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Research Article

A Validated Method for the Determination of Firocoxib in Equine Tissues

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Abstract

A new reversed-phase High Performance Liquid Chromatography (HPLC) method was developed and validated for the determination of firocoxib in equine tissue. Firocoxib was detected by ultraviolet detection at 290nm after undergoing a liquid extraction using ethyl acetate: hexane (40:60) and separation on a Sunfire C18 column. The mobile phase consisted of water with 0.025% trifluoroacetic acid and acetonitrile (50:50), with a flow rate 1.1 mL/min. A concentration range of 5-1500ng/gm produced a linear curve with r2>0.99. The lower limit of quantification was 5ng/gm. The intra and inter assay variability was <10% and the average recovery was 97%.

Keywords: Firocoxib; HPLC; Tissue; UV detection

Introduction

Firocoxib, 3-(cyclopropylmethoxy)-4-(4-(methyl-sulfonyl) phenyl)-5, 5-dimethylfuranone, is a coxib-class Non-Steroidal Anti-Inflammatory Drug (NSAID) approved for use in horses and dogs [1]. It is a Cyclo-Oxygenase (COX) inhibitor that is highly specific for COX-2 and has little effect on COX-1 enzymes. Cyclo-oxygenase plays an important role in the production of prostaglandins and thromboxanes [1]. The inhibition of COX blocks prostaglandin synthesis and prostaglandin-mediated effects including inflammation.

Placentitis is well established as a significant cause of pregnancy loss in mares. In a study looking at 1800 mares during a 24-year period it was found that 64% of the pregnancy losses were due to placentitis [2]. Data in horses support the addition of an anti-inflammatory agent to the antimicrobial therapy for prevention of preterm delivery after placental infection [3]. The specificity of firocoxib for COX-2 inhibition, and its potential anti-inflammatory effects, has made it a desirable drug for the treatment of placentitis. To validate the use of firocoxib in mares with placentitis, it is critical to determine if the drug attains therapeutic concentrations in target tissues.

There are methods for detecting firocoxib in plasma, urine and milk [1,4-8]. However, a literature search revealed no published methods for the determination of firocoxib in tissue. A simple and reliable method to determine the concentration of firocoxib in equine tissue has been developed in the Pharmacology laboratory at the University of Tennessee using a liquid extraction technique, ultraviolet detection, and reverse phase HPLC.

Materials and Methods

Equipment

The chromatography system consisted of a 2695 separation module and a 2487 ultraviolet detector (Waters, Milford, MA). Empower software (Waters) was used for data acquisition and processing. Firocoxib separation was achieved on a Waters Sunfire C_{18} (4.6mm x 150 mm, 3.5 µm) column preceded by a 3.5µm Sunfire C_{18} guard column (20 mm x 3.9 mm).

Chromatographic conditions

The mobile phase was an isocratic 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 (22°C).Ultraviolet detection occurred at 290 nm.

Preparation of standard solutions

Firocoxib was a gift from Merial (Duluth, GA). Deracoxib, the internal standard, was a gift from Novartis (East Hanover, NJ). All reagent grade chemicals and solvents were purchased from Fisher Scientific (Pittsburg, PA). Water was obtained from a Barnstead Nanopure Infinity (Dubuque, IA) ultrapure water system. Firocoxib and deracoxib (internal standard) were dissolved in methanol to

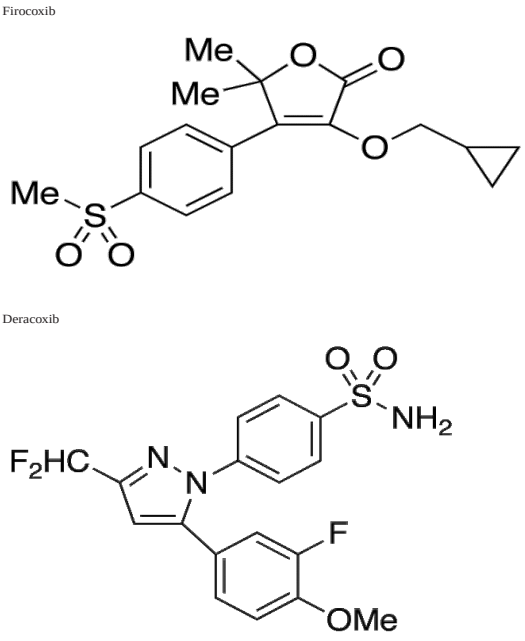

Figure 1: Structures of firocoxib and deracoxib.

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produce stock concentrations of 100 µg/mL. Dilutions of firocoxib were prepared in methanol to produce 10, 1 and 0.1 µg/mL working stock solutions. Standards were aliquoted into 2-mL vials to prevent evaporation and cross contamination. All solutions were protected from light in bottles wrapped in aluminum foil and stored at 4°C. By comparing standard areas over time, it was determined that they were stable for a minimum of 6 months.

Sample preparation

Previously frozen equine tissue was thawed then 0.5 gm weighed and placed into Potter-Elvehjem glass homogenizers with a PTFE pestle. Fifteen microliters of internal standard was added (deracoxib, 100 µg/mL) followed by 5 mL of ethyl acetate: hexane (40:60). The tissue was ground by hand for 3 min. The tissue and extraction solution were transferred to a 15mL glass screw top tube. The homogenizer tube and probe were rinsed with 1mL of extraction solution and the rinse added to the screw top tube. The tubes were vortexed for 60 seconds then centrifuged for 20 minutes at 1000xg. The supernatant was removed to a clean 16x100 glass tube and evaporated under a stream of nitrogen. They were re-dissolved in 300 µL of mobile phase (50:50), loaded into HPLC vials, and 50 µL were injected into the HPLC system.

Preparation of calibration standards

For preparation of calibration standards and quality control samples, appropriate volumes of stock solutions were added to untreated tissue. The final concentrations for our tissue calibration standard curve were 5, 10, 25, 50, 100, 250, 500, 1000, and 1500 ng/gm with quality control standards of 7.5, 175, 750, and 1250 ng/gm. Calibration standards and control samples were treated the same as test samples (Figure 1).

Method validation

Specificity: For specificity testing, untreated cervical star and amnion tissue were prepared in the same manner as study samples.

Linearity: The tissue peak ratio (area of firocoxib divided by the internal standard area) versus the concentration was plotted and linearity 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%.

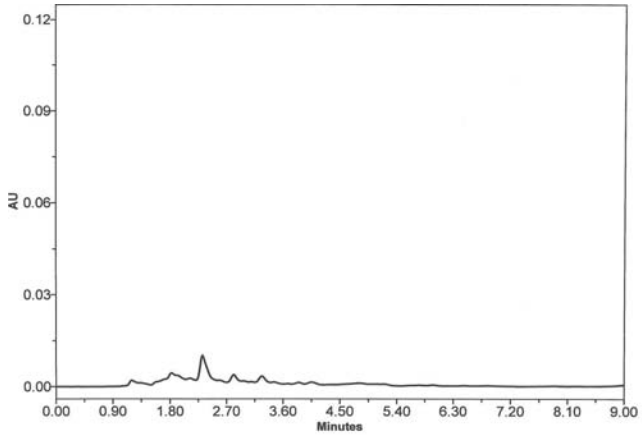

Figure 2: Chromatograms for firocoxib. (A) blank cervical star tissue.

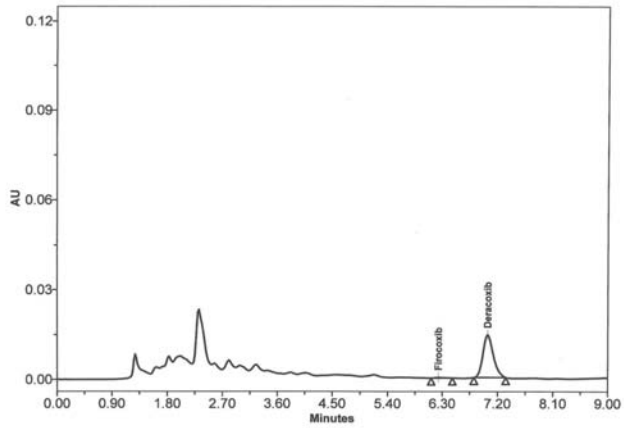

Figure 2: (B) 7.5 ng/gm spiked cervical star tissue standard.

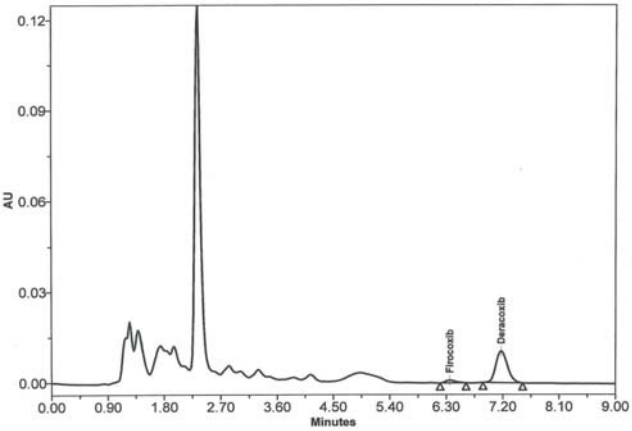

Figure 2: (C) cervical star tissue sample from a horse after oral administration of 0.1 mg/kg firocoxib.

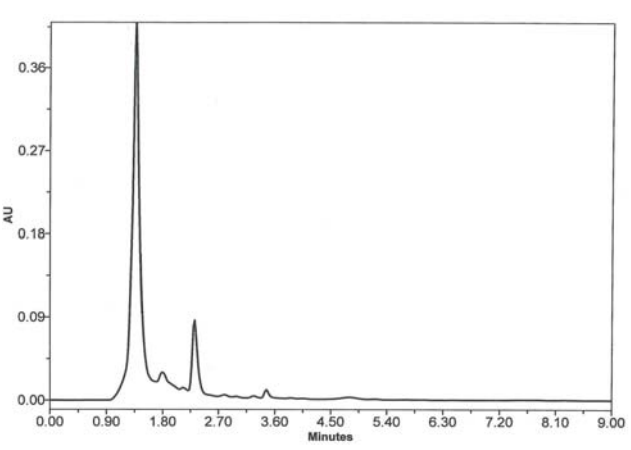

Figure 2: (D) blank amnion tissue.

Precision and accuracy: Five replicates of quality control standards at concentrations of 7.5, 175, 750, and 1250 ng/gm were used to determine the precision and accuracy of this assay.

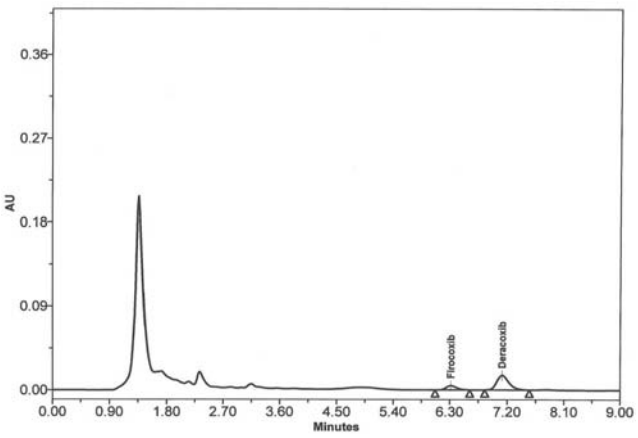

Figure 2: (E) 175 ng/gm spiked amnion tissue sample.

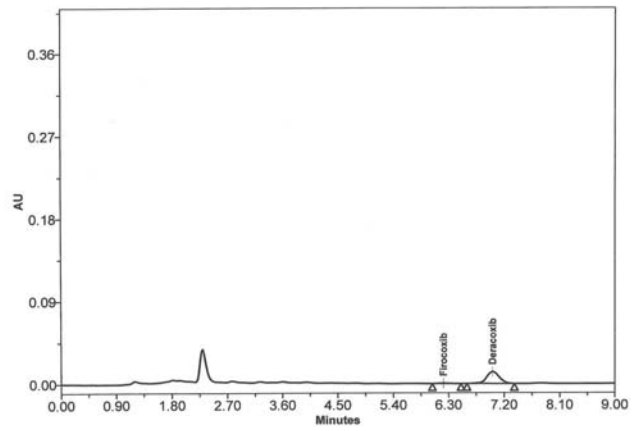

Figure 2: (F) an amnion tissue sample from a horse after oral administration of 0.1 mg/kg firocoxib.

Results and Discussion

For specificity testing, six different blank cervical star tissue samples were used in the pre-validation process. Endogenous tissue components did not interfere with elution of the compounds of interest. Figure 2 shows chromatograms of (A) a blank cervical star, (B) a 7.5 ng/gm spiked tissue standard and (C) a cervical star tissue sample from a horse after oral administration of 0.1 mg/kg firocoxib. The retention times for firocoxib and deracoxib were 6.31 and 7.12 minutes, respectively.

The tissue area ratio versus the concentration produced a linear curve for the concentration used (5-1500 ng/gm) with resulting correlation coefficients of >0.99. A typical equation for the calibration curve was y = 0013x + 0.0122, where y represents the peak area ratios of firocoxib to internal standard and x represents the tissue concentration of firocoxib in nanograms per gram. The intra and inter-assay Coefficient of Variation (CV) for tissue spiked with specific concentrations of firocoxib was used to determine accuracy and precision which ranged from 2.3-10%. A typical dose response curve for cervical star tissue is illustrated in Figure 3. The precision was below the set ± 15% for all quality control samples as shown in Table 1 and Table 2.

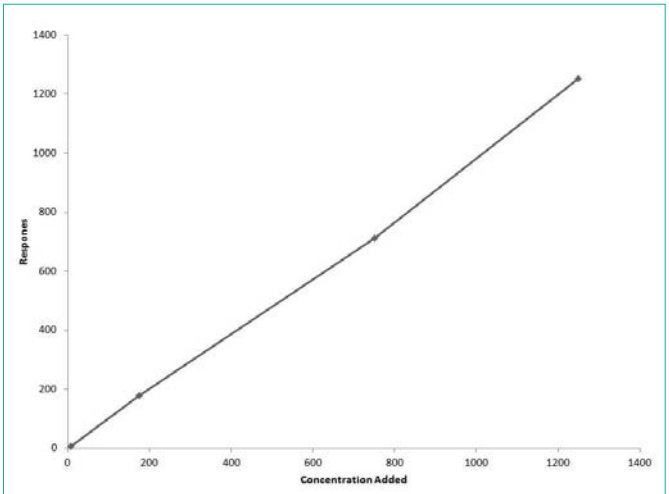

Figure 3: Dose response curve for cervical star tissue fortified with quality control concentrations.

The recovery (Table 2) of firocoxib from spiked tissue was determined by comparing the extracted areas with the directly injected analytes areas and ranged from 96% to 98%. The relative standard deviation was less than 10% for all values. The recovery of deracoxib was 95% and its relative standard deviation was 8.9%. The use of deracoxib as an internal standard corrects for intra- and inter-day assay variability in the assay. The Lower Limit of Quantification (LLOQ) was 5 ng/gm, which represents a peak approximately five times baseline noise.

Testing of short term stability of the quality control standards indicated there was no loss of drug after 24 hours in the auto sampler and no loss after 24 hours in the refrigerator at 4°C. Therefore, if there were a power or equipment failure, samples could be reanalyzed. After storage at -80°C for 30 days, there was less than 1% drug loss for the same quality control standards. This is two weeks longer than study samples were stored for analysis. Samples in our studies were thawed one time and analyzed; however, there was no loss of drug after two freeze-thaw cycles.

This method was also validated in amnion tissue. The chromatograms of (D) a blank amnion tissue, a (E) 175 ng/gm spiked

Table 1: Intra-assay accuracy, precision and assay linearity for firocoxib in cervical star tissue.

Intra-assay variability (n = 5)		
Concentration added (ng/gm)	Concentration measured (ng/gm) (mean ± S.D.)	R.S.D. (%)
7.5	6.7± 0.26	3.9
175	180 ± 5	2.8
750	733 ± 26	3.5
1250	1255 ± 29	2.3
Assay linearity (n = 5)		
	Mean ± S.D.	R.S.D. (%)
Y-Intercept	0.0122 ± 0.0002	1.4
Slope	0.0013 ± 0.00009	7.4
r^2	0.9989 ± 0.0007	0.07

SD: Standard Deviation; n: number of samples; RSD: Relative Standard Deviation

Table 2: Inter-assay variability and recovery for firocoxib in cervical star tissue and (n = 5).

Concentration added (ng/gm)	Concentration measured (ng/gm) (mean ± S.D.)	R.S.D. (%)	Recovery (%)
7.5	6.2 ± 0.64	10.0	98
175	179 ± 13	7.3	97
750	712 ± 54	7.6	96
1250	1251 ± 56	4.5	97

SD: Standard Deviation; n: number of days; RSD: Relative Standard Deviation

amnion tissue sample, and (F) an amnion tissue sample from a horse after oral administration of 0.1 mg/kg firocoxib are illustrated in Figure 2. The same number of blank amnion tissues were used for specificity testing as for the cervical star tissue and no endogenous components from this tissue were found to interfere with the elution of firocoxib or deracoxib. The retention times were 6.30 and 7.12 for firocoxib and deracoxib, respectively. A typical equation for this calibration curve was $y = 0.0013x + 0.0036$ with an r^2 of 0.999. The intra-assay coefficient of variation ranged from 1.4% to 6.2% while the inter-assay variation ranged from 0.9% to 8.7%. The recovery of firocoxib ranged from 94% to 102% while the average recovery of the internal standard was 95% ± 7%. The LOD and LLOQ were the same in the amnion tissue as the cervical star.

There are no other tissue methods in the literature to compare the one developed here but there are a few that have analyzed firocoxib in plasma [1,4,7] and milk [8]. Our lower limit of quantification (5 ng/gm) is better than the UV method of Kavaternick et al. [1], which is 25 ng/ml, and is the same as Hovanessian et al. [4] however; methods using mass spectrometry do have lower values (1 and 2 ng/ml) than our method. The analysis time for our method is shorter than the methods of Kavaternick et al. [1] and Hovanessian et al. [4] but slightly longer than the mass spectrometry methods. Mass spectrometry is expensive and not always available in all laboratories. If a lower limit of quantification is necessary the injection volume could be increased and if a shorter run time is needed then the mobile phase could be adjusted. Some potential factors that could impact sample analysis would include sample matrix, the condition of the patient (disease processes, renal or hepatic disease), insufficiently treated samples which could lead to the production of interfering peaks. The concurrent administration of other drugs may also impact study results. Any drugs that were going to be administered should be checked for interfere with the assay.

Table 3: Equine tissue results after oral administration of 0.1 mg/kg firocoxib.

Sample	Cervical Star (ng/gm)	Amnion (ng/gm)
P	79	41
T	232	35
D	92	45

Conclusion

This method is capable of quantifying firocoxib consistently and reliably in equine tissues. The procedure has been authenticated in terms of recovery, linearity, LLOQ, precision, and accuracy. Our results indicate that the HPLC method is reproducible and provides consistent quantification of firocoxib in tissue. This method has been successfully used to determine firocoxib concentrations in tissue samples analyzed at this institution (Table 3). The development of this method will allow investigators to determine if firocoxib reaches therapeutic concentrations in target tissues making it a potential candidate for the treatment of placentitis in horses. To our knowledge this is the first method developed to analyze firocoxib in tissues.

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