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Quantitative HPLC-UV method for the determination of firocoxib from horse and dog plasma

Valerie Kvaternick [a,*], Thomas Malinski [b], Jill Wortmann [b], James Fischer [a]

[a] *Merial Limited, Pharmacokinetics and Drug Metabolism, 631 Route 1, North Brunswick, NJ 08902, United States*
[b] *Merial Limited, Clinical Operations, 6498 Jade Road, Fulton, MO 65251, United States*

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Abstract

A sensitive reversed-phase HPLC-UV method was developed for the determination of firocoxib, a novel and highly selective COX-2 inhibitor, in plasma. A 1.0 mL dog or horse plasma sample is mixed with water and passed through a hydrophobic–lipophilic copolymer solid-phase extraction column to isolate firocoxib. Quantitation is based on an external standard curve. The method has a validated limit of quantitation of 25 ng/mL and a limit of detection of 10 ng/mL. The validated upper limit of quantitation was 2500 ng/mL for horses and 10,000 ng/mL for dogs. The average recoveries ranged from 88–93% for horse plasma and 96–103% for dog plasma. The coefficient of variation in all cases was less than 10%. This method is suitable for the analysis of clinical samples from pharmacokinetic and bioequivalence studies and drug monitoring.

Keywords: Firocoxib; HPLC-UV; Plasma; Method validation; Coxib; NSAID; Horse; Dog

1. Introduction

Inflammation is a beneficial response that protects tissues affected by injury or pathogens; yet, left unchecked, inflammation (and inflammatory mediators) can contribute to the pathogenesis of numerous diseases including arthritis, cancer, periodontitis-induced bone loss, and colitis [1–4]. Firocoxib (Fig. 1), a veterinary analgesic and anti-inflammatory drug, is administered orally once daily for the control of pain and inflammation associated with osteoarthritis in dogs (NADA 141–230) and horses (NADA 141–253). Dosages of firocoxib are species dependent, with the recommended dosage for dogs being 5 mg/kg every 24 h and for horses 0.1 mg/kg every 24 h.

Firocoxib belongs to an important class of nonsteroidal anti-inflammatory drugs (NSAID) known as coxibs that are selective for cyclooxygenase-2 (COX-2) and sparing for cyclooxygenase-1 (COX-1). Development of this new phase of NSAIDs began in the 1990s after the discovery of a mitogen-inducible form of the cyclooxygenase enzyme (also known as prostaglandin G/H synthase) [5]. Two groups of researchers independently discovered this second cyclooxygenase gene that appeared, based on its pattern of regulation and expression, to be the sole isozyme that produced prostaglandins responsible for potentiating inflammatory processes [6]. The recognition that inhibition of COX-2 might be sufficient to achieve the anti-inflammatory benefits of NSAIDs, and that the side effects commonly associated with NSAIDs was due at least in part to the indiscriminate inhibition of COX-1, resulted in the development of new chemical entities that would selectively inhibit only COX-2. This class of drugs, coxibs, which firocoxib belongs to, has now evolved to second generation drugs [7,8] that are highly selective for the COX-2 isozyme, thus sparing COX-1 at therapeutic levels and potentially reducing the incidence of COX-1 side effects, such as gastrointestinal irritation [9–11]. Firocoxib's mode of action is similar to that of other NSAIDs, and is through inhibition of the arachidonic acid enzyme cascade that synthesizes various prostanoids, such as prostaglandins, thromboxanes, and lipoxins, from arachidonic acid via the COX isozymes, and it specifically interrupts the biosynthetic pathway of prostaglandin formation, an inflammation mediator, by inhibition of COX-2 [3,12,13].

While a variety of studies have been published on the cyclooxygenase potency and selectivity of this new genera-

* Corresponding author. Tel.: +1 732 729 5706; fax: +1 732 729 5821.
E-mail address: valerie.kvaternick@merial.com (V. Kvaternick).

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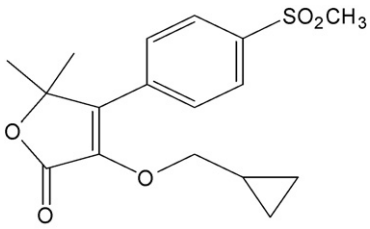

Fig. 1. Structure of firocoxib (3-cyclopropylmethoxy)-4-(4-(methylsulfonyl) phenyl)-5,5-dimethylfuranone).

tion of NSAIDs, including firocoxib [14–16], there are, to the best of our knowledge, no comprehensive published bioanalytical procedures for the detection and quantification of firocoxib in plasma. There are, however, a large number of published methods (HPLC-UV, LC/MS/MS, LC and chemometric methods) for the determination of various other COX-2 inhibitors such as valdecoxib and rofecoxib in plasma and as bulk drug [17–20] of which the chromatographic conditions have been succinctly summarized in a review paper by Rao et al. [21]. Firocoxib is a small nonionizable molecule with a molecular weight of 336.4 amu. It has a conjugated ring system similar to other NSAIDs/coxibs making it amenable to UV and fluorescence detection. While the LC/MS/MS methods generally reported much lower limits of quantitation, ranging from <1–10 ng/mL, sufficient limits of quantitation (5–25 ng/mL) were reported for HPLC-UV methods of related compounds [20,22,23]. Rao et al. [24] also reported a multianalyte reversed-phased HPLC method using a photodiode array detector, however, the quantitation limits were high, e.g., greater than 1000 ng/mL for valdecoxib and rofecoxib. For sample preparation, these methods typically employed protein precipitation, liquid–liquid extraction, solid-phase extraction, and/or filtration, however, one method using a first derivative spectrophotometric method noted that it circumvents the need for extensive sample clean-up such as filtration, thus saving time [17]. Typically solvents such as diethyl ether/methylene chloride or acetonitrile were reported for liquid–liquid extraction or protein precipitation, respectively, [22,23] while others reported the use of solid-phase extraction, mainly reversed-phase, for the separation of NSAIDs from plasma. These sample preparation steps, alone or in combination, are intended to improve selectivity and recovery, but can be onerous and require the use and disposal of organic solvents. Thus, presented here is the first bioanalytical method (validated) for the determination and quantification of firocoxib, a new chemical entity, that involves a simple one step extraction (copolymer-based solid-phase extraction) and HPLC-UV analysis that reduces the quantity of solvent used and chromatographically separates firocoxib in under 10 min from horse and dog plasma with excellent accuracy. Although a lower LOQ can be achieved with MS detection, an acceptable LOQ (25 ng/mL) is achieved with UV detection which is more widely available in labs. This method was demonstrated suitable for the analysis of the large number of samples generated by pharmacokinetic studies in dogs and horses.

2. Experimental

2.1. Chemicals

Firocoxib (of known purity) was provided internally by the Formulations Development department (Merial North Brunswick, NJ, USA). HPLC grade acetonitrile (ACN) and trifluoroacetic acid (TFA) were obtained from Burdick and Jackson (Muskegon, MI, USA) and J.T. Baker (Phillipsburg, NJ, USA), respectively. Water was obtained from an in-house Barnstead water purification system.

2.2. Liquid chromatography apparatus and conditions

The HPLC system consisted of a SCL-10A$_{VP}$ System Controller, a SIL-10A autosampler, LC-10AS liquid chromatographic pumps, a SPD-10A series UV-vis detector, a CTO-10A column oven, and Class VP Data Acquisition Software, version 5.03, all from Shimadzu® Scientific Instruments, Inc. (Columbia, MD, USA). Separation was achieved on an Inertsil™ ODS-3 column (150 mm × 4.6 mm, 5 μm, 100 Å pore diameter, ES Industries, West Berlin, NJ, USA) maintained at 40 °C and protected by one or two 2-μm frits (Upchurch, Oak Harbor, WA, USA). The mobile phase consisted of 45:55:0.025 acetonitrile/water/trifluoroacetic acid at a flow rate of 1.0 or 1.2 mL/min. The mobile phase was prepared daily and degassed by an in-line membrane degasser. The detector wavelength was set at 290 nm.

2.3. Standard preparation

A stock standard (nominal 50 μg/mL) was prepared by dissolving 5.0 mg (corrected for purity) of firocoxib in 100 mL of acetonitrile. Calibration standards (10,000, 5000, 2500, 1000, 500, and 50 ng/mL) were prepared by serial dilution of the stock standard. An aliquot of each calibration standard was evaporated to dryness, reconstituted in an equal volume of 40% acetonitrile/water, and transferred to an autoinjector vial for injection (50 μL). Standard solutions were also used to prepare fortified or quality control samples. Standard solutions were stored at −20 °C.

2.4. Sample preparation

Aqueous plasma samples (1.0 mL of plasma plus 2.0 mL of water) were transferred to preconditioned (2.0 mL of acetonitrile followed by 2.0 mL of water) solid-phase extraction columns (Waters Oasis®, HLB, 3cc/60 mg) and allowed to elute by gravity. A minimum amount of vacuum (<5 in. Hg) or positive pressure was used if necessary. The columns were rinsed with 2.0 mL of 5% acetonitrile in water (gravity elution) and a vacuum of ∼10 in. of Hg was used to remove the residual solvent. Firocoxib was eluted with 2.0 mL of acetonitrile that was evaporated to dryness with nitrogen while heating the samples to 35–40 °C. The residue was reconstituted in 0.25 mL of 40% acetonitrile/water with vortex mixing for 10 s. An aliquot sufficient to allow the injection of 50 μL was transferred to an autoinjector vial for analysis.

Fortified samples were prepared by adding the appropriate amount of standard to a control (blank) plasma sample after the addition of 2.0 mL of water. Samples were fortified at 25–2500 ng/mL for horse plasma and 25–10,000 ng/mL for dog plasma.

Firocoxib was identified by comparing retention times of peaks in the samples to retention times of peaks in the standards within sets.

2.5. Method validation

To yield reliable results that could be properly interpreted, this method was validated according to Good Laboratory Practice standards for Nonclinical Laboratory Studies promulgated by the FDA as well as the bioanalytical method validation guideline #145 [25,26].

2.5.1. Selectivity

Potential endogenous and exogenous interferences were evaluated by assaying control plasma from at least 20 different animals as well as 7 potential concomitant medications. None of the animals had been treated with NSAIDs or other anti-inflammatory drugs for at least 30 days prior to treatment or blood collection for blank plasma analysis.

2.5.2. Accuracy and precision

Validation samples were prepared and analyzed in quintuplicate on 3 or more separate days to evaluate the intra- and interday accuracy and precision. Accuracy, the measure of the closeness of agreement between the observed value and the "true" value, was determined by the use of plasma samples fortified with known amounts of firocoxib. The amount of the recovered analyte was compared to the amount added and expressed as percent recovery. In this method, accuracy and extraction recovery (absolute recovery) are the same as unknown amounts in matrix are quantified from a nonmatrix-based external standard curve. Precision, defined as the closeness of agreement among multiple independent observations, was expressed as the coefficient of variation or percent relative standard deviation around the mean. Intra- or interday precision was evaluated by determining the coefficient of variation for each fortification level for each set (intraday) or across all sets (interday). Horse plasma was fortified at the following concentrations: 25, 50, 100, 500, 1000, and 2500 ng/mL. Dog plasma was fortified at the following concentrations: 25, 50, 500, 1000, 2500, 5000, and 10,000 ng/mL.

2.5.3. Limit of detection and quantification

The limit of detection (LOD) and quantification (LOQ) were determined by signal-to-noise ratio evaluations of samples fortified from 5–50 ng/mL. The LOD was defined as the lowest concentration with a signal-to-noise ratio of at least 5. The LOQ was defined as the lowest concentration with a signal-to-noise ratio of at least 10 and acceptable accuracy (±20%) and precision (≤20%). Responses from control samples were also used to statistically evaluate the LOD and LOQ, calculated as the mean of the blanks plus three times the standard deviation of the mean for the LOD and the mean of the blanks plus six times the standard deviation of the mean for the LOQ.

2.5.4. Linearity

A calibration curve covering the LOQ was prepared with each set and included concentrations of 50, 500, 1000, 2500, 5000, and 10,000 ng/mL. This set of standards was run before and after the samples; both sets of standard peak areas were used to calculate the linear regression equation as well as the coefficient of determination (r^2). Blank samples were included with each set.

2.5.5. Stability

Standard solutions prepared at the beginning of the study and stored at −20 °C when not in use were compared to freshly prepared standard solutions at the end of the study. Stability of firocoxib in plasma was evaluated during storage at −20 °C for more than 2 years and after three, six, or eight freeze–thaw cycles at −20 °C. Post-preparation (extract) storage stability was assessed at room temperature after approximately 16 h.

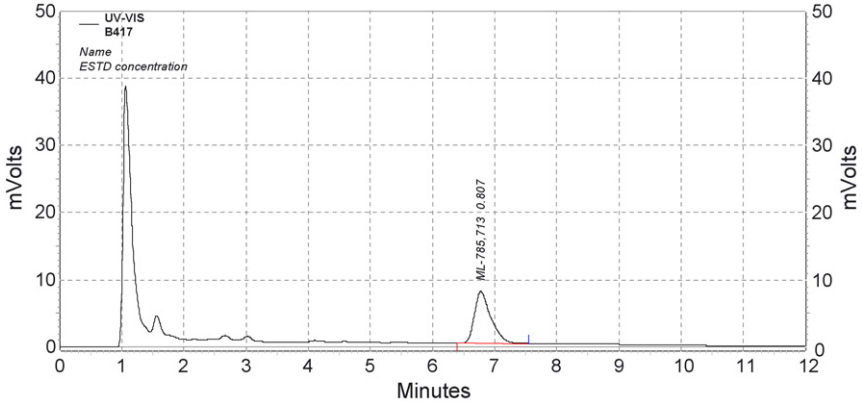

Fig. 2. Chromatogram of a fortified plasma sample showing peak tailing related to column deterioration prior to adding an in-line filter and mobile phase modifier (earlier retention time due to slightly different mobile phase composition evaluated during method development and validation, standard and sample retention time matched within set).

3. Results/discussion

3.1. Method development

This method was based on the facts that firocoxib is a non-ionizable molecule that is soluble in polar solvents, amenable to reversed-phase HPLC, and has a chromophore sufficient to allow UV detection at 290 nm. A sample preparation technique, protein precipitation, was evaluated because of its simplicity, but the resulting sample was not suitable for HPLC-UV analysis without additional clean-up. Liquid–liquid extraction was not tried as it is more time consuming and generates more solvent waste. A single solid-phase extraction clean up was found to be suitable. Internal standards were not investigated since the external standard method initially employed yielded acceptable accuracy, precision, and linearity, and correction by an internal standard was not necessary. Various combinations of organic solvents (methanol and acetonitrile) and water were evaluated as mobile phase components. Finally, a 55% water/acetonitrile-based mobile phase was selected as one that yielded sufficient resolution in a reasonable time, retention time less than 10 min. Mobile phase was prepared by either the volume/volume or dilution (quantity sufficient (qs) to volume) technique. Flow rate of either 1.0 or 1.2 mL/min was evaluated and resulted in acceptable chromatography and incorporated into the method. Depending on the mobile phase, flow rate, and chromatographic system used the retention time ranged from ~8 to 9.5 min. Three analytical columns were also tested during method development and the Inertsil™ ODS-3 found to produce acceptable chromatography. Increased column backpressure and unacceptable

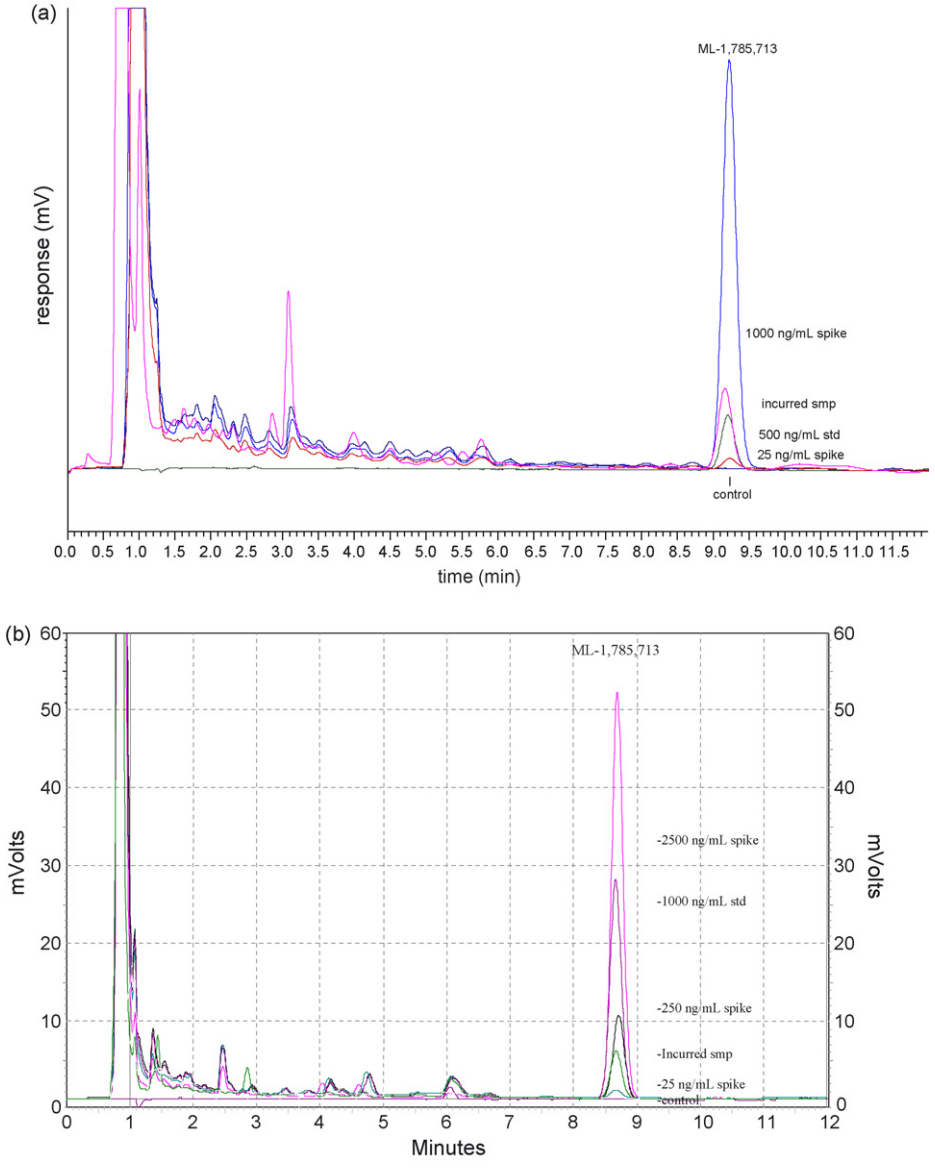

Fig. 3. (a) Chromatographic overlay representing a control (blank) horse plasma sample; 25 and 1000 ng/mL firocoxib (ML-1,785,713) fortified horse plasma samples; an incurred firocoxib plasma sample (1 min) from a horse administered a single intravenous dose of firocoxib (ML-1,785,713) at 0.1 mg/kg; and a 500 ng/mL standard. (b) Chromatographic overlay representing a control (blank) dog plasma sample; 25, 250, and 2500 ng/mL firocoxib (ML-1,785,713) fortified dog plasma samples; an incurred firocoxib plasma sample (6 h) from a dog administered a single oral dose of firocoxib (ML-1,785,713) at 5 mg/kg; and a 1000 ng/mL standard.

peak symmetry (Fig. 2) were observed after several injection sets requiring the analytical column to be replaced. First, 2-μm frits were placed in-line prior to the analytical column. This initially helped extend the life of the analytical column, but a longer column life was desirable. Second, trifluoroacetic acid was added to the mobile phase which appeared to completely resolve the problem. Several different concentrations of TFA were evaluated and a concentration of 0.025% had the desired effect of extending column life and improving peak symmetry. Concentrations below this had no effect and at higher concentrations the low pH impacted column life. Subsequently, during routine analyses a packed guard column/precolumn (Inertsil™ ODS-3), in conjunction with the TFA, was also used. In addition, it was found that the 10,000 ng/mL standard could be excluded from the standard curve and 100 and 250 ng/mL level standards included since sample responses were typically in the lower half of the curve.

3.2. Method validation

3.2.1. Selectivity

Under the method and chromatographic conditions used, no interferences were noted in the control samples around the retention time of firocoxib.

Control samples from 20 different horses and 45 different dogs were analyzed during the validation. Additionally, seven drugs commonly used in veterinary medicine and one used in human medicine were tested and found not to interfere chromatographically with firocoxib. The drugs tested were ketoprofen, carprofen, acepromazine, phenylbutazone, ivermectin, praziquantel, ketamine, and rofecoxib. Representative chromatograms are shown in Fig. 3.

3.2.2. Accuracy and precision

Excellent accuracy, defined as absolute recovery, and precision for both interday and intraday evaluations were established for concentration ranges of 25–2500 ng/mL for horses and 25–10,000 ng/mL for dogs. Typically, recoveries were in the 90% range and coefficients of variation were less than 5%. The interday results for horse and dog samples are summarized in Table 1 and correspond to work from different analysts on different days, that is, intermediate precision or reproducibility. Representative intraday results from one analyst demonstrating repeatability for horse and dog samples are also presented in Table 1.

3.2.3. Limit of detection and quantification

The established LOD and LOQ were 10 and 25 ng/mL, respectively. Acceptable accuracy and precision (88 ± 6%) were obtained at the LOQ which is contained within the standard curve. The signal-to-noise ratios for the defined LOD and LOQ were ~16 and 30, respectively. For comparison, the statistically calculated LOD and LOQ values were 5 and 10 ng/mL. The statistical limit of detection was calculated as the mean of the noise ($n = 20$) + 3x standard deviation of the mean, where the noise (response) was determined at the retention time of firocoxib in control matrix extracts. The equivalent concentration was determined using the mean calibration curve obtained from the linearity portion of the study. The limit of quantitation was determined similarly except using 6x the standard deviation.

3.2.4. Linearity and response function

Both unweighted and 1/x weighted standard curves were evaluated and shown to yield acceptable and comparable results (Table 2), so the simplest linear regression model (unweighted)

Table 1
Intra- and interday accuracy (percent recovery) and precision (coefficient of variation) of the method for the determination of firocoxib from horse and dog plasma

Horse							
Fort level (ng/mL)	Intraday						Interday ($n \geq 49$)
	Rep 1 (% rec)	Rep 2 (% rec)	Rep 3 (% rec)	Rep 4 (% rec)	Rep 5 (% rec)	Avg ± CV (%)	Avg ± CV (%)
25	91	99	91	88	82	90 ± 6	88 ± 6
50	92	90	93	91	96	92 ± 2	91 ± 5
100	96	98	96	94	99	97 ± 2	92 ± 5
500	95	96	90	97	98	95 ± 3	93 ± 5
1,000	96	96	96	95	95	95 ± 1	92 ± 2
2,500	94	93	98	98	95	96 ± 2	92 ± 3
Dog							
Fort level (ng/mL)	Intraday						Interday ($n \geq 15$)
	Rep 1 (% rec)	Rep 2 (% rec)	Rep 3 (% rec)	Rep 4 (% rec)	Rep 5 (% rec)	Avg ± CV (%)	Avg ± CV (%)
25	90	90	93	88	85	89 ± 3	103 ± 3
50	104	91	94	89	94	94 ± 6	100 ± 4
500	93	95	96	95	93	95 ± 1	96 ± 1
1,000	95	94	96	95	95	95 ± 1	96 ± 1
2,500	96	95	96	97	98	96 ± 1	96 ± 3
5,000	96	96	96	97	96	96 ± 1	96 ± 1
10,000	97	98	103	102	102	100 ± 3	98 ± 2

Fort: fortification; rep: replicate; % rec: percent recovery; avg: average (unit: %); CV: coefficient of variation (unit: %); n: number of samples.

Table 2
Comparison of results from fortified horse plasma samples (Set A) using either a 1/*x* weighted or unweighted linear regression analysis of the standard curve ranging from 50 to 5000 ng/mL and inter-assay linearity statistics

Fortification level = 50 ng/mL	Percent recovery	
	1/*x* weighted	Unweighted
Sample 1	101	103
Sample 2	95	98
Sample 3	100	102
Sample 4	96	98
Sample 5	101	103
Average ± coefficient of variation	98 ± 3	101 ± 3
Curve parameters		
Slope	1.10E-05	1.10E-05
Intercept	−0.0001	0.0052
Coefficient of determination	1.00	1.00

Inter-assay linearity ($n = 4$) (average ± standard deviation)

Coefficient of determination (r^2)	Slope	Intercept
0.9998 ± 0.0003	1.102E-05 ± 1.882E-07	0.00448 ± 0.00216

(Fig. 4) was chosen for routine use. Coefficients of determination were >0.99 in all cases and the slopes were significantly ($p < 0.05$) different from 0.

Response linearity was determined by regression analyses of calculated concentration *versus* theoretical concentration for fortified horse and dog plasma samples. The output (Fig. 5) provides evidence that a linear model describes the relationship between the calculated (response) and theoretical concentrations over several orders of magnitude, 25–5000 ng/mL. The coefficient of determination was >0.99 for both horse and dog.

3.2.5. Stability

The standard solutions were stable for almost 2 years when stored at ∼−20 °C. Except for the 100 ng/mL concentration standard, the means of the standard solutions at the beginning and the end of the study were equivalent (Table 3). Based on the results, the authors conclude the difference observed for the 100 ng/mL standard may be attributed to sample preparation error rather than lack of stability. Inherent variability of response at the lower level may also have been a contributor. Long-term stability studies showed no significant degradation of firocoxib in horse or dog plasma samples stored >2 years at −20 °C. The average percent difference between initial results and stability results was less than 15%. Overnight (∼16 h) stability at room temperature was also demonstrated for the post-preparation samples (extracts), Table 4. Triplicate fortified samples (500 ng/mL) were prepared for each freeze–thaw cycle scenario and the average stability results (Table 5) were ≥86%. Horse plasma was used to perform the freeze–thaw test, but similar results would

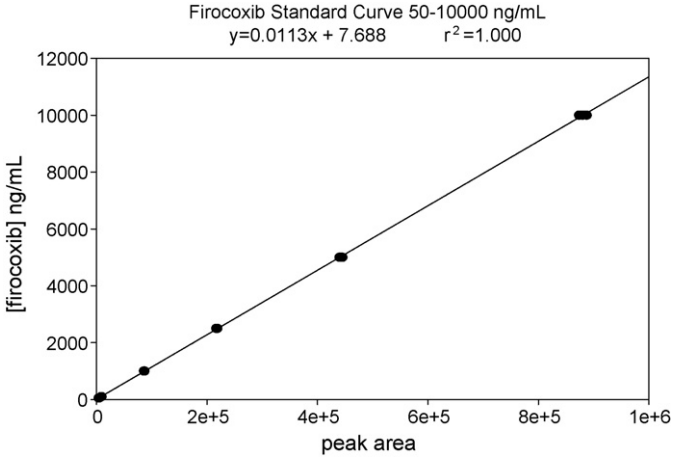

Fig. 4. Dog Set B Firocoxib Standard Curve from 50–10000 ng/mL (unweighted linear regression) with a Normal Distribution and a p-value < 0.0001 for the Slope Based on ANOVA.

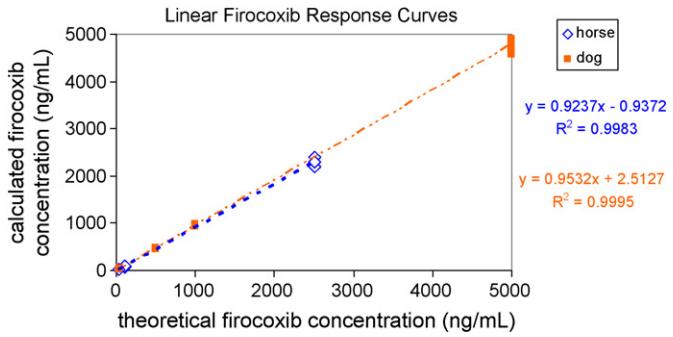

Fig. 5. Dose response curve for horse plasma fortified at 25, 100, and 2500 ng/mL ($n = 12$ each level) and dog plasma fortified at 25, 50, 500, 1000, and 5000 ng/mL ($n = 14$ or 15 each level).

Table 3
Evaluation of firocoxib standard solution stability using the Student's *t*-Test (95% confidence level)

Standard solution (ng/mL)	Initial mean value (ng/mL)	Reassay mean value (ng/mL)*	*p*-value
50	48.8	54.8	0.07
100	97.5	103	<0.01
500	505	498	0.37
1000	989	1010	0.18
2500	2510	2470	0.13
5000	4990	5000	0.54

p-value > 0.05 indicates no statistically significant difference.
* Standard solutions reassayed 23 months later.

Table 4
Post-preparation (extract) stability of firocoxib at room temperature

	50 ng/mL		1000 ng/mL	
	Original inj (% rec)	Reinj (% rec)	Original inj (% rec)	Reinj (% rec)
Sample 1	104	95	95	96
Sample 2	91	96	94	97
Sample 3	94	99	96	98
Sample 4	89	97	95	97
Sample 5	94	103	95	96
Avg ± CV	94 ± 6	98 ± 3	95 ± 1	97 ± 1
Percent difference of average		4		2

Inj: injection; reinj: reinjection; % rec: percent recovery; avg: average; CV: coefficient of variation.

Table 5
Freeze–thaw sample stability of firocoxib using fortified (500 ng/mL) horse plasma

Number of freeze/thaw cycles	Average firocoxib concentration (ng/mL)	Average percent recovery ± coefficient of variation
0 Cycles ($n = 3$)	450	90 ± 8
3 Cycles ($n = 3$)	431	86 ± 7
6 Cycles ($n = 3$)	475	95 ± 2
8 Cycles ($n = 3$)	448	90 ± 7
Overall ($n = 12$)	451	90 ± 7

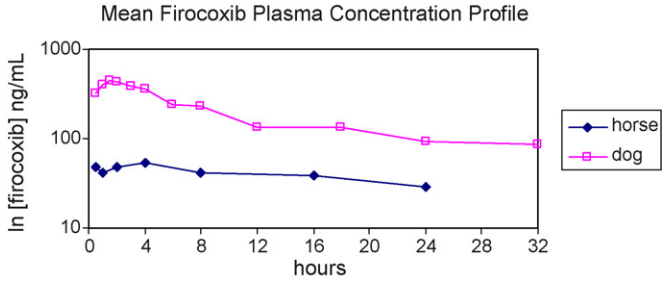

Fig. 6. Mean firocoxib plasma concentration profile after a single oral dose of 5 mg/kg to dogs ($n = 8$) or 0.1 mg/kg to horses ($n = 12$).

be expected using dog plasma. Overall, the results indicate that firocoxib is a very stable compound for extended periods of time.

3.2.6. *Application to pharmacokinetic studies*

The validated method was applied to numerous pharmacokinetic studies following oral and intravenous administrations to dogs and horses and the peak plasma concentrations were within the validated concentration ranges, *i.e.*, <1500 ng/mL for the dog and <200 ng/mL for the horse (Fig. 6). After oral administration, firocoxib is relatively well absorbed (oral bioavailability greater than 75% in the horse) [27] and distributed, biotransformed into dealkylated compounds and glucuronide conjugates with no or low activity, and eliminated in the urine and feces with a half-life of approximately 8 h in dogs. Also, based upon efficacy studies completed during the registration process, the threshold for effect (approximately 100 ng/mL for the dog and 30 ng/mL for the horse) levels were also within the validated limits of the method [28].

4. Conclusion

A facile and robust method, with a single solid-phase extraction step that reduces the sample preparation time and solvent usage relative to liquid–liquid extraction, was developed and validated and is reported for the first time. It uses common, commercially available equipment and reagents, and is suitable for the analysis of pharmacokinetic or drug monitoring samples (~50 samples can be prepared in an 8 h day with overnight analysis by HPLC-UV). The method is selective, accurate, precise, and practical for the quantitation of firocoxib from horse and dog plasma. It achieves a limit of quantitation (25 ng/mL) similar to that reported for other single drug HPLC-UV methods (5 and 10 ng/mL for valdecoxib and rofecoxib, respectively) and better than those reported for a multi-drug method (over 1000 ng/mL for both valdecoxib and rofecoxib) [20,22,23]. Moreover, if a 2 mL plasma sample is used, as would be available from horses, the LOQ can be lowered to 7.5 ng/mL. Additionally, the extraction recovery of firocoxib using this method was much higher, namely ~90% or higher versus ~76%, compared to other methods for related compounds such as etoricoxib [23], and with very good precision. Accordingly, an internal standard was not used in this method as is reported in so many methods in the literature [20,22,23,29]. The exclusion of an internal standard also simplifies the standard and sample preparation as well as the data processing, and in some instances improves the precision [30].

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