Effect of Flunixin Meglumine on Selected Physiologic and Performance Parameters of Athletically Conditioned Thoroughbred Horses Subjected to an Incremental Exercise Stress Test

Patrick T. Colahan, DVM, DACVS
James E. Bailey, DVM, MS, DACVA
Chi-Chung Chou, BVSc, MS
Martha Johnson, MS
Brett L. Rice, BS
Galin L. Jones, MStat, PhD
Joseph P. Cheeks

University of Florida
Box 100136
Gainesville, Florida 32610

a Department of Large Animal Clinical Sciences
College of Veterinary Medicine

b Department of Statistics
Institute of Food and Agricultural Sciences

ABSTRACT

Twelve clinically sound, healthy, athletically conditioned Thoroughbred horses were subjected to an incremental exercise stress test to determine the effects and period of detection of a single dose of flunixin meglumine (1.1 mg/kg by intravenous injection) in serum and urine by ELISA. Flunixin concentrations, performance, and hematologic and clinical chemical parameters were measured. All horses were rotated through four treatment groups of a Latin-square design providing for each horse to serve as its own control. Flunixin meglumine reduced prostaglandin F$_{1a}$ and thromboxane concentrations that had been increased by intense exercise. Performance parameters did not improve and prostaglandin concentrations did not significantly correlate with total run time. Exercise did not change the flunixin elimination profile in either serum or urine, and concentrations were found to be below the detection limit of the ELISA test within 36 hours in serum and 120 hours in urine.

INTRODUCTION

Flunixin meglumine, an NSAID, is a potent inhibitor of the enzyme cyclooxygenase and suppresses thromboxane and prostaglandin (PG) production. Analgesic, anti-PG, and antipyretic properties have made flunixin meglumine one of the most commonly used treatments for horses with sepsis, musculoskeletal pain, or visceral pain associated with abdominal
crises and/or endotoxic shock.\textsuperscript{1–4} Because flunixin is often used in competing athletic horses, the question arises as to whether this drug actually affects the physiologic responses to exercise in a manner that could enhance performance.

Because of this potential for performance enhancement, flunixin is banned by some organizations that regulate equestrian competitions and racing. The use of flunixin to treat horses that subsequently participate in competitions in which this drug is prohibited would be greatly facilitated by knowledge of the time required for flunixin concentrations in urine and/or blood to fall below levels that are detectable by sensitive detection methods. Previous studies have shown that after intravenous administration of flunixin meglumine at dosages up to 1.1 mg/kg, the elimination half-life is relatively short, reportedly from 1.5 to approximately 4 hours.\textsuperscript{5–10} The elimination half-life was significantly faster in horses younger than 5 years of age.\textsuperscript{6}

The effects of NSAIDs in exercising athletic horses have been studied,\textsuperscript{11–15} and the use of phenylbutazone is permitted by some regulatory organizations.\textsuperscript{4} The effects of flunixin in exercising horses are not as well established. Except for studies on thromboxane B\(_2\) (TXB\(_2\)),\textsuperscript{13} the effect of exercise on pharmacokinetics,\textsuperscript{6} and the role of PG in exercise-induced pulmonary problems,\textsuperscript{14,15} most studies of potential physiologic, hematologic, and hormonal changes induced by flunixin have been carried out in sedentary horses.\textsuperscript{2,3,8,9,16–18} Nevertheless, it has been suggested that flunixin improved gait in standardbred horses, increased heart rate, and reduced lactate accumulation but did not increase maximal speed.\textsuperscript{19} This information implies that flunixin administration may improve athletic performance to some degree.

The purposes of this study were to determine the elimination period of flunixin meglumine from serum and urine as determined by a sensitive ELISA test and to investigate the hematologic, serochemical, hormonal, and performance effects of flunixin meglumine in athletically conditioned horses similar to those engaging in pari-mutuel sanctioned athletic competitions.

\section*{MATERIALS AND METHODS}

\textbf{Animals}

Twelve Thoroughbred horses, including three stallions, four mares, and five geldings 3 to 8 years of age, were selected for participation in the study. Prior to inclusion in the study, all horses were examined to ensure they were orthopedically sound and free of any respiratory and cardiovascular diseases. Additional ancillary examinations included upper-airway endoscopy, cardiopulmonary auscultation, electrocardiography, serum chemistry, hematology, and musculoskeletal and physical examinations. Routine prophylactic health procedures, including control of internal parasites, vaccinations for infectious diseases, and daily monitoring of health, were performed in all horses. During the exercise testing, a second experienced veterinarian observed the horses for signs of lameness. The goal of these examinations was to alleviate humane concerns and to select a study group that was in a state of health comparable to horses racing in pari-mutuel-sanctioned competitions. Records were kept for each horse for exercise duration and dates and times of drug administration, sample collection, and preventive medical procedures. The horses were maintained at pasture except when stalled for sample collection or training on the treadmill.

For 6 to 18 months before the study, the horses were physically conditioned by galloping on a high-speed treadmill. Five days a week, the horses were exercised using a regimen of trotting (4 m/s) on a level treadmill for 0.8 km (0.5 mile) followed by galloping (8 m/s) for 3.24 km (2
miles) and then trotting (4 m/s) again for another 0.4 km (0.25 mile). On Mondays, Wednesdays, and Fridays, the treadmill was elevated to a 10% incline during the gallop exercise. On Tuesdays and Thursdays, no incline of the treadmill was implemented. The horses were considered physically fit when they could gallop a mile in 2 minutes without undue stress and could maintain a forward position on the treadmill without urging from the treadmill operator. Each horse was subjected to two periods of intense exercise to the point of exhaustion before the initiation of the study, including one within 8 weeks of the initiation of the exercise tests of the study. The University of Florida Institutional Animal Care and Use Committee approved all protocols for animal use.

**Treatments**

The 12 horses were evenly and randomly allocated to four treatment groups as follows: no exercise and no flunixin (control), exercise and no flunixin, exercise and flunixin administered before exercise, and no exercise and flunixin administered before exercise.
no flunixin, no exercise plus flunixin, and exercise plus flunixin. The previously described conditioning regimen was performed for each 4-week trial period for horses in the exercised groups. The horses were rotated through all four treatment groups with at least 4 weeks between trials. This design allowed each horse to serve as its own control. Horses in the nonexercised groups were maintained at pasture.

The exercise stress test was an incremental test conducted to exhaustion. The horses were placed on the treadmill, and an air-flow mask was placed over the nose. The mask was attached to a calibrated, industrial mass-flow unit by 20-cm-diameter, wire-reinforced, rubber-coated, nylon-mesh tubing. Following a 2-minute warm-up period at 4 m/s, the treadmill was raised to an incline of 6° (10% incline), and the horses were exercised for 1 minute at each of the following speeds: 8, 9, 10, 11, and 12 m/s. The horses were exercised until they would not stay in place on the treadmill without intense urging by the treadmill operator. They were then trotted (4 m/s) for 0.8 km (0.5 mile) on the flat and hand walked to cool out. The person conducting the exercise test was blinded to the treatment groups.

**Samplings**

On the first Monday of the test period, pretreatment urine and blood samples were collected from each horse at 6:00 AM. Immediately prior to testing (designated Time 0), either flunixin meglumine at 1.1 mg/kg or an identical volume of sterile isotonic saline (control) was injected into the right jugular vein. The laboratory personnel and the venipuncturist were blinded to the material administered. Horses in the group receiving both flunixin and exercise were injected with flunixin 1 hour before the exercise stress test was initiated. Blood samples were collected via a jugular catheter according to the schedule in Table 1. The 1-hour sample was taken immediately prior to the exercise test. Serum was analyzed for flunixin, electrolyte, cortisol, corticotropic (ACTH), insulin, β-endorphin, glucose, TXB₂, PGE₂, PGF₁α, and creatinine concentrations. Urine obtained by free catch was analyzed for flunixin and creatinine concentrations. All horses in the study were conditioned to urinate on a signal such that all samples were collected within 10 minutes of the assigned sampling time.

**Flunixin Detection**

Blood collected in 10-ml glass vacuum tubes was allowed to clot. The samples were centrifuged and the serum harvested. A one-step ELISA for measurement of flunixin meglumine was prepared as described by Chou and coworkers.²⁰ Briefly, 96-well plates were coated with antibody from rabbits sensitized to flunixin meglumine over a period of 6 months. Optimal antibody and antigen concentrations were determined by use of the checkerboard method. A standard curve for flunixin meglumine was established over a range of 0.1 to 5000 ng/ml in the appropriate matrix (urine or serum) and was run on each plate along with the unknown samples. Twenty microliters of sample (or standards) was combined with 100 µl of 1:1000 flunixin-horseradish peroxidase conjugate in each well and incubated at 37°C for 1 hour. The plates were then washed three times with 300 µl of phosphate-buffered saline containing 0.05% Tween 20 (pH 7.4) before tetramethylbenzidine substrate was added to each well and the samples were incubated for another 15 minutes at 37°C for color formation. The intensity of blue color was read at 650 nm on a microplate reader. To estimate the concentration of flunixin meglumine in experimental samples, competitive inhibition of antigen/antibody binding by immunoreac-
tive flunixin in the samples was calculated and compared with that of the standards. The limit of quantification (LOQ) was set at 10 times the standard error of the mean above the average response in the Time-0 samples. The LOQ values were determined to be 25 ng/ml for serum and 190 ng/ml for urine samples. Mean intra- and interassay variations through five replicates were found to be 3.5% and 8.3%, respectively.

**Inspired/Expired Gas Analysis**

Oxygen consumption (VO\(_2\)) and carbon dioxide production (VCO\(_2\)) were measured using a paramagnetic oxygen sensor and a spectrophotometric CO\(_2\) sensor. Expired gases were collected for sampling via nasal mask and an open-flow system connected to an industrial mass-flow collection unit. All expired gases were vented from the building. The sensors and the mass-flow unit were calibrated before each use following the manufacturer’s recommendations. Sampling and analysis were done at 10-second intervals. The final sample measured at each speed and at failure was used for analysis. Airflow through the mass-flow unit was maintained at 4000 L/min or higher while the treadmill was at speeds of 4 m/s and at 12,000 L/min or higher while the treadmill was at speeds of 8 m/s or higher. Run time was measured with a stopwatch.

**Hematologic, Serum Electrolyte, and Glucose Analysis**

Plasma samples were collected in 2-ml glass tubes containing lithium EDTA for determination of hematologic values. The hemogram was performed using automated cell and differential cell counts with manual screening for accuracy and manual, spun hematocrit. Total plasma protein was estimated by refractometer, and fibrinogen was estimated by heat precipitation. Electrolytes were measured by a commercial electrolyte analyzer.

Serum glucose concentrations were measured using an enzymatic procedure described by Raabo and Terkildsen,\(^{21}\) with minor changes in the quantity of chromogen to increase sensitivity.

**Serum Cortisol, Insulin, ACTH, and \(\beta\)-endorphin Analysis**

Cortisol, insulin, ACTH, and \(\beta\)-endorphin were assayed on serum harvested from cold 10-ml glass vacuum tubes. Cortisol and insulin were measured using a solid-phase radioimmunoassay (RIA). Endogenous ACTH was measured using a double-antibody\(^{125}\) I-labeled RIA. The \(\beta\)-endorphin immunoassay was a solid-phase, two-site, immunoradiometric assay. Based on samples paired in a single assay, these RIAs had an intraassay coefficient of variability (CV) ranging from 3% to 9%. The interassay CV ranged from 4% to 10% based on three samples paired between assays and conducted 1 week apart. The sensitivity of each assay was as follows: insulin: 1.2 IU/ml; cortisol: 0.2 ng/ml; ACTH: 3.5 pg/ml; and \(\beta\)-endorphin: 14 pg/ml.

**Serum/Urine Creatinine**

Both serum and urine creatinine concentrations were measured using a colorimetric procedure. This is a method developed with improved specificity by Slot\(^{22}\) and simplified by Heinegard and Tiderstrom.\(^{23}\)

**Plasma Prostaglandins and Thromboxane**

Plasma samples for assay of PGs and TXB\(_2\) were collected into cold 10-ml glass vacuum tubes containing 0.2 ml of 15% EDTA and 0.02% aspirin. The samples were kept on ice and centrifuged at 4°C; the plasma was collected within 15 minutes. The PGs and TXB\(_2\) were first extracted from plasma as described by Bottoms and coworkers\(^{24}\) and then measured using
competitive RIAs. The intraassay CV of all RIAs ranged from 3% to 9% based on samples paired in a single assay, and the interassay CV ranged from 4% to 10% based on three samples paired between assays and conducted 1 week apart. The sensitivity of each assay was as follows: PGE\(_2\): 0.8 pg/ml, PGF\(_{1\alpha}\): 20 pg/ml, and TXB\(_2\): 20 pg/ml.

**Data Analysis**

A mixed-effect linear model was fit using generalized least squares. This explicitly modeled the correlation structure between the repeated measures on each horse. Each parameter was modeled as a function of treatment, time, and the interaction of these factors. In addition, a random effect was included for each horse. When the interaction was not significant, contrasts testing the difference between the presence versus absence of drug and exercise were included. If the interaction was significant, these contrasts were performed for each fixed time. In addition, an overall F-test of the simple effects for each fixed time was included. To reduce the possibility that random variation would lead to a spurious finding of significance in view of the large number of parameters analyzed, significance level was set at \( P < .01 \). To determine whether there was any association between the total run time and the maximal values of TXB\(_2\) and PGF\(_{1\alpha}\), Spearman's rank correlation coefficient, \( r \), was calculated for the entire data set and for each treatment (drug or no drug). These tests were conducted at the .05 level of significance.

**RESULTS**

**Flunixin Immunoreactivity**

Mean serum flunixin immunoreactivity was significantly \( (P < .01) \) elevated with a peak of 695 ng/ml in the first sample (1 hour) and then decreased quickly to baseline level approximately 8 hours after intravenous injection (Figure 1A). Flunixin became undetectable in serum between 18 and 36 hours and was undetectable in serum in all horses 36 hours after administration.

After flunixin meglumine injection, mean urine flunixin immunoreactivity reached a peak of 3.3 \( \mu \)g/ml at 2 hours (Figure 1B). The mean urinary immunoreactivity was significantly \( (P < .01) \) elevated above pretreatment level for 24 hours and remained detectable until 36 hours. Exercise did not change the elim-
ination profile of flunixin in either serum or urine. Flunixin became undetectable in urine in all horses 120 hours after administration.

Run Time and Inspired/Expired Gas Analysis

Mean total run time in the incremental exercise stress test was not significantly affected by flunixin administration ($P = .91$). The mean run time for horses receiving flunixin was $232.5 \pm 7.5$ seconds, and the run time in horses not receiving flunixin was $231.5 \pm 5.3$ seconds (Table 2). Flunixin administration had no significant effect on mean peak oxygen con-

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Mean $\pm$ SEM $231.5 \pm 13.0$ $232.5 \pm 7.0$ $10 \pm 6$

*Net change = run time with flunixin administration minus the run time without flunixin.

Figure 2. Serum (A) and urinary (B) creatinine concentrations in Thoroughbreds at various times after a single intravenous injection of flunixin meglumine at 1.1 mg/kg. Data points represent mean $\pm$ SEM values for 12 horses.
sumption ($P = .9701$) or CO$_2$ production ($P = .7845$) (Table 3).

**Hemogram, Serum Electrolytes, and Glucose**

No significant effect of flunixin administration was observed on any red blood cell parameter, total white blood cell counts, differential counts, platelet counts, or mean platelet volume at any sampling time. No significant effect of flunixin administration was noted after exercise on serum sodium ($P = .6667$), potassium ($P = .4832$), chloride ($P = .6582$), calcium ($P = .7399$), glucose ($P = .8456$), CO$_2$ content ($P = .0332$), or anion gap ($P = .481$).

**Hormonal and Creatinine Concentrations**

Flunixin administration caused no significant changes in serum cortisol ($P = .6485$), ACTH ($P = .0997$), insulin ($P = .2529$), β-endorphin ($P = .5736$), or creatinine ($P = .2208$) concentrations after exercise. Urine creatinine concentrations increased at 4 hours after flunixin administration ($P = .0023$) (Figure 2A). Mean serum creatinine concentrations at the 4-hour sampling were $111.04 \pm 11.2$ mg/dl in horses that were exercised but not given flunixin and $128.12 \pm 11.1$ mg/dl in horses that were exercised and given flunixin ($P < .05$) (Figure 2B).

**Thromboxane and Prostaglandin Concentrations**

Exercise increased TXB$_2$ and PGF$_{1\alpha}$ concentrations at 1.5 and 2.5 hours (i.e., immediately and 1 hour after exercise) (Figure 3). Flunixin administration prevented these exercise-induced elevations in serum TXB$_2$ and PGF$_{1\alpha}$ concentrations. Exercise caused no observable effect on PGE$_2$ concentrations. Evaluation of TXB$_2$ and PGF$_{1\alpha}$ concentrations using Spear-
man’s rank correlation coefficient revealed no correlation between TXB$_2$ ($r = .041, P = .8578$) or PGF$_{1\alpha}$ ($r = .059, P = .7986$) concentrations and the performance of the horse in the exercise stress test as measured by run time. Thromboxane concentrations were reduced for 8 hours after flunixin and returned to baseline at 12 hours (Figure 3). Prostaglandin F$_{1\alpha}$ concentrations were significantly reduced over the 1.5- to 2.5-hour period and were not different from baseline by 12 hours (Figure 3). Mean PGE$_2$ concentrations averaged over all sample times were significantly lower in horses given flunixin than in those not given flunixin ($P = .0021$). However, comparisons at any specific sampling time between mean PGE$_2$ concentrations in serum of horses given flunixin and those not given flunixin were not significant.

**DISCUSSION**

A single intravenous administration of flunixin meglumine at 1.1 mg/kg produced mean concentrations of the drug not detectable by ELISA in serum after 8 hours and in urine after 36 hours. This is within the ranges of values reported previously. However, flunixin was found in serum samples of individual horses for up to 36 hours and in urine samples for up to 120 hours. This variation in the duration of detectable levels of flunixin among individual horses is of practical importance. Earlier recommendations for horses younger than 5 years of age were 24 hours for elimination from serum and 48 hours for elimination from urine. An additional 24 hours for each recommendation was suggested for horses older than 5 years. The range of ages for horses in the present study was 3 to 8 years, spanning the 5-year mark previously used as a milestone. The goal of this study was not to address clearance variations caused by age but to develop elimination information for mature horses (older than 2 years of age) engaging in pari-mutually sanctioned competitions. The previously recommended 48 hours for elimination to below detectable concentrations in serum would cover all the horses in this study. In urine, however, three of 24 samples in this study had concentrations of 19 to 27 ng/ml at 96 hours.
Other studies have found detectable concentrations in urine up to 15 days.\textsuperscript{13}

While most horses may eliminate flunixin to below detectable concentrations and be able to meet medication regulations near the previously recommended times, some individual horses will have detectable concentrations for considerably longer periods, particularly in urine. Determining the causes of this variation in elimination periods and the consistency of elimination periods within individual horses was beyond the scope of this study. However, this study and that of Soma and coworkers\textsuperscript{13} indicate that assessment of timing to enter horses in competitions forbidding detectable concentrations of flunixin in their urine cannot be based on reported mean elimination periods without some risk of rule violation. Individual testing of a horse, with its attendant economic and logistical difficulties, is the only certain method of ensuring compliance with medication regulations. As in other studies, exercise had no effect on the elimination of flunixin meglumine.\textsuperscript{6}

Various techniques have been developed for testing the athletic fitness of humans and horses. The most commonly used method to evaluate combined submaximal and maximal exertion has been the incremental stress test conducted on a treadmill.\textsuperscript{26} Although not identical to exercise performance in a competition, the incremental exercise stress test is a reproducible measure of athletic condition, in which the intensity of exercise is confirmed by measurement of physiologic parameters such as oxygen consumption and CO\textsubscript{2} production during exercise.\textsuperscript{27} Oxygen consumption values in this study approached the ranges reported by others for thoroughbred horses in intense short-term exercise.\textsuperscript{26}

Flunixin administration did not improve selected performance parameters when given at the recommended therapeutic dose 1 hour before the incremental exercise stress test. Any effect of flunixin was obscured by a sequence effect ($P = .0058$). All but one horse performed better on the second exercise stress test; however, the cause of the sequence effect was not obvious. The frequency of exercise to exhaustion in the period during which the studies were conducted ranged from 4 to 8 weeks. The horses had been subjected to intense exercise episodes within the 8 weeks before their participation in the study and were given a minimum rest period of 4 weeks between intense exercise periods to avoid any influence of one period of intense exercise on performance in a subsequent one.

An improvement in performance following flunixin administration has not been reported but has been investigated.\textsuperscript{28} However, during kinematic analysis on a track, standardbred horses have been reported to improve their gait 4 hours after flunixin administration.\textsuperscript{19} The gait improvement noted was an increased swing phase and decreased stance phase with constant stride duration. An improvement in maximal speed or changes in metabolic responses did not accompany this gait improvement.\textsuperscript{19} These changes in gait mimic those reported by Buchner and coworkers\textsuperscript{29} in horses with induced lameness. In that study, lameness induction led to increased stance time.\textsuperscript{28} Kinematic analysis was not undertaken in the present study, but no improvement in speed or endurance was noted in this study of clinically sound horses.

The effects of exercise on TXB\textsubscript{2} and PGF\textsubscript{1a} concentrations found in this study were similar to those previously reported,\textsuperscript{30,31} but an elevation of PGF\textsubscript{1a} has not been reported consistently.\textsuperscript{12,28} This variation may be related to the intensity and duration of the exercise or the location of the catheter used for blood collection.\textsuperscript{30,31} The source of these cyclooxygenase-related mediators is unknown, but possible
sources are platelets and lung or muscle blood vessel endothelium. Prostanoids have been reported to be involved in the mediation of bronchodilation after furosemide administration and have been explored as mediators of hemodynamic changes associated with exercise. The kinematic changes noted after flunixin administration may indicate a musculoskeletal source. While the sources, sites of action, and effects of these mediators on exercising horses are not known, they potentially could contribute to fatigue or exercise-induced pain. However, in this study, the concentrations of these mediators failed to significantly correlate with performance in an incremental exercise stress test. Flunixin administration caused depression of TXB, PGF and PGE. These effects have been reported previously and are commonly accepted effects of NSAID administration.

Increase in serum creatinine after exercise has been reported to be a result of hemoconcentration and increased turnover of phosphocreatine. However, the decrease in urine creatinine 24 hours after exercise has not been previously reported and no clear cause was identified. Alteration of renal function and redistribution of body fluids are possible explanations, but a definitive explanation requires further investigation.

Flunixin meglumine administration led to significantly elevated urinary creatinine concentration in exercised horses for 4 hours. To the authors’ knowledge, this has not been reported before. Submaximal exercise in unfit horses alters renal function, including increased urine flow, but glomerular filtration does not change. Because creatinine is not reabsorbed and is only minimally secreted in the renal tubule, the changes in creatinine concentration occur mainly in response to changes in water resorption in the renal tubule and creatinine concentration in the plasma. Alteration of PG concentrations in the kidneys is associated with changes in renal blood flow and with changes in the permeability of the collecting ducts. Flunixin alteration of renal physiology, specifically inhibition of PGE-mediated diuresis and PGF-mediated water and sodium chloride excretion, may have influenced creatinine concentrations in urine after strenuous exercise by reducing urine water content. The practical significance of this finding is unknown, and elucidation of a mechanism requires further investigation.

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REFERENCES


