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Pharmacokinetics and effects on thromboxane B_2 production following intravenous administration of flunixin meglumine to exercised thoroughbred horses

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Flunixin meglumine is commonly used in horses for the treatment of musculoskeletal injuries. The current ARCI threshold recommendation is 20 ng/mL when administered at least 24 h prior to race time. In light of samples exceeding the regulatory threshold at 24 h postadministration, the primary goal of the study reported here was to update the pharmacokinetics of flunixin following intravenous administration, utilizing a highly sensitive liquid chromatography–mass spectrometry (LC-MS). An additional objective was to characterize the effects of flunixin on COX-1 and COX-2 inhibition when drug concentrations reached the recommended regulatory threshold. Sixteen exercised adult horses received a single intravenous dose of 1.1 mg/kg. Blood samples were collected up to 72 h postadministration and analyzed using LC-MS. Blood samples were collected from 8 horses for determination of TxB_2 and PGE_2 concentrations prior to and up to 96 h postflunixin administration. Mean systemic clearance, steady-state volume of distribution and terminal elimination half-life was 0.767 ± 0.098 mL/min/kg, 0.137 ± 0.12 L/kg, and 4.8 ± 1.59 h, respectively. Four of the 16 horses had serum concentrations in excess of the current ARCI recommended regulatory threshold at 24 h postadministration. TxB_2 suppression was significant for up to 24 h postadministration.

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INTRODUCTION

Flunixin meglumine is a nonsteroidal anti-inflammatory drug (NSAID) used commonly for treating musculoskeletal injuries in horses (Lee *et al.*, 1985). Currently, flunixin is one of three NSAIDs with an authorized threshold level in serum or plasma. Flunixin is classified as a Class 4 foreign substance by the Association of Racing Commissioners International (ARCI) and concentrations in excess of the threshold will result in a Class C penalty. The current ARCI recommended threshold for flunixin in regulatory samples is 20 ng/mL with a corresponding withdrawal time of 24 h when administered at the label dose of 1.1 mg/kg i.v. Many individuals interpret this to mean that administration of a single i.v. injection, 24 h prior to race time, will not result in a violation; however, recently, regulatory samples collected from one racing jurisdiction, that exceeded the threshold concentration for flunixin at 24 h, when the drug was administered at the recommended dose (1.1 mg/kg i.v.) have been found.

There are a number of previous reports describing the pharmacokinetics of flunixin following i.v. administration in the horse (Semrad *et al.*, 1985; Soma *et al.*, 1988; Jensen *et al.*, 1990; Coakley *et al.*, 1999; Lee & Maxwell, 2013). With respect to regulatory samples, however, only one of the aforementioned studies (Lee & Maxwell, 2013) utilized instrumentation sensitive enough to measure flunixin concentrations at or below the current ARCI recommended regulatory threshold and in that study sampling was terminated 24 h postdrug administration. As previous reports describing the pharmacokinetics of flunixin either used less sensitive analytical instrumentation or described plasma or serum concentrations for a shorter period of time than is necessary to establish when levels no longer exceed the regulatory threshold, these studies are not particularly amenable to establishing withdrawal times for

the 20 ng/mL regulatory threshold. In the study described here, we describe the pharmacokinetics of flunixin in exercised horses following intravenous administration of the label dose of 1.1 mg/kg, utilizing a highly sensitive liquid chromatography–mass spectrometry (LC-MS/MS) method currently employed by drug testing laboratories to analyze biological samples collected from racehorses.

The anti-inflammatory effects of NSAIDs are due to inhibition of cyclooxygenase enzymes (COX-1 and COX-2), and numerous studies have described the effectiveness of COX inhibition by flunixin in horses (Semrad *et al.*, 1985; Lees *et al.*, 1987; Soma *et al.*, 1992; Galbraith & McKellar, 1996; Brideau *et al.*, 2001; Beretta *et al.*, 2005). In one such study, Semrad *et al.* (1985) reported significant inhibition of COX-1 by flunixin following intravenous administration of 1.1 mg/kg for up to 12 h. Based on thromboxane B_2 (TxB_2) production, the authors estimated the minimum effective inhibitory concentration of flunixin to be 25 ng/mL; however, it is important to note that the limit of detection (LOD) of the assay in this study was 50 ng/mL (Semrad *et al.*, 1985). Therefore, a secondary objective of this study was to use a highly sensitive assay, capable of quantifying flunixin concentrations below the current ARCI recommendation and correlating those concentrations with COX inhibition.

MATERIALS AND METHODS

Animals

For the drug administration study, sixteen university-owned healthy-exercised adult Thoroughbred horses including eight geldings and eight mares (4–7 years of age; mean ± SD weight of 535 ± 33 kg) were studied. Eight of these horses (five mares and three geldings) were randomly selected by use of a random number generator for determination of COX-1 activity. Prior to and throughout the course of the study, horses were exercised 5 days a week, following standard protocols established by our laboratory. The general exercise protocol for these horses consists of 3 days per week on an Equineciser (Centaur Horse Walkers Inc., Mira Loma, CA, USA) (5 min walk; 20 min trot; 5 min walk) and 2 days per week on a high-speed treadmill (Mustang 2200; Graber AG, Fahrwangen, Switzerland) (5 min at 1.9 m/s; trot 5 min at 4 m/s; canter 2 min at 10 m/s; walk 5 min at 1.9 m/s or 5 min at 1.9 m/s; trot 5 min at 4 m/s at 4 degree incline; walk 5 min at 1.9 m/s).

Before beginning the study, horses were determined healthy and free of disease by physical examination, complete blood count, and a serum biochemistry panel that included aspartate aminotransferase, creatinine phosphokinase, alkaline phosphatase, total bilirubin, sorbitol dehydrogenase, blood urea nitrogen, and creatinine. Blood analyses were performed by the Clinical Pathology Laboratory of the William R. Pritchard Veterinary Medical Teaching Hospital of the University of California, Davis, using their standard protocols. Horses did not receive any other medications for at least 2 weeks prior to commencement of this study. This study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

Instrumentation and drug administration

A 14-gauge catheter was aseptically placed in each external jugular vein. The right jugular vein catheter was used for drug administration while the contralateral catheter was used for sample collection. Each horse was weighed immediately prior to drug administration and received a single intravenous dose of 1.1 mg/kg flunixin meglumine (Banamine; Merck Animal Health, Whitehouse Station, NJ, USA), followed by 5–10 mL of a heparinized saline flush (10 IU/mL). The right jugular vein catheter was removed following dosing.

Sample collection

Blood samples were collected at time 0 (immediately prior to drug administration) and at 5, 10, 15, 30, and 45 min and at 1, 2, 3, 4, 6, 8, 12, 18, 24, 30, 36, 48, 72, and 96 h postdrug administration. Prior to drawing each sample of blood for analysis of drug concentrations, 10 mL of blood was aspirated from the catheter and T-port extension set (combined internal volume <2 mL) and discarded. The catheter was flushed with 10 mL of a dilute heparinized saline solution (10 IU/mL) following each sampling. Catheters used for sample collection were removed following collection of the 18-h sample and the remaining samples collected by direct venipuncture. Blood samples were collected into serum separator tubes and placed at room temperature prior to centrifugation at 1620 ***g*** for 10 min. Serum was immediately transferred into storage cryovials and stored at −20 °C until analyzed (approximately 48 h from the final collection).

Urine samples were collected at time 0 (immediately prior to drug administration) and at 24, 48, and 72 h postdrug administrations. Urine samples were collected either by free catch or urinary catheterization (mares) when necessary. Urine samples were stored at −20° C until analysis (approximately 48 h from the final collection).

Flunixin meglumine serum sample analysis

The analytical reference standard flunixin meglumine was purchased from U.S. Pharmacopeia (Rockville, MD, USA) and internal standard D3-flunixin was purchased from Sigma-Aldrich (St. Louis, MO, USA). The stock solution of flunixin was prepared in acetonitrile and water, and internal standard D3-flunixin was prepared in methanol; both were prepared at 1 mg/mL and each analyte concentration was calculated using the free base (flunixin) molecular weight. Acetonitrile (ACN) and water were purchased from Burdick and Jackson (Muskegon, MI, USA). Methanol and buffer reagents were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic Acid, 97%, was purchased from Alfa Aesar (Ward Hill, MA, USA). The solvents were HPLC grade or better.

Serum sample analysis. Flunixin working solutions were prepared by dilution of the 1 mg/mL stock solutions with water to concentrations of 0.01, 0.1, 1, 10, and 100 ng/μL. Serum calibrators were prepared by dilution of the working standard solutions with drug-free equine serum to concentrations of 0.5, 1, 2.5, 5, 10, 25, 100, 250, 500, 1000, 2000, 3000, 4000, 6000, and 8000 ng/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples (serum fortified with analyte at three concentrations within the standard curve) were included with each sample set as an additional check of accuracy.

Prior to analysis, 500 μL of serum was diluted with 500 μL of ACN:1M Acetic Acid (9:1, v:v) containing 10 ng/mL of D3-flunixin internal standard, to precipitate proteins. Water was added to bring the final volume to 1050 μL. The samples were vortexed on a Glas-Col Large Capacity Mixer (Terre Haute, IN, USA) for 2 min to mix, refrigerated for 30 min, vortexed for an additional 1.5 min, and centrifuged in a Sorvall Super T21 centrifuge (Thermo Scientific, San Jose, CA, USA) at 3830 ***g*** for 10 min at 4° C and 40 μL injected into the LC/MS system.

The concentration of flunixin was measured in serum by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using positive heated electrospray ionization (HESI(+)). Quantitative analysis of serum was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific) coupled with a turbulent flow chromatography system (TFC TLX4 Thermo Scientific) having 1100 series liquid chromatography systems (Agilent Technologies, Palo Alto, CA, USA) and operated in laminar flow mode. The spray voltage was 3501 V. The vaporizer temperature was 292 °C, and the sheath and auxillary gas were 40 and 40, respectively (arbitrary units). Product masses and collision energies of each analyte were optimized by infusing the standards into the TSQ Vantage. Chromatography employed an ACE 3 C18 10 cm × 2.1 mm column (Mac-Mod Analytical, Chadds Ford, PA, USA) and a linear gradient of ACN in water with a constant 0.2% formic acid at a flow rate of 0.40 mL/min. The initial ACN concentration was held at 5% for 0.5 min, ramped to 90% over 2.33 min, held at that concentration for 0.50 min, before re-equilibrating for 3.5 min at initial conditions.

Detection and quantification was conducted using selective reaction monitoring (SRM) of initial precursor ion for flunixin (mass to charge ratio 297.065 (*m/z*)) and the internal standard D3-flunixin (mass to charge ratio 300.072 (*m/z*)). The response for the product ions for flunixin (*m/z* 109.1, 264.1, 279.2) and the internal standard D3-flunixin (*m/z* 264.1, 282.2) were plotted and peaks at the proper retention time integrated using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantitate flunixin in all samples by linear regression analysis. A weighting factor of 1/X was used for all calibration curves.

The response for flunixin was linear and gave correlation coefficients (R^2) of 0.99 or better. The intraday, interday, analyst to analyst precision, and accuracy of the assay were determined by assaying quality control samples in replicates ($n = 6$) for flunixin. Accuracy was reported as percent nominal concentration and precision was reported as percent relative standard deviation. The technique was optimized to provide a limit of quantitation (LOQ) of 1.0 ng/mL and a limit of detection (LOD) of approximately 0.25 ng/mL for flunixin.

Urine sample analysis. Flunixin working solutions were prepared by dilution of the 1 mg/mL stock solutions with water to concentrations of 0.1, 1, and 10 ng/μL. Urine calibrators were prepared by dilution of the working standard solutions with drug-free urine, collected from the same horses prior to commencement of the study, to concentrations of 0.5, 1, 2.5, 5, 10, 50, 100, 250, 500, 750, 1000, 1500, and 2000 ng/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples (urine fortified with analyte at three concentrations within the standard curve) were included with each sample set as an additional check of accuracy.

Prior to analysis, 0.5 mL of urine was diluted with 0.1 mL of water containing 0.1 ng/μL of D3-flunixin internal standard and 0.2 mL of *β*-glucuronidase enzyme, (Sigma-Aldrich) at 10 000 Units/mL in pH 5, 1.6 M acetate buffer. The pH was adjusted to 5 ± 0.5 with 2 N NaOH or 2 N HCl, as necessary, and heated in a sonicating Branson water bath (Danbury, CT, USA) at 65 °C for 2 h with 99 min of sonication. After cooling to room temperature, the pH was adjusted to 4 ± 0.5 with 2 mL of 0.2% formic acid in water. Samples were mixed gently and centrifuged at 3310 ***g*** for 5 min at 4 °C. The samples were subjected to solid-phase extraction using IST Isolute HAX 3 cc (200 mg) columns (Biotage, Charlotte, NC, USA). In brief, the columns were conditioned with 2 mL of methanol and then 2 mL of 0.2% formic acid in water before the samples were loaded onto the column. The samples were passed through the column using a CEREX system 48 Processor with positive pressure SPE manifold (SPE Ware, Baldwin Park, CA, USA); no <2 min for samples to pass through column. The columns were rinsed with 2 mL of (50:50, v:v) methanol:water prior to elution with 2 mL (98:2, v:v) methanol:acetic acid. Samples were dried under nitrogen in a Zymark TurboVap (McKinley Scientific, Sparta, NJ, USA) at 50 °C and dissolved in 200 μL of 5% acetonitrile in water, both with 0.2% formic acid. 30 μL was injected into the LC-MS/MS system. Detection and quantification was the same as described above for serum samples.

The response for flunixin was linear and gave correlation coefficients (R^2) of 0.99 or better. Accuracy was reported as percent nominal concentration, and precision was reported as percent relative standard deviation. The technique was optimized to provide a LOQ of 0.5 ng/mL and a LOD of approximately 0.25 ng/mL for flunixin.

Determination of COX-1 activity

Thromboxane B_2 analysis. Samples for determination of enzyme activity were collected at time 0 (immediately prior to drug

administration) and 2, 4, 8, 24, 30, 48, 72, and 96 h postdrug administration.

For determination of COX-1 activity, blood samples were collected into vacutainer tubes containing no anticoagulant and placed at 37° for 1 h. Samples were subsequently centrifuged at 720 ***g*** for 10 min and serum removed for determination of TxB_2 by LC-MS/MS.

The analytical reference standards for Thromboxane-B_2 (TxB_2) and internal standard d_4-Thromboxane-B_2 (d_4-TxB_2 at 100 ng/μL) were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). A stock solution of TxB_2 was prepared at 1 mg/mL in ethanol (Sigma-Aldrich). TxB_2 working solutions were prepared by dilution of the 1 mg/mL stock solutions with methanol (Fisher Scientific) to concentrations of 0.001, 0.01 0.1, and 1 ng/μL. Due to endogenous levels of TxB_2 in serum, calibrators were prepared by dilution of the working standard solutions in charcoal stripped serum at concentrations of 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, 5, 10, 20, and 50 ng/mL. Calibration curves were prepared fresh for each quantitative assay. In addition, quality control samples (at three concentrations within the standard curve) were included with each sample set as an additional check of accuracy. Prior to analysis, 0.25 mL of sample was mixed with 20 μL of methanol containing 0.625 ng/μL of d_4-TxB_2 internal standard solution and extracted with 2 mL Hexane:Ethyl Acetate (1:1, v:v) (Fisher Scientific) in 13 × 100 mm screw cap tubes. Samples were vortexed for 2 min, then rotated on a Glas-Col Rotator (Terre Haute, IN, USA) for 5 min at 40 revolutions per minute, before centrifugation at 1860 ***g*** for 2 min at 4 °C. The organic layer was transferred to a 12 × 75 mm tube, and the extraction procedure was repeated a second time with the first and second extracts being combined. The extracts were dried under nitrogen at 35 °C in a Zymark TurboVap LV Evaporator (Zymark Corp., Hopkinton, MA, USA). All samples were re-constituted in 120 μL of methanol:water (1:1, v:v); 50 μL was injected into the LC/MS system.

The concentration of TxB_2 was measured in serum by LC-MS/MS using negative heated electrospray ionization (HESI(-) at 270 °C. Quantitative analysis of serum was performed on a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific) coupled with a turbulent flow chromatography system (TFC TLX2 Thermo Scientific) having LC-10ADvp liquid chromatography systems (Shimadzu, Kyoto, Japan) and operated in laminar flow mode. The spray voltage was 3500 V, the vaporizer temperature was 46 °C, and the sheath and auxillary gas were 30 and 15, respectively (arbitrary units). Product masses and collision energies of each analyte were optimized by infusing the standards into the mass spectrometer. Chromatography employed an EZ:faast 4 μ AAA:MS 250 × 2 mm column (Phenomenex, Torrance, CA, USA) and a linear gradient of acetonitrile (Burdick and Jackson) in water (Burdick and Jackson) with a constant 0.2% formic acid (Alfa Aesar) at a flow rate of 0.25 mL/min. The initial acetonitrile concentration was held at 40% for 0.50 min, ramped to 75% over 7.5 min, ramped to 95% over 1 min, before re-equilibrating for 4.5 min at initial conditions.

Detection and quantification was conducted using selective reaction monitoring (SRM) of initial precursor ion for TxB_2 ((*m/z)* 369.169) and the internal standard d_4-TxB_2 ((*m/z)* 373.194). The response for the product ions for TxB_2 (*m/z* 168.936) and the internal standard d_4-TxB_2 (*m/z* 173.036) were plotted and peaks at the proper retention time integrated using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantitate TxB_2 in all samples by linear regression. A weighting factor of 1/X was used for all calibration curves. The response for TxB_2 was linear and gave correlation coefficients (R^2) of 0.99 or better. Accuracy was 114%, 106%, and 99% for 0.3, 1, and 8 ng/mL, respectively. The precision was 3%, 3%, and 5% for 0.3, 1, and 8 ng/mL, respectively. The technique was optimized to provide a limit of quantitation of 0.05 ng/mL for TxB_2.

Pharmacokinetic/pharmacodynamic calculations

Pharmacokinetic and PK/PD modeling were performed using commercially available software (Phoenix WinNonlin Version 6.0; Pharsight, Cary, NC, USA). Pharmacokinetic modeling was performed using compartmental analysis. Coefficient of variation, Akaike information criterion (Yamaoke *et al.*, 1978) and visual inspection of the residual plots were used to select the most appropriate compartmental model. The area under the curve and area under the first moment curve were calculated using the log-linear trapezoidal rule and extrapolation to infinity using the last measured serum concentration divided by the terminal slope λ_z. The PK/PD relationship was modeled using an indirect response model.

Statistical analysis

Pharmacokinetic parameters are presented as mean ± SD (micro and macro rate constants) or individual values. Estimation of the likelihood of the general racehorse population exceeding the 20 ng/mL threshold concentration at 24 h was determined with a 95% confidence interval calculated using the Jeffreys interval method (Epitools Epidemiological Calculators (http://epitools.ausvet.com.au).

Data from TxB2 analysis are expressed as mean ± SD. Statistical analyses were performed using SAS statistical software (version 9.3; SAS Institute, Cary, NC, USA) to assess significant differences in TxB2 concentrations relative to baseline (pretreatment) for individual horses. The residual errors for each variable was checked for normality using the Wilk–Shapiro test and then log-transformed as necessary to bring the residual distribution into close agreement with a normal distribution. Data for all variables were subsequently analyzed using a repeated measures (blocked ANOVA) model. Post hoc comparisons against baseline levels were conducted using Dunnetts method. Significance was set at $P < 0.05$ Inhibition of TxB_2 production was calculated using the equation, percentage of inhibition (control serum TxB_2—sample TxB_2/control TxB_2) × 100.

RESULTS

Accuracy and precision data for the LC-MS/MS assay used for determination of flunixin concentrations met all pre-set criterion (Table 1). The mean serum concentration–time curve for flunixin following intravenous administration of 1.1 mg/kg is depicted in Fig. 1. Serum flunixin concentrations were above 20 ng/mL at 24 h in samples collected from 4 of 16 horses. The 95% confidence interval suggests that the proportion of the general racehorse population that would have serum flunixin concentrations exceeding 20 ng/mL at 24 h postadministration of 1.1 mg/kg is between 9 and 49%. Serum flunixin concentrations were below 20 ng/mL in all horses at 30 h, the next sampling point. (Fig. 1). A three-compartment model ($C_p = Ae^{-\alpha t} + Be^{-\beta t} + C^{-\Upsilon t}$) with a weighting factor of 1 gave the best fit to flunixin concentration data points from individual animals. Serum concentration profiles were characterized by both a rapid and slow distribution phase (alpha and beta) and a slower elimination phase (gamma). Selected pharmacokinetic parameters for flunixin are listed in Tables 2 and 3.

The average urine concentrations of flunixin are depicted in Fig. 2. Urinary flunixin concentrations remained above the LOD of the assay (0.25 ng/mL) in all horses at 72 h postadministration.

Serum TXB_2 concentrations with respect to time before and after flunixin administration are presented in Fig. 3 and Table 4. A wide degree of variability was noted between individual horses at each time point sampled. Relative to baseline, maximum COX-1 inhibition was observed at 2 h postdrug administration with statistically significant inhibition, relative to baseline observed for 24 h. The IC_{50} based on the indirect pharmacodynamic response model was 58.5 ± 40.9.

Table 2. Micro constants, coefficients, and macro constants generated from compartmental analysis of serum flunixin concentrations following intravenous administration of 1.1 mg/kg of flunixin meglumine to 16 exercised Thoroughbred horses

	Mean ± SD	Range
K12 (1/h)	2.35 ± 1.76	1.02–8.30
K13 (1/h)	0.051 ± 0.035	0.009–0.145
K21 (1/h)	3.88 ± 1.40	2.72–7.45
K31 (1/h)	0.164 ± 0.051	0.083–0.258
K10 (1/h)	0.625 ± 0.115	0.500–0.957
A (ng/mL)	6248 ± 1,726	2.753–10.940
B (ng/mL)	8078 ± 663	7.162–9.438
C (ng/mL)	604 ± 385	70.0–1.378
Alpha (1/h)	6.49 ± 3.13	4.12–16.3
Beta (1/h)	0.431 ± 0.077	0.306–0.568
Gamma (1/h)	0.143 ± 0.037	0.081–0.202

DISCUSSION

The purpose of the current study was to describe the pharmacokinetics of flunixin meglumine following intravenous administration of the label dose to exercised Thoroughbred horses and more specifically to describe serum concentrations in light of postrace regulatory sample concentrations exceeding the ARCI recommended regulatory threshold at 24 h postdrug administration. A secondary goal was to describe COX-1 and COX-2 inhibition at various times postdrug administration, including, but not limited to, the time at which serum drug concentrations reach the current regulatory threshold of 20 ng/mL.

In the current study, a 3-compartment model gave the best fit to serum flunixin concentrations. This is in agreement with a previous report describing a prolonged elimination phase following flunixin administration to horses (Lee & Maxwell, 2013) but in contrast to the 2-compartment model described in numerous earlier reports (Semrad *et al.*, 1985; Soma *et al.*, 1988; Jensen *et al.*, 1990; Coakley *et al.*, 1999). The discrepancy between pharmacokinetic models is most likely attributable to the increased sensitivity of the analytical assay used in the report by Lee and Maxwell (2013) and the current study, which allowed for prolonged detection of flunixin in serum samples. The average alpha half-life was in close agreement with that reported in the previous study while the beta half-life was more prolonged in the current study (beta: 1.60 h) relative to the previous study (beta: 0.81 h) (Lee & Maxwell, 2013). Similar to the beta half-life, the gamma half-life in the presently reported study was slightly longer (4.83 (average)) compared to that reported by Lee and Maxwell (2013) (3.38 h). While the difference is small, the most likely explanation for the discrepancy between the two studies is the absence of samples beyond 24 h in the previous study, at which time plasma concentrations were low but still quantifiable in 5 of 8 the horses studied.

The current ARCI regulatory threshold for flunixin is 20 ng/mL in serum or plasma administered 24 h or more

Table 1. Accuracy and Precision Values for LC-MS/MS analysis of flunixin meglumine in equine serum and urine

Matrix	Concentration (ng/mL)	Intraday accuracy (% nominal conc)	Intraday precision (% relative SD)	Interday accuracy (% nominal conc)	Interday precision (% relative SD)
Serum	3.0	114	7.0	109	7.0
	750.0	105	2.0	103	2.0
	5000.0	105	2.0	104	1.0
Urine	1.5	109	6.0	109	6.0
	50.0	106	4.0	106	4.0
	500.0	102	3.0	102	3.0

Table 3. Pharmacokinetic parameters for flunixin meglumine following intravenous administration of 1.1 mg/kg to 16 exercised Thoroughbred horses. All values in this table were generated using compartmental analysis

	$AUC_{0\text{-inf}}$ (h·ng/mL)	AUMC (h·ng/mL)	MRT (h)	Vdss (L/kg)	Alpha HL (h)	Beta HL (h)	Gamma HL (h)	Cl (mL/min/kg)
Horse 1	20 720	54 824	2.65	0.140	0.128	1.22	3.55	0.884
Horse 2	27 322	78 586	2.88	0.116	0.163	1.73	5.60	0.671
Horse 3	25 282	74 423	2.94	0.128	0.123	1.53	4.61	0.725
Horse 4	22 478	61 700	2.75	0.134	0.102	1.50	4.33	0.816
Horse 5	25 511	87 674	3.44	0.148	0.142	1.86	6.87	0.719
Horse 6	23 857	64 730	2.71	0.125	0.127	1.54	4.23	0.768
Horse 7	22 833	69 807	3.06	0.147	0.108	1.56	4.00	0.803
Horse 8	20 354	56 883	2.79	0.151	0.042	1.25	3.43	0.901
Horse 9	20 394	59 454	2.92	0.157	0.074	1.38	5.37	0.899
Horse 10	24 961	88 453	3.54	0.156	0.072	1.69	4.66	0.734
Horse 11	26 776	86 456	3.23	0.133	0.097	1.83	4.86	0.685
Horse 12	23 935	68 005	2.84	0.131	0.161	1.60	3.95	0.766
Horse 13	29 902	106 969	3.58	0.132	0.149	2.19	7.63	0.613
Horse 14	26 754	80 015	2.99	0.123	0.163	1.97	7.63	0.685
Horse 15	27 433	93 648	3.41	0.137	0.134	2.26	8.51	0.668
Horse 16	19 666	48 425	2.46	0.138	0.168	1.40	4.19	0.932
Mean ± SD	24 261 ± 3014	73 754 ± 16 119	3.01 ± 0.337	0.137 ± 0.012	0.107* ± 0.037	1.61* ± 0.306	4.83* ± 1.59	0.767 ± 0.098

$AUC_{0\text{-inf}}$, area under the serum concentration–time curve from 0 to infinity; AUMC, area under the first moment curve; MRT, mean residence time; Vd_{ss}, volume of distribution at steady-state; Cl, systemic clearance. *Harmonic mean.

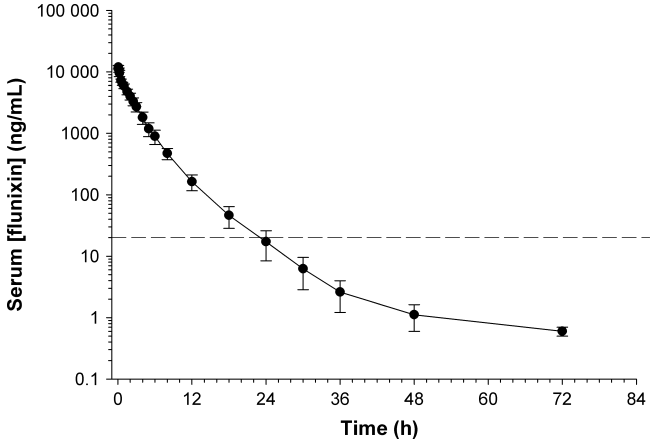

Fig. 1. Serum concentration vs. time curve following administration of 1.1 mg/kg i.v. flunixin meglumine to 16 exercised Thoroughbred horses. Data are presented as mean ± SD. The dotted line represents the current ARCI recommended regulatory threshold.

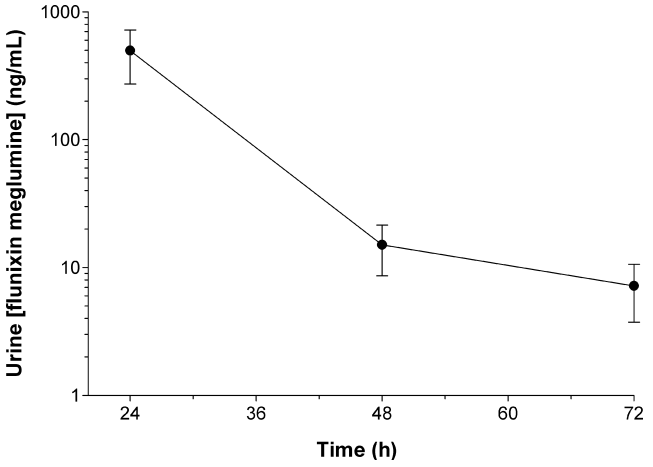

Fig. 2. Urine concentration vs. time curve following administration of 1.1 mg/kg flunixin meglumine to 16 exercised Thoroughbred horses. Data are presented as mean ± SD.

prior to race time. In the study reported here, flunixin concentrations were below the ARCI recommended regulatory threshold (20 ng/mL) by 24 h postdrug administration in the majority of horses studied (12/16); however, concentrations exceeded this threshold level in the other four horses studied. This is in agreement with a previous report by Stanley *et al.* (2006) whereby serum flunixin concentrations were below the recommended threshold in 12 horses but remained above 20 ng/mL in 2 of the horses at 24 h postadministration of a single i.v. dose of 500 mg. Additionally, while sampling was terminated at 24 h postdrug administration in the study conducted by Lee and Maxwell (2013), based on the flunixin plasma concentration–time curve, the average concentration in the five horses in which drug was still quantifiable at 24 h, appears to be slightly higher than 20 ng/mL. These findings indicate that a withdrawal time for flunixin of 24 h at 1.1 mg/kg i.v. is inadequate to avoid exceeding the threshold concentration of 20 ng/mL and incurring a violation.

Pharmacokinetic/pharmacodynamic (PK/PD) studies are considered the gold standard for determination of physiologically relevant concentrations of a drug and ideally results of these studies would be used to establish appropriate regulatory recommendations for drugs used in performance horses. Based on their ability to inhibit COX-1 and COX-2 enzymes, TxB_2

Table 4. Serum flunixin concentration and corresponding percent inhibition of serum TxB_2 following intravenous administration of 1.1 mg/kg flunixin meglumine to 8 exercised Thoroughbred horses. All values in this table are mean ± SD

Time (h)	[flunixin] (ng/mL)	[TxB_2] (ng/mL)
0	0	0
2	4232.2 ± 331.6	99.0 ± 0.5*
4	2309.5 ± 343.9	97.9 ± 1.7*
8	654.8 ± 196.3	94.0 ± 4.7*
24	22.3 ± 13.3	51.0 ± 14.3*
30	10.5 ± 8.73	30.6 ± 10.1
48	2.61 ± 3.14	46.9 ± 24.4
72	ND	35.2 ± 30.2
96	ND	22.0 ± 14.7

*Indicates statistically significant difference ($P < 0.01$) from baseline.

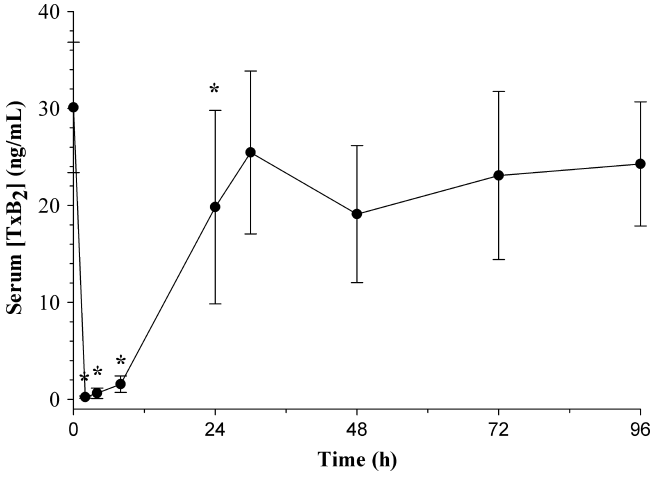

Fig. 3. Mean ± SD Serum TxB2 concentrations as a function of time after administration of 1.1 mg/kg flunixin meglumine to eight horses. *indicates a statiscally significant ($P < 0.01$) change from baseline.

and PGE_2 serum concentrations postdrug administration are commonly used as indicators of the effectiveness of NSAIDs. Both *in vitro* and *ex vivo* methods for assessing this inhibition have been described in the literature (Semrad *et al.*, 1985; Lees *et al.*, 1987; Soma *et al.*, 1992; Landoni & Lees, 1995; Galbraith & McKellar, 1996; Brideau *et al.*, 2001; Blain *et al.*, 2002; Beretta *et al.*, 2005; Araujo *et al.*, 2013). In the current study, an *ex vivo* approach was chosen in an attempt to account for factors affecting drug disposition in a whole animal model.

In the current study, administration of flunixin-induced rapid and complete inhibition of TxB_2 and this is in agreement with previous reports (Semrad *et al.*, 1985; Lees *et al.*, 1987; Soma *et al.*, 1992). Previous studies have described prolonged inhibition of TxB_2 production following flunixin administration (Semrad *et al.*, 1985; Lees *et al.*, 1987; Soma *et al.*, 1992). Semrad *et al.* (1985) and Soma *et al.* (1992) reported significant TxB_2 inhibition for up to 12 and 8 h postdrug administration, respectively, with TxB_2 concentrations returning to baseline levels by 24 h postdrug administration in both of these studies. Lees *et al.* (1987) reported a slightly longer duration of TxB_2 inhibition, more consistent with that observed in the currently reported study. Investigators have suggested that differences between studies is attributable to the use of ponies by Lees *et al.* (1987) and horses in the other two investigations (Semrad *et al.*, 1985 and Soma *et al.*, 1992), however, as horses were used in the current study and the duration of effect was similar to that reported for ponies (Lees *et al.*, 1987), this is likely at least not the sole explanation. The difference is more likely due to individual differences between animals, irrespective of whether they are horses or ponies and/or differences in environment, husbandry practices or fitness level. Soma *et al.* (1992) reported no significant differences in TxB_2 concentrations between sedentary and fit horses following administration of flunixin; however, the sample size was limited and it is possible that this hindered their ability to detect of differences between the 2 groups. Age-related differences in TxB_2 have also been reported (Soma *et al.*, 1992) and is another plausible explanation for the discrepancy between studies. Lastly, differences between studies may be due to differences in data modeling. Many of the earlier investigations utilized an effect compartment model; however, as NSAIDS are known to exhibit hysteresis between serum drug concentrations and enzyme inhibition, indirect response models are likely more appropriate (Lees *et al.*, 2003).

The average serum flunixin concentration at 24 h postadministration, for the eight horses in which TxB_2 inhibition was studied, was 22.3 ± 1.35 ng/mL. The corresponding percent inhibition was 51.0 ± 14.0% (range: 35.6–65.2%) for TxB_2. Although Toutain *et al.* (1994) have previously demonstrated that *ex vivo* measurements, such as TxB_2 production can be used as an index of the therapeutic effect of flunixin in horses they are still considered surrogate markers of clinical effects. In another study, Toutain *et al.* (1994) assessed the effects of flunixin on stride length in an experimental model of arthritis in the horse. In that study, investigators described an IC_{50} of 0.93 ± 0.35 μg/mL and concluded that the duration of clinical effect (improvement in stride length) persisted for 16 and 24 h following administration of a flunixin dose of 1 and 2 mg/kg, respectively (Toutain *et al.*, 1994). While the sample size is limited compared to the general Thoroughbred racehorse population, results of this study suggest that by 24 h postflunixin administration (1.1 mg/kg) clinical effects are no longer present.

In the current study, 4 of the 16 horses administered the labeled dose of flunixin meglumine had serum concentrations in excess of the current ARCI recommended regulatory threshold (20 ng/mL) at 24 h suggesting that this drug should be used cautiously in race horses and an extended withdrawal time may be warranted. Based on the results of this study and the 95/95% confidence interval method, the Racing Medication and Testing Consortium has calculated that a withdrawal time of 32 h would be more appropriate to avoid a positive regulatory finding following administration of 1.1 mg/kg of flunixin.

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