therefore 1 ng/mL. The limit of detection (LOD) was 0.25 ng/mL in both dog and horse plasma. The mean S/N for six replicates of 0.25 ng/mL of firocoxib in dog plasma was 8.26, and the samples had a CV of 12.2%. The mean S/N for six replicates of 0.25 ng/mL of firocoxib in horse plasma was 17.3, and the samples had a CV of

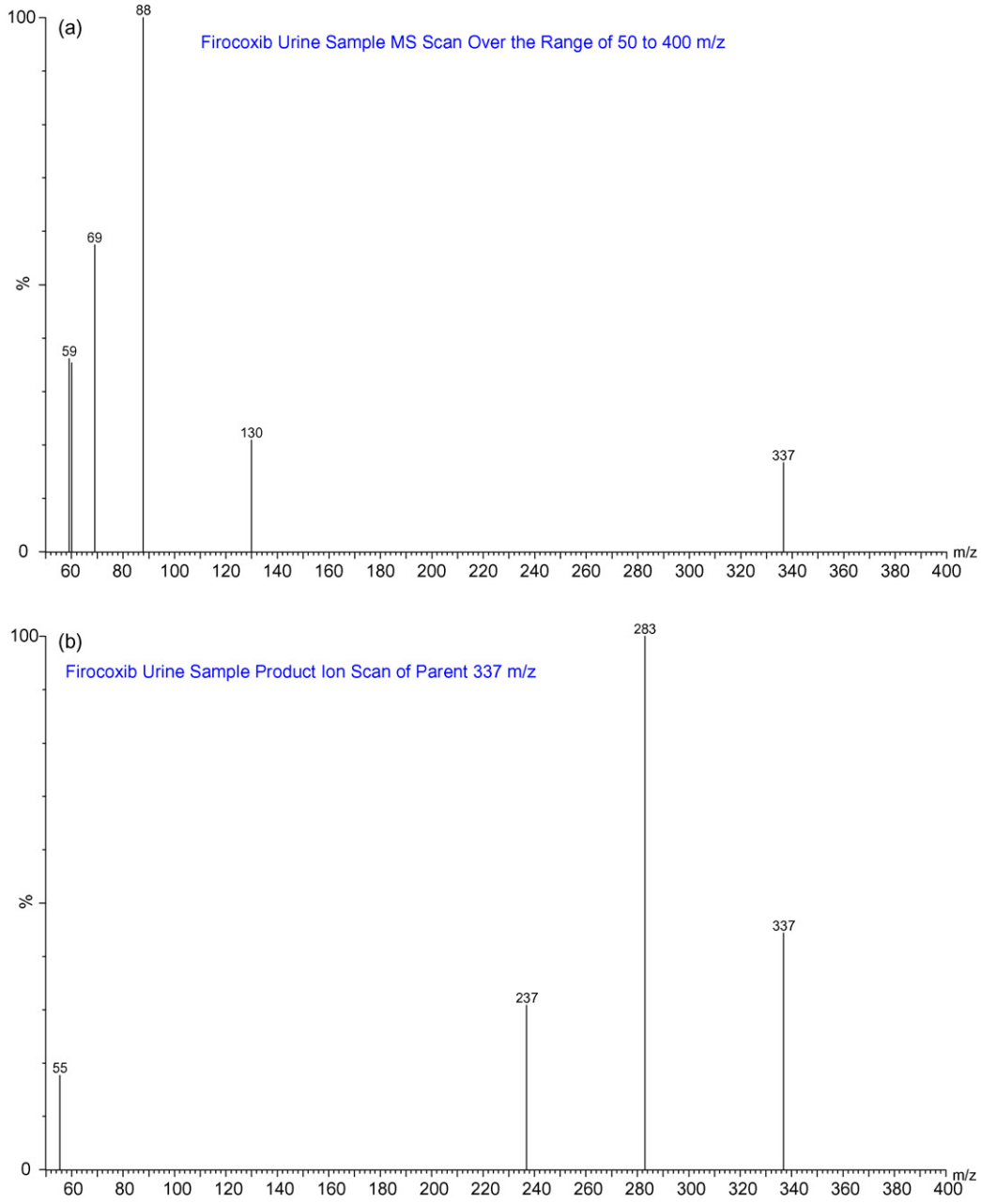

Fig. 6. Typical MS (a) and MS/MS (b) spectra of firocoxib in urine. The same product ion fragments were obtained from the scan of a neat firocoxib standard.

26.3%. At this level many peaks had to be integrated manually. Interday precision and accuracy for the LLOQ is given in Table 3.

Intraday accuracy and precision for the 5 ng/mL urine QC was 87.8 ± 4.7% (dog) and 101 ± 8% (horse). Signal-to-noise for the 5 ng/mL urine QC was always greater than 10. The LOD was 3.0 ng/mL for dog and 1.0 ng/mL for horse. The CV for six replicate dog urine samples was 38% for 1.0 ng/mL and 10% for 3.0 ng/mL. The mean S/N for six replicates of firocoxib in dog urine was 17 and 58 for 1.0 and 3.0 ng/mL samples, respectively. The mean S/N for six replicates of 1.0 ng/mL of firocoxib in horse urine was 12, and the samples had a CV of 30%. Interday precision and accuracy for the LLOQ is given in Table 3.

3.6. Dilution accuracy

The accuracy and precision of samples diluted 10-fold in biological matrix prior to the extraction procedure was 105 ± 1 and 95.9 ± 2.1% for dog and horse urine, respectively, and 101 ± 2 and 98.9 ± 3.0% for dog and horse plasma, respectively.

3.7. Recovery

Table 4 lists the ionization efficiency (matrix effect), extraction efficiency, and overall recovery of firocoxib from plasma and urine. Extraction efficiencies were greater than 92% in all cases. Ionization efficiencies ranged from 72 to 103% and were

Table 5
Accuracy and precision of ruggedness samples

Sample preparation condition tested	Nominal concentration					
	25 ng/mL Dog plasma			50 ng/mL Dog urine		
	Mean measured concentration (ng/mL)	Mean accuracy (%)	Precision, CV (%) ($n=3$)	Mean measured concentration (ng/mL)	Mean accuracy (%)	Precision, CV (%) ($n=3$)
Diluting each sample with 0.1% acetic acid instead of 5.0% acetic acid	27.2	109	5	53.7	108	5
Eliminating the 1 mL 5% acetic acid rinse	23.7	94.6	10.5	52.6	105	2
Rinsing SPE plate with 10% methanol instead of 25% methanol	25.7	103	4	55.1	110	3
Rinsing SPE plate with 35% methanol instead of 25% methanol	27.4	110	6	51.0	102	6
Eliminating the second 0.5 mL acetonitrile elution step	24.6	98.3	6.2	53.7	107	3
Mixing reconstituted samples for only 5 min instead of 10 min	25.6	102	5	50.5	101	2
Mixing reconstituted samples for 20 min instead of 10 min	23.8	95.2	14.6	52.0	104	5
	Nominal concentration					
	25 ng/mL Horse plasma			50 ng/mL Horse urine		
	Mean measured concentration (ng/mL)	Mean accuracy (%)	Precision, CV (%) ($n=3$)	Mean measured concentration (ng/mL)	Mean accuracy (%)	Precision, CV (%) ($n=3$)
Diluting each sample with 0.1% acetic acid instead of 5.0% acetic acid	24.0	95.9	5.9	54.2	109	2
Eliminating the 1 mL 5% acetic acid rinse	**20.5**	**81.9**	**9.1**	53.6	107	1
Rinsing SPE plate with 10% methanol instead of 25% methanol	23.7	94.8	5.2	52.3	105	3
Rinsing SPE plate with 35% methanol instead of 25% methanol	26.1	104	1	54.6	109	3
Eliminating the second 0.5 mL acetonitrile elution step	24.0	96.1	5.1	51.7	103	2
Mixing reconstituted samples for only 5 min instead of 10 min	**19.9**	**79.6**	**12.4**	51.3	103	3
Mixing reconstituted samples for 20 min instead of 10 min	**19.6**	**78.3**	**2.6**	50.9	102	2

Bold entries did not pass the acceptance criteria.

greatest in dog urine, similar in horse urine and plasma, and lowest in dog plasma.

3.8. Ruggedness

All dog (13 sets) and horse (12 sets) urine sets passed the acceptance criteria. Several sets had to be reinjected before they passed due to LC–MS instrument problems. Ten out of 11 horse and all of the dog plasma (13 sets) sets met the acceptance criteria. As many as 200 samples were run in a single day. To test the ability to quantitate firocoxib in actual study samples, horse plasma study samples were assayed, and the results were comparable to those obtained using the LC-UV method [21], suggesting that matrix effects due to the dosing vehicle were negligible. A typical chromatogram following an IV dose of firocoxib in horse is given in Fig. 7.

Table 6
Post-extraction and short-term (benchtop) stability of firocoxib samples

	25 ng/mL		250 ng/mL		2500 ng/mL	
	Mean concentration (ng/mL)	Mean accuracy (%)	Mean concentration (ng/mL)	Mean accuracy (%)	Mean concentration (ng/mL)	Mean accuracy (%)
Dog urine ($n=2$ per level)						
DE[a], RT[b]	28.5	114	245	98.0	2600	104
DE, −20 °C	27.6	111	254	102	2620	105
PP[c], RT	30.2	**121**	241	96.2	2040	**81.6**
PP, −20 °C	26.2	105	240	95.9	2380	95.2
Reinjection[d] 5 °C						
Inj.[e] #1	27.7	111	254	101	2620	105
Inj. #2	25.4	101	260	104	2241	89.7
Short-term urine[f] RT ($n=3$)	26.5	106			2760	110
Horse urine ($n=2$ per level)						
DE, RT	27.9	112	281	113	2710	109
DE, −20 °C	27.3	109	278	111	2690	108
PP, RT	26.5	106	269	108	2600	104
PP, −20 °C	26.7	107	265	106	2650	106
Reinjection 5 °C						
Inj. #1	27.3	109	279	111	2690	108
Inj. #2	28.6	115	281	112	2630	106
Short-term urine RT ($n=3$)	25.9	104			2530	101
Dog plasma ($n=3$ per level)[g]						
DE[a], RT[b]	25.0	100			2580	103
DE, 5 °C	28.6	115			2680	107
PP[c], RT	24.3	97.1			2340	93.7
PP, 5 °C	26.0	104			2540	102
Reinjection 5 °C						
Inj. #1	28.6	115			2680	107
Inj. #2	27.9	112			2680	107
Horse plasma ($n=3$ per level)[g]						
DE, RT	28.3	113			2810	112
DE, 5 °C	26.8	107			2840	113
PP, RT	27.1	108			2800	112
PP, 5 °C	24.8	99.3			2800	112
Reinjection 5 °C						
Inj. #1	28.3	113			2810	112
Inj. #2	26.5	106			2553	102

Bold samples were out of specification.
[a] DE: dried eluate—samples were extracted via solid phase extraction and evaporated but not reconstituted. Stability of the dried residue is tested here.
[b] RT: room temperature.
[c] PP: post-preparative—samples were extracted via solid phase extraction, evaporated, and reconstituted. Stability of the reconstituted sample is tested here.
[d] Reinjection: samples were injected onto the LC–MS and analyzed, stored at 10 °C in the autosampler for 72 h and then reinjected. The first injection was on the MICROMASS QUATTRO ULTIMA and the second was on the MICROMASS QUATTRO MICRO.
[e] Inj.: injection.
[f] Short-term urine: stability of firocoxib in urine for 4 h at room temperature.
[g] The CV is less than 9% for plasma samples at each level and under all conditions.

The calculated concentration for this sample was 807.8 ng/mL, 4.2% different from results obtained via the LC-UV method. Horse urine study samples have also been quantified using this method.

The method was accurate and precise despite minor changes, such as using different lots of analytical columns and solid phase extraction plates or analyzing via different LC–MS systems. Accuracy and precision of the method were also maintained with small changes in the sample preparation procedure as shown in Table 5. The resulting urine analysis was rugged to all conditions tested. The dog plasma method was also rugged to all conditions tested. Mean accuracy of the horse plasma QC samples was out of specification (<15% of nominal) when the 1 mL acetic acid rinse was eliminated (82%) and when the mixing time was changed (78–80%).

The effect of pH change on the assay was also investigated. A standard curve was analyzed using the mobile

Table 7
Freeze–thaw stability of firocoxib in dog and horse urine at two QC levels

	25.0 ng/mL		2500 ng/mL	
	Freshly prepared (ng/mL)	After three F/T cycles (ng/mL)	Freshly prepared (ng/mL)	After three F/T cycles (ng/mL)
Dog ($n = 3$ per level)				
Mean	24.3	26.4	2760	2800
CV (%)	2.7	3.0	1	3
P-value	0.02		0.71	
Horse ($n = 3$ per level)				
Mean	24.8	25.3	2740	2480
CV (%)	7.3	3.8	1	2
P-value	0.40		0.09	

The *P*-value was determined by a two sample Student's *t*-test.

phase described in Section 2.4 with the pH changed from 4.0 to 4.5 or 3.5. A previously injected standard curve for each species was run under each condition. The retention time and sensitivity were similar for all pH conditions. The method is therefore robust to changes in pH in the range of 4.0 ± 0.5.

3.9. Post-extraction and short-term stability

Extracted, reconstituted dog urine samples were out of specification after being left at room temperature for 72 h. It is therefore recommended that extracted dog urine samples be stored at 10 °C or below. The accuracy of firocoxib levels in extracts under other post-extraction stability conditions was well within the acceptance criteria under all other conditions. Precision of samples with $n = 3$ replicates was less than 9.0%. Mean accuracies are listed in Table 6.

Short-term stability was established in dog and horse urine left on the benchtop at room temperature for 4 h prior to extraction. Good accuracy and precision were obtained for samples analyzed against a freshly prepared standard curve (not subjected to 4 h at room temperature), indicating that the urine can be left on the benchtop for up to 4 h prior to analysis. Accuracies of short-term stability samples ranged from 101 to 110% (see Table 6).

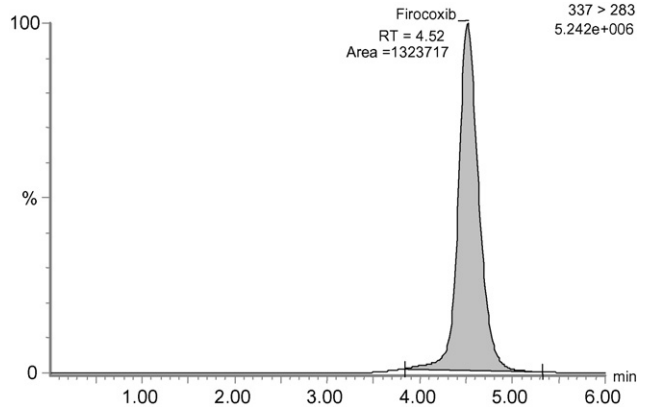

Fig. 7. Chromatogram from an incurred firocoxib horse plasma sample collected 1 min after an intravenous injection.

3.10. Freeze–thaw stability

A comparison of concentrations for freshly prepared and extracted QC samples to aliquots extracted after three F/T cycles is given in Table 7. *P*-values, determined using a two sample Student's *t*-test, were greater than 0.05 for horse urine samples, indicating with 95% confidence that the concentrations of the freshly prepared QC samples and samples assayed after three F/T cycles were the same. For dog urine samples, the *P*-value was greater than 0.05 for the 2500 ng/mL fortification level. For the 25 ng/mL fortification level, the *P*-value was 0.02 due to the low variability within each group, but the percent difference between the two averages is 8.3% which is within the tolerance for variability in the analysis. Firocoxib in urine has therefore been determined to be stable for up to three F/T cycles at low and high concentrations in dog and horse urine.

4. Conclusions

The LC–MS/MS method presented here for quantitation of firocoxib in horse and dog urine and plasma is efficient, selective, sensitive, and rugged. The 96-well plate solid phase extraction procedure has been automated and at least 200 samples can be processed in 1 day by a single scientist. The 5.5 min run time allowed for good separation of firocoxib from residual matrix components in urine and plasma. MS/MS detection may be preferable to UV analysis when greater sensitivity is needed for the assay, because the plasma MS/MS method is 25 times more sensitive than the UV method. The LLOQ in both plasma and urine was comparable to LC–MS/MS methods validated for other NSAIDs which ranged from 0.25 to 10 ng/mL. The method is suitable for use by racing commissions for illicit drug control screening and for pharmacokinetic studies.

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