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Short communication

Determination of firocoxib in equine plasma using high performance liquid chromatography

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ABSTRACT

A new method of analysis has been developed and validated for the determination of firocoxib, a new nonsteroidal anti-inflammatory drug (NSAID) approved for use in horses and dogs to control pain and inflammation associated with osteoarthritis. Following a liquid extraction using ethyl acetate:hexane (40:60), samples were separated by isocratic reversed-phase HPLC on a Sunfire C_{18} column and quantified using UV detection at 290 nm. The mobile phase was a mixture of water with 0.025% trifluoroacetic acid and acetonitrile, with a flow-rate of 1.1 ml/min. The procedure produced a linear curve over the concentration range 5–1500 ng/ml with a lower limit of quantification of 5 ng/ml. Intra- and inter-assay variability was less than 7%. The average recovery was 98%. The method is suitable for the analysis of clinical samples from pharmacokinetic studies and can also be used for small volume sample sizes.

1. Introduction

Firocoxib, 3-(cyclopropylmethoxy)-4-(4-(methylsulfonyl)phenyl)-5,5-dimethylfuranone, is part of the coxibs which are non-steroidal anti-inflammatory drugs (NSAIDs). It has been approved for use in horses and dogs to control pain and inflammation associated with osteoarthritis [1]. It has been used in the treatment of food producing animals in Europe [2]. It is a fast-acting cyclo-oxygenase (COX) inhibitor that is highly specific for COX-2 and has little effect on COX-1 isozymes. Cyclo-oxygenase plays an important physiologic role in the production of prostaglandins and thromboxanes, which are highly active biologic compounds [3]. COX is a heme-containing enzyme that catalyzes reactions leading to molecules that regulate physiologic function, such as mediation of inflammatory response, gastrointestinal mucosal blood flow, regulation of blood flow in the kidney and platelet aggregation [4]. Thus inhibition of COX blocks prostaglandin synthesis and prostaglandin-mediated effects, such as inflammation.

Traditional NSAIDs are nonselective for cyclooxygenase and consequently have a narrow therapeutic window, with the consequences of gastric ulcers, renal damage, and prolonged bleeding time as the primary side effects. Newer NSAIDs with some selectivity for COX-2 have an improved therapeutic window but may still frequently inhibit COX-1 at therapeutic concentrations [5]. Coxibs are selective for COX-2 and should not inhibit COX-1 at therapeutic levels. Data from human studies with coxibs indicates that selective COX-2 inhibitors have fewer serious gastrointestinal side effects associated with their use than non-selective NSAIDs, such as naproxen, ibuprofen or aspirin [5]. Firocoxib exhibits high selectivity for the inhibition of COX-2. In vitro canine whole blood assays have demonstrated firocoxib to be 350–430-fold more selective for COX-2 than for COX-1 [6].

A literature search revealed only three methods [1,2,7] of analysis for firocoxib in plasma, two involve the use of mass spectrometry [2,7] and all involve the use of solid phase extraction cartridges. We describe a reliable, less time consuming, cost effective, highly selective and reproducible chromatographic method for use in pharmacokinetic studies in horses. The utilization of a liquid extraction eliminates the use of costly solid phase extraction cartridges and our limit of quantification is more than sufficient for firocoxib studies and does not require the use of mass spectrometry. The method could also be used for small volume samples making it useful for toy breeds and small exotic animals. This method has been used to successfully determine firocoxib concentrations in studies conducted at this institution.

2. Experimental

2.1. Reagents and chemicals

HPLC grade acetonitrile, methanol, and trifluoroacetic acid as well as all reagent grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Firocoxib (Fig. 1) was a gift from Merial (Duluth, GA) while deracoxib (Fig. 1) was a gift

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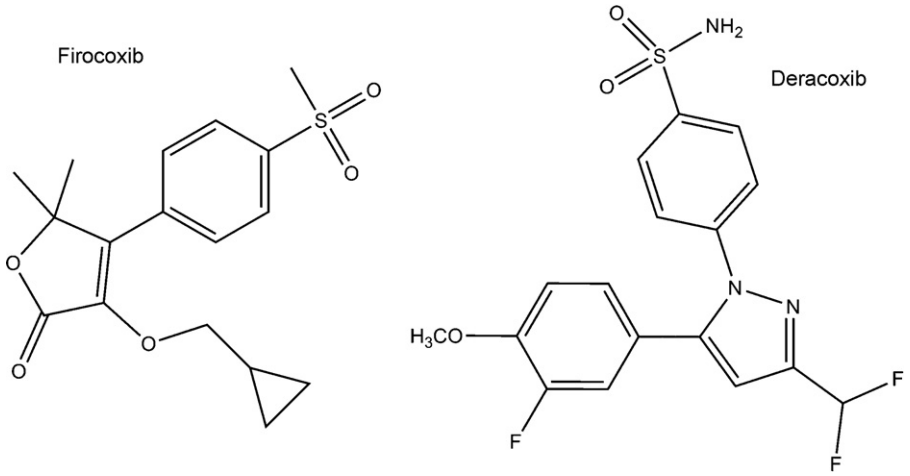

Fig. 1. Structure of firocoxib and deracoxib.

from Novartis (East Hanover, NJ). Water was obtained from a Barnstead (Dubuque, IA) Nanopure Infinity ultrapure water system.

2.2. Chromatography

The HPLC system consisted of a 2695 separation module and a 2487 UV detector (Waters, Milford, MA). Empower software (Waters) was used for data acquisition and processing. Firocoxib was separated on a Waters Sunfire C_{18} (150 mm × 4.6 mm, 5 μm) column with a Sunfire C_{18} (20 mm × 3.9 mm) guard column. The mobile phase was a mixture of water with 0.025% trifluoroacetic acid and acetonitrile (50:50, v/v). All solutions were filtered through a 0.22 μm filter and degassed before their use. The flow rate was 1.1 ml/min and the column temperature was ambient. UV absorbance was measured at 290 nm.

2.3. Preparation of calibration standards

Firocoxib and deracoxib were dissolved in methanol to produce stock concentrations of 100 μg/ml. Appropriate dilutions of stock concentrations were prepared to produce working stock solutions. Standards were stored at 4 °C and were stable for eight months. For preparation of calibration standards and quality control samples, appropriate aliquots of the stock solutions were added to untreated plasma. The final concentrations were 5, 10, 25, 50, 100, 250, 500, 1000 and 1500 ng/ml for the calibration standards and 7.5, 175, 750 and 1250 ng/ml for quality control samples. Calibration standards and control samples were treated the same as samples. Linearity was assessed by linear regression analysis. The calibration curve had to have a correlation coefficient of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except lower limit of quantification (LLOQ) which was set at 20%.

2.4. Sample preparation

Firocoxib was extracted from plasma using a liquid extraction. Previously frozen plasma samples were thawed and 1 ml of plasma placed into 15 ml screw cap tubes. Fifteen microliters of deracoxib (internal standard, 100 μg/ml) was added followed by 6 ml of ethyl acetate:hexane (40:60). Tubes were vortexed for 1 min then centrifuged for 20 min at 1000 × *g*. The supernatant was removed to glass tubes and evaporated with nitrogen. Residues were re-dissolved in 300 μl of mobile phase and placed into chromatography vials. A 50 μl sample was injected into the HPLC.

For 0.1 ml plasma samples 15 μl of internal standard (10 μg/ml deracoxib) and 3 ml of ethyl acetate:hexane (40:60) were added. The vortexing, centrifugation, and evaporation steps were the same. Residues were re-dissolved in 125 μl of mobile phase and 100 μl was injected.

3. Results

Endogenous plasma components did not interfere with the elution of the compounds of interest. Blank plasma samples for specificity testing were prepared in the same way as study samples. Six different blank plasma samples were used in the pre-validation process and a blank sample from each subject was included in the analysis. Fig. 2 shows chromatograms of a (A) blank plasma sample, (B) a 500 ng/ml plasma standard and (C) a plasma sample from a subject 2 h after a 0.1 mg/kg dose of firocoxib was administered. Retention times were 6.9 min for firocoxib and 7.9 min for deracoxib.

The plasma peak area ratio (area of firocoxib divided by internal standard area) versus concentration was plotted which produced a linear curve for the concentration range used (5–1500 ng/ml) with the correlation coefficients ranging from 0.998 to 0.999. The mean slopes, intercepts and r^2 values are reported in Table 1. Intra-assay RSD for plasma spiked with specific concentrations of firocoxib ranged from 3.3 to 7.2% (Table 1). The LLOQ was 5 ng/ml. The inter-assay RSD ranged from 1.7% to 6.6%. The individual values are reported in Table 2. The area of firocoxib from spiked plasma was

Table 1
Intra-assay accuracy, precision and assay linearity for firocoxib in horse plasma.

Concentration added (ng/ml)	Concentration measured (ng/ml) (mean ± SD)	RSD (%)
Intra-assay variability (n = 4)		
7.5	7.2 ± 0.5	7.2
175	175 ± 11	6.1
750	760 ± 25	3.3
1250	1252 ± 75	5.9
	Mean ± SD	RSD (%)
Assay linearity (n = 4)		
Intercept	0.00151 ± 0.00018	12.4
Slope	0.00143 ± 0.00126	0.88
r^2	0.99995 ± 0.00570	0.005

SD: standard deviation; n: number of samples and number of curves; RSD: relative standard deviation.

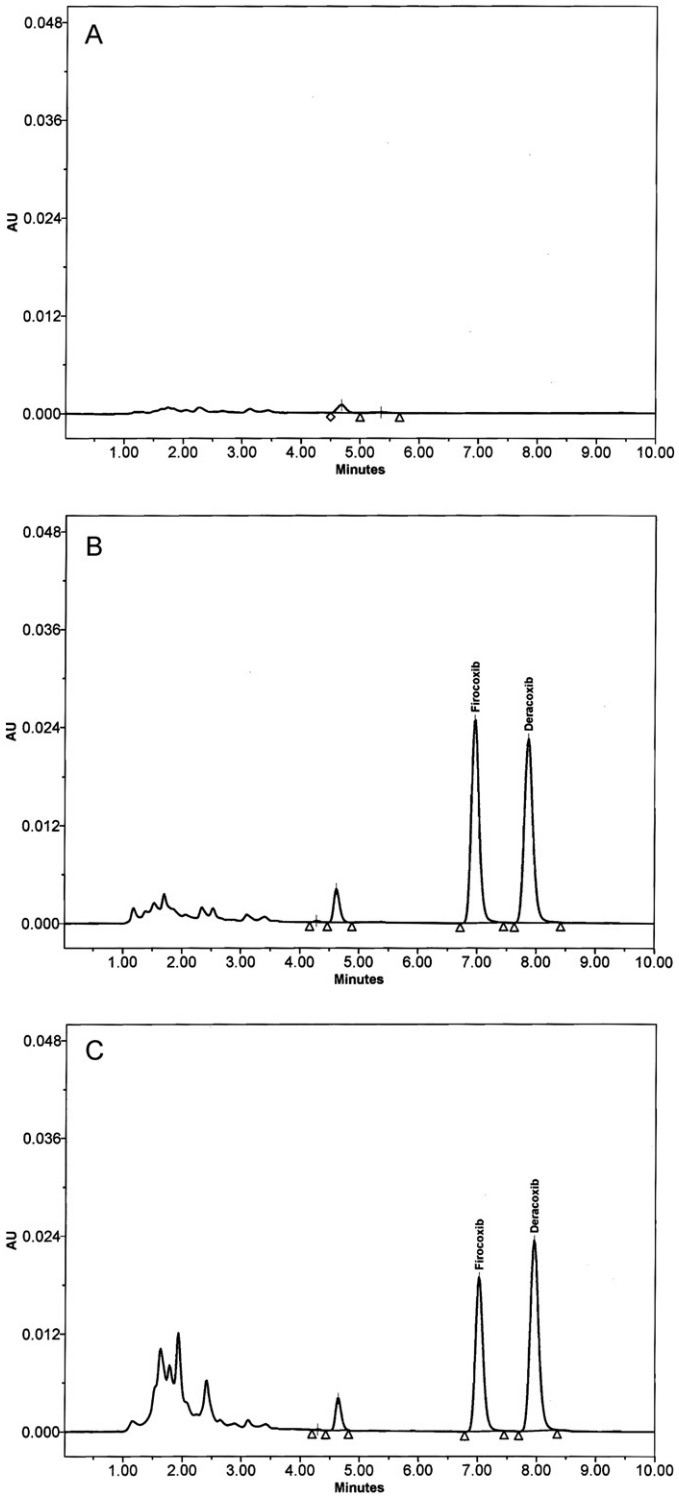

Fig. 2. Chromatograms for firocoxib. (A) Blank plasma sample, (B) 250 ng/ml plasma standard and (C) a plasma sample after a 0.1 mg/kg dose of firocoxib (195 ng/ml).

compared with the directly injected analyte at concentrations 7.5, 175, 750 and 1250 ng/ml to calculate recovery. Values ranged from 94 to 99% (Table 2). The recovery of the internal standard, deracoxib was 92% for the concentration used in the assay (100 μg/ml). The limit of detection for firocoxib in plasma was 2.5 ng/ml. This represents a peak approximately three times baseline noise.

Values for the 100 μl sample size were very similar to those seen for the 1 ml sample. Recovery ranged from 94 to 100% for the same quality control concentrations. The recovery of the internal standard (10 μg/ml) was 94%. Inter- and intra-assay variability were less than 10% and the LLOQ was still 5 ng/ml.

Table 2
Inter-assay variability and recovery for firocoxib in horse plasma ($n = 4$).

Concentration added (ng/ml)	Concentration measured (ng/ml) (mean ± SD)	RSD (%)	Recovery (%)
7.5	7.4 ± 0.5	6.6	94
175	174 ± 3	1.7	99
750	750 ± 14	1.9	99
1250	1249 ± 36	2.9	99

SD: standard deviation; n: number of days; RSD: relative standard deviation.

Testing of autosampler and short term stability of standards for 24 h showed that extractions were stable. The testing revealed that extracted plasma samples were stable for 24 h in an autosampler at ambient temperature. For the concentrations of 7.5, 175, 750 and 1250 ng/ml, there was less than 1% drug loss after 24 h in the autosampler and after 24 h of short term storage in the refrigerator at 4 °C. After storage in a −80 °C freezer for 30 days there was less than 1% drug loss for the same concentrations.

4. Discussion

To be useful for pharmacokinetic investigations, a method must be simple as well as sensitive and reproducible. Firocoxib was quantified in equine plasma by combining a liquid extraction with isocratic reversed-phase HPLC analysis. We tried solid phase extraction cartridges (Oasis HLB) as well as 5, 6, and 7 ml volumes of chloroform:isopropyl alcohol (80:20) as possible extraction methods. However, both methods extracted constituents that interfered with the elution of the compounds of interest and the recovery was much lower than that of the ethyl acetate hexane mixture. The combination of ethyl acetate and hexane (40:60) produced the greatest recovery and optimum peak shape. We also varied the volume of the ethyl acetate:hexane mixture. Five ml of the mixture did not produce adequate recovery while 7 ml produced recoveries similar to the 6 ml volume used. Therefore a 6 ml extraction volume was chosen. We have added deracoxib as an internal standard which corrects for intra- and inter-assay variability in the extraction. Our lower limit of quantification (5 ng/ml) is better than the UV method of Kvaternick et al. [1] on the other hand, methods [2,7] using mass spectrometry do have lower values (1.0 and 2.0 ng/ml) than our method. However, mass spectrometry is expensive and not available in all laboratories and our limit of quantification and recovery are more than adequate for use in pharmacokinetic studies. If a lower limit of quantification is necessary the injection volume could be increased.

The stability studies indicate that samples were stable for 24 h after extraction. If there was an equipment malfunction, samples could be reanalyzed. Samples in our studies were thawed one time and analyzed; therefore, freeze–thaw studies were not conducted. Also studies conducted by Kvaternick et al. [1] indicated that plasma samples were stable for up to 2 years in the freezer (−20 °C) and could undergo several freeze–thaw cycles without degradation. We did evaluate short term storage of 30 days in a −80 °C freezer and found similar results to those of Kvaternick et al. [1].

This analytical procedure was validated in terms of selectivity, recovery, linearity, LLOQ, precision, and accuracy. Our procedure eliminates the need for expensive solid phase extraction cartridges, and mass spectrometry, which is not always readily available. It also provides the addition of an internal standard which can be used to correct for extraction variability. The limit of quantification and recovery are more than adequate for use in pharmacokinetic

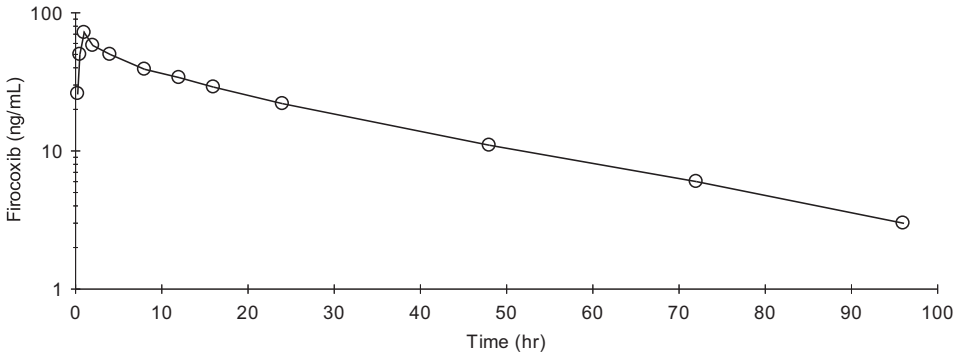

Fig. 3. Concentration time profile of firocoxib after a 0.1 mg/kg oral dose to a horse.

studies. The procedure could also use a 100 μl sample size making it useful for toy breeds and small exotic animals if necessary.

5. Conclusion

In conclusion, the results of the study indicate that this HPLC procedure represents a highly specific and reproducible method that provides consistent quantification of firocoxib. The method was found to be suitable for the generation of data from pharmacokinetic studies conducted at this institution which involved the analysis of over 144 samples. Fig. 3 is a representative concentration-time profile from one of the horses involved in the study after receiving 0.1 mg/kg orally. The half-life, T_{max}, C_{max} and AUC (area under concentration–time curve) were 24 h, 1 h, 72 ng/ml and 1628 h*ng/ml, respectively.

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