Pharmacokinetic Report

Plasma and urine pharmacokinetics of intravenously administered flunixin in greyhound dogs
Short Title: Pharmacokinetics of flunixin in greyhounds

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ABSTRACT

Medication control in greyhound racing requires information from administration studies that measure drug levels in the urine as well as plasma, with time points that extend in to the terminal phase of excretion. To characterise the plasma and the urinary pharmacokinetics of flunixin and enable regulatory advice for greyhound racing in respect of both medication and residue control limits flunixin meglumine was administered intravenously on one occasion to six different greyhounds at the label dose of 1 mg/kg and the levels of flunixin were measured in plasma for up to 96 hours and in urine for up to 120 hours. Using the standard methodology for medication control the Irrelevant Plasma Concentration was determined as 1 ng/mL and the Irrelevant Urine Concentration was determined as 30 ng/mL. This information can be used by regulators to determine a Screening Limit, Detection Time and a Residue Limit. The greyhounds with the highest average urine pH had far greater flunixin exposure compared with the greyhounds that had the lowest. This is entirely consistent with the extent of ionisation predicted by the Henderson-Hasselbalch equation. This variability in the urine pharmacokinetics reduces with time and at 72 hours post administration, in the terminal phase, the variability in urine and plasma flunixin concentrations are similar and should not affect medication control.

Keywords: flunixin, greyhound, pharmacokinetics, plasma, urine

INTRODUCTION

Flunixin is a non-steroidal anti inflammatory drug used in veterinary medicine. In the context of this study to inform control of its use for Greyhound racing, in Europe flunixin is licensed for use in farm animals and horses, in Australia is also licensed for use in dogs. As such, information is required to inform for its medication control when it is used in racing greyhounds, or control of residues if meat is fed to racing greyhounds.

There have been a number of previous studies of the pharmacokinetics of flunixin in dogs. Plasma pharmacokinetics have been reported by Hardie et al (1985) for up to 12 hours after IV administration and by McKellar et al (1989) and Ogino et al (2005) for 24 hours after oral and subcutaneous administration, respectively. The range of levels of detection were 10-50 ng/mL in these studies.

For medication control of drugs for animals used in sport urine is the sample matrix of choice and the standard methodology to define sensitivity control is described by Toutain and Lassourd (2002). Here it is especially important to understand the need for an administration study with contemporaneous plasma and urine levels that defines the terminal phases, with a limit of detection that is appropriate for medication control rather than therapeutics.

Brady et al (1997), using enzyme linked immunosorbent assay (ELISA) methodology, measured the concentration of flunixin in urine after administration to greyhounds with a limit of detection of 25 ng/ml. Dumasia et al (1988) administered a therapeutic dose of 1 mg/kg flunixin and fed meat containing residue amounts of 0.1, 0.5 or 2.0 mg, and then measured using high performance liquid chromatography/mass spectrometry (HPLC/MS) methodology the concentration of flunixin in urine after administration to greyhounds with detection down to at least 2 ng/mL for up to 96 hours after administration."
In addition to the therapeutic use of flunixin, the role of exposure via feeding meat from livestock that contains residues of flunixin to greyhounds has been recognised (Dumasia et al 1988) and this dictates the need for a limit of detection that is also appropriate for residue control.

The aims of this study were: to characterise the plasma and the urinary pharmacokinetics of flunixin when given intravenously to greyhounds at a dose and formulation relevant to its use by trainers of racing greyhounds; ensure analytical detection extended into the terminal phase and at a higher sensitivity than previous studies; derive irrelevant plasma and urine concentrations; and so enable regulatory advice for greyhound racing in respect of both medication and residue control limits.

MATERIALS AND METHODS

Flunixin meglumine (Ilium Flunixil® Injection 50 mg/mL, Troy Laboratories Australia Pty. Ltd) was administered intravenously on one occasion to six different greyhounds at the label dose of 1mg/kg (0.6 mg/kg flunixin free acid). Three female and three male animals were studied, with a dose of 1 mg/kg product, a mean bodyweight of 33.1 kg (range 28.0-39.4kg) and mean age of 3.8 years (range 3-5 years). Blood and urine were collected before drug administration. Blood samples were collected after 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 hours. Urine samples were collected after 2, 4, 6, 8, 10, 12, 24, 48, 72, 96, and 120 hours. The blood samples were heparinised and plasma obtained by centrifugation. All samples were stored at -20°C. Dogs were fed a commercial dry dog food (Dogpro PLUS Working Dog, Hypro Petcare P/L) with an additional portion of fresh meat, with the daily feed ration as two meals and had access to water at all times. The morning feed was not given on the treatment day prior to drug administration. The study was conducted in accordance to the principles of the VICH GCP guidelines (International Co-operation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products, Good Clinical Practice, June 2000, effective July 2001). Ethics approval was obtained from the Secretary's Animal Care and Ethics Committee of the NSW Department of Primary Industry.

The frozen samples were transported on dry ice to the analytical laboratory. Concentrations of flunixin were measured in the pre- and post-administration urine and plasma. Urine and plasma samples were extracted using liquid/liquid extraction. For urine; samples (250 µL) were diluted with deionised water (750 µL) followed by the addition of sodium hydroxide (2 M, 150 µL). Samples were then vortexed and allowed to hydrolyse at room temperature for 30 minutes. Sodium acetate buffer (pH 3.3, 300 µL) was added to samples and the pH adjusted to 3. A solvent mix of hexane/dichloromethane/ethyl acetate (40:30:30) was added prior to mixing on a rotating mixer for 15 min-

utes. After centrifugation, the organic layer was removed and liquid/liquid extracted with saturated sodium bicarbonate (1 mL) for 10 minutes. Samples were again centrifuged before the organic layer was removed and passed through anhydrous sodium sulphate. Extracts were evaporated to dryness under nitrogen at 60°C, followed by reconstitution in ammonium fluoride (0.2 mM) and methanol (50:50, 100 µL). For plasma; samples (200 µL) were diluted with deionised water (800 µL), followed by the addition of hydrochloric acid (6.4%, 300 µL). Samples were then vortexed, followed by liquid/liquid extraction with diethyl ether (3 mL) for 15 minutes. After centrifugation, the organic layer was removed and evaporated to dryness under nitrogen at 60°C. Dried residues were reconstituted in ammonium fluoride (0.2 mM) and methanol (50:50, 50 µL).

Samples were analysed by liquid chromatography mass spectrometry using a Shimadzu 8050 triple quadruple mass spectrometer (Shimadzu Corp., Kyoto, Japan) coupled to a Nexera LC-30AD (Shimadzu Corp., Kyoto, Japan) liquid chromatograph. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with positive polarity. Electrospray ionisation was carried out with heater block, interface and DL temperatures of 300°C, 300°C and 275°C respectively. The nebulizer, heating and drying gas flow rates were 3, 5 and 15 L/min respectively. Flunixin was monitored using the precursor ion m/z 297.1 and daughter ions m/z 259.1 (for quantitation), m/z 264.1 and m/z 279.1 (for identification). The internal standard (Tan & Awaiye 2013), flunixin-d_3, was monitored using the transition m/z 300.1 > m/z 282.1.

Chromatographic separation was achieved using a Poroshell 120 EC-C18 column (3 mm x 50 mm, 2.7 µm particle size) (Agilent Technologies, CA, USA). The mobile phase consisted of 0.2 mM ammonium fluoride (A) and methanol (B). The initial composition was 50% B, which was held for 0.3 minutes and then ramped to reach 98% B at 2.5 minutes. This was held for 2.5 minutes before being returned to 50% B and equilibrated for 1.5 minutes.

Calibration and quality control samples were used in the range of 2.5-1000 ng/mL for urine and plasma, and the methods were shown to be linear with correlation coefficients greater than 0.99. The urine and plasma methods produced a lower limit of quantification (LLOQ) of 1 ng/mL, a Limit of Detection (LOD) of 0.2 ng/ml, and were shown to be accurate and reproducible with acceptable inter-batch variability of precision and accuracy. The accuracy of the urine method was demonstrated to be 8.4% at LLOQ, and the precision 18.1% at LLOQ. The accuracy of the plasma method was demonstrated to be 13.9% at LLOQ, and the precision 6.7% at LLOQ.

Urine pH was measured with a Mettler Toledo SevenEasy pH meter (Mettler Toledo, Schwerzenbach, Switzerland). The area under the urine flunixin concentration-time curve was computed and compared to the time weighted average urine pH for each dog using a non-compartmental approach.

Plasma data were fitted to a 2 compartmental pharmacokinetic model using Phoenix WinNonlin 6.0 (Pharsight Corporation, Cary, NC). The area under the urine flunixin concentration-time curve was computed and compared to the time weighted average urine pH for each dog using a non-compartmental approach also using Phoenix WinNonlin Version 6.0.

RESULTS

Following intravenous administration to six greyhounds of flunixin, plasma pharmacokinetic parameters are summarised in Table 1. Mean distribution half-life (alpha half-life) was 0.54 hour; elimination half-life (beta half-life) was 5.81 hours; volume of distribution at steady state

was 0.26 L/kg; volume of the central compartment was 0.085 L/kg; and total body clearance was 1.20 mL/min/Kg. The terminal urine half-lives for flunixin were 6.77±1.69 hours. Plasma concentrations fell to 0.5 ng/mL at 48 hours, and urine concentrations fell to 1 ng/mL at 96 hours, as illustrated in Figure 1a and 1b. At later time points flunixin was not detected.

DISCUSSION

The plasma concentrations of flunixin and the fit to a 2-compartment model gave similar pharmacokinetic parameters to what has previously been observed (Hardie et al 1985; McKellar et al 1989; Ogino et al 2005). Hardie et al. (1985) carried out an IV pharmacokinetic study of flunixin meglumine in 6 intact, awake dogs. Plasma samples were obtained up to 12 hours after IV administration of flunixin meglumine. Plasma data best fitted a 2-compartment model. Distribution half-life was 0.55 hour; elimination half-life was 3.7 hours; volume of distribution at steady state was 0.18 L/kg; volume of the central compartment was 0.079 L/kg; and total body clearance was 1.07 mL/min/kg. The pharmacokinetic parameters obtained for the herein Ilium Flunixil® IV Injection greyhound study, as shown in Table 1, are entirely consistent with the results from the Hardie et al. (1985) publication. Plasma concentrations fell to as low as 0.5 ng/mL at 48 hours, below the range of 10-50 ng/mL levels of detection in these previous studies. The urine concentrations of flunixin were similar to those previously found for studies up to 96 hours in urine (Brady et al 1997: Dumasia et al 1988). Concentrations fell to as low as 1 ng/mL at 96 hours, below the range of 2-25 ng/mL in these previous studies. Using the standard methodology for medication control as described by Toutain and Lassourd (2002) the Irrelevant Plasma Concentration (IPC) was determined as 1 ng/mL and the Irrelevant Urine Concentration (IUC) was determined as 30 ng/mL. The actual Screening Limit and Detection Time used by regulators will not necessarily be absolutely based on these Irrelevant Concentrations. Regulatory Risk Management is not a scientific exercise but it should be scientifically sound, and the Screening Limit may be (slightly) higher or lower than the IPC/IUC to take into account other relevant factors other than residual drug efficacy as the common goal to achieve harmonisation (Toutain 2010).

A field study on meat from farm animals potentially to be fed to dogs found concentrations of flunixin of up to 92 µg/kg (Dumasia et al 1988). Twelve hours after exposure of cattle to a final dose of 3.3 mg/kg gave mean total residue concentrations for flunixin of 3800, 2500, 60 and 10 µg equivalents/kg in liver, kidney, fat and muscle, respectively (Committee for Veterinary Medicinal Products 1999). Plasma bioavailability following oral administration of flunixin is reported as 97% (Committee for Veterinary Medicinal Products 1999). Therefore in any animal by products from treated farm animals or horses (Committee for Veterinary Medicinal Products 1999), which might include liver and kidney as well as meat, being fed to greyhounds, there is potential for that greyhound to be exposed to a significant fraction (0.01-3.8 mg/dog based on 1kg of meat) of the therapeutic dose used in this study (19.8 mg flunixin free acid/dog). Therefore any regulatory Screening Limit for medication control of flunixin, derived from the IPC and IUC, can also serve as a Residue Limit for flunixin exposure via feeding animal by products (Morris 2015).

While the terminal urine half-lives for flunixin are similar between dogs (6.77±1.69) it is clear from Figure 1b that there is a large variation in the flunixin concentrations for time points between 8 and 48 hours post dose. From analysis of the data from individual dogs it appears that there are two groups of dogs, with three having much higher flunixin exposure compared to the remaining three dogs. Flunixin is a drug of the weak acid class and therefore can ionise from its neutral form (HA) to a charged form (A^-) and a dissociated proton. The degree of ionisation will be dependent on the

pKa of flunixin, 5.82, (Odensvik 1995) and the pH of the environment according to the Henderson-Hasselbalch equation: pH = pKa + log[A^-/HA]. If the pH of urine is less than the pH of blood then HA dominates in urine resulting in greater reabsorption of flunixin from urine back into blood and therefore reduced levels of flunixin in the urine. On the other hand, if the pH of urine is greater than the pH of blood then A^- dominates in urine resulting in the trapping of flunixin in the urine and therefore increased levels of flunixin in the urine. The area under the urine flunixin concentration-time curve is a measure of flunixin exposure in the urine. Figure 2 shows that there is a relationship between the flunixin exposure in urine and the average urine pH. The three dogs with the highest average urine pH have far greater flunixin exposure compared with the dogs that have the lowest. This is entirely consistent with the extent of ionisation predicted by the Henderson-Hasselbalch equation where A^- dominates for pH above 5.82 leading to trapping of flunixin in the urine and HA dominates for pH below 5.82 leading to greater reabsorption of flunixin back into blood. The urine pH is therefore an important consideration with regard to variability in the urine pharmacokinetics of flunixin and can be clearly seen when the urine and plasma pharmacokinetics are compared. Correcting urine concentrations for pH is not straightforward as only the flunixin reabsorption from urine to blood component of the pharmacokinetics will be affected. A possible explanation for the single decay phase in urine for the three dogs with higher urinary pH may be due to negligible reabsorption of flunixin from urine to blood whereas the first decay phase for the three dogs with lower urinary pH may be due to a significant component of reabsorption from urine to blood. Nevertheless, the variability in the urine pharmacokinetics reduces with time and at 72 hours post administration, in the terminal phase, the variability in urine and plasma flunixin concentrations are similar.

This administration study enabled the IPC and IUC to be determined after the exposure of greyhounds to clinical doses of flunixin. This information will allow greyhound regulators to provide regulatory advice for greyhound racing in respect of both screening limits for medication control and residue limits if meat containing residues is fed.

CONFLICT OF INTEREST STATEMENT

Greyhound Racing Victoria funded this study, the administration study was performed at Eurofins SCEC, the chemical analysis was performed at Racing Analytical Services Limited and the pharmacokinetic analysis was performed at the University of Nottingham. Tim Morris is Independent Scientific Adviser to the Greyhound Board of Great Britain and receives fees for this activity and holds an unpaid appointment the University of Nottingham. Steven Karamatic is Chief Veterinarian at the Greyhound Racing Victoria and receives payment this activity. Paul Zahra and Eric Li are employees of Racing Analytical Services Limited. Stuart Paine is an employee of the University of Nottingham and has received fees for advice from the Greyhound Racing Victoria. Sally Colgan is an employee of Eurofins SCEC.

AUTHOR CONTRIBUTION STATEMENT

Tim Morris coordinated the interpretation and reporting of this study, Stuart Paine performed pharmacokinetic analysis and interpretation, Paul Zahra and Eric Li developed methodology and provided chemical analysis. Steven Karamatic designed the study and contributed to regulatory interpretation. Sally Colgan was the principal investigator for the in-vivo study. All authors contributed to and reviewed the manuscript and are accountable for its contents.

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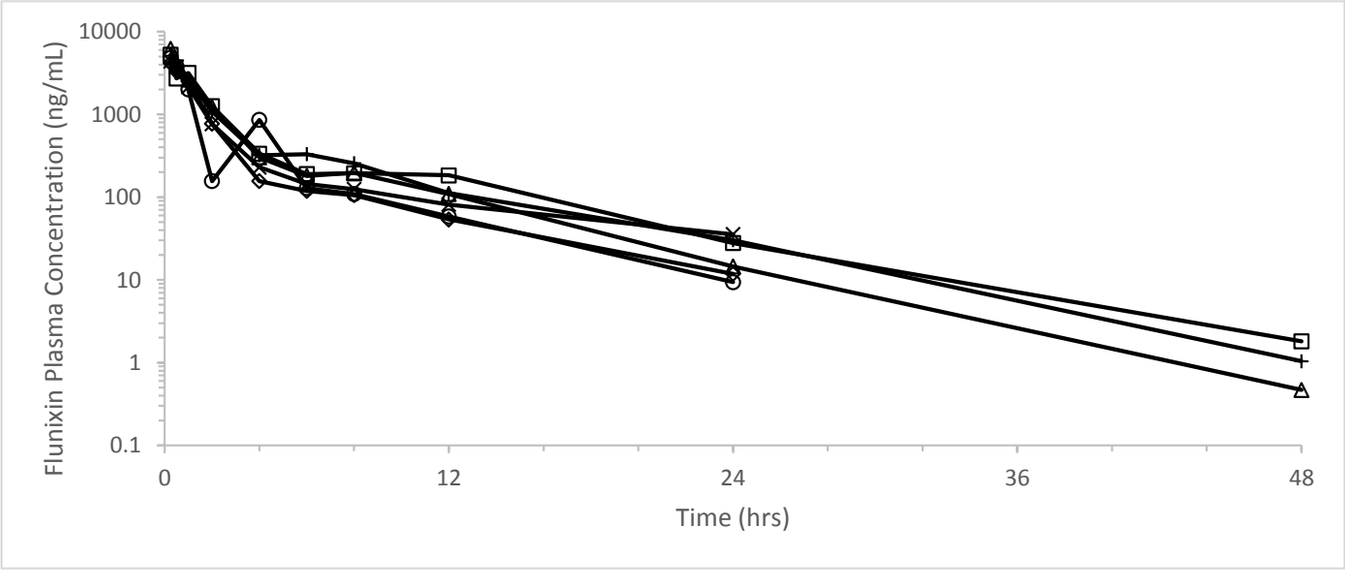
Table 1: Plasma pharmacokinetic parameters for flunixin following a single intravenous 1 mg/kg dose to 6 greyhounds fitted to a 2 compartmental pharmacokinetic model. V1 and V2 are the physiological volumes of each respective compartment. Alpha and beta HL are the respective half-lives of the first and second decay phases; MRT is the mean residence time (average time spent in animal) of flunixin; Vss is the steady-state volume of distribution for flunixin; CL is the plasma clearance for flunixin.. * = Geometric mean

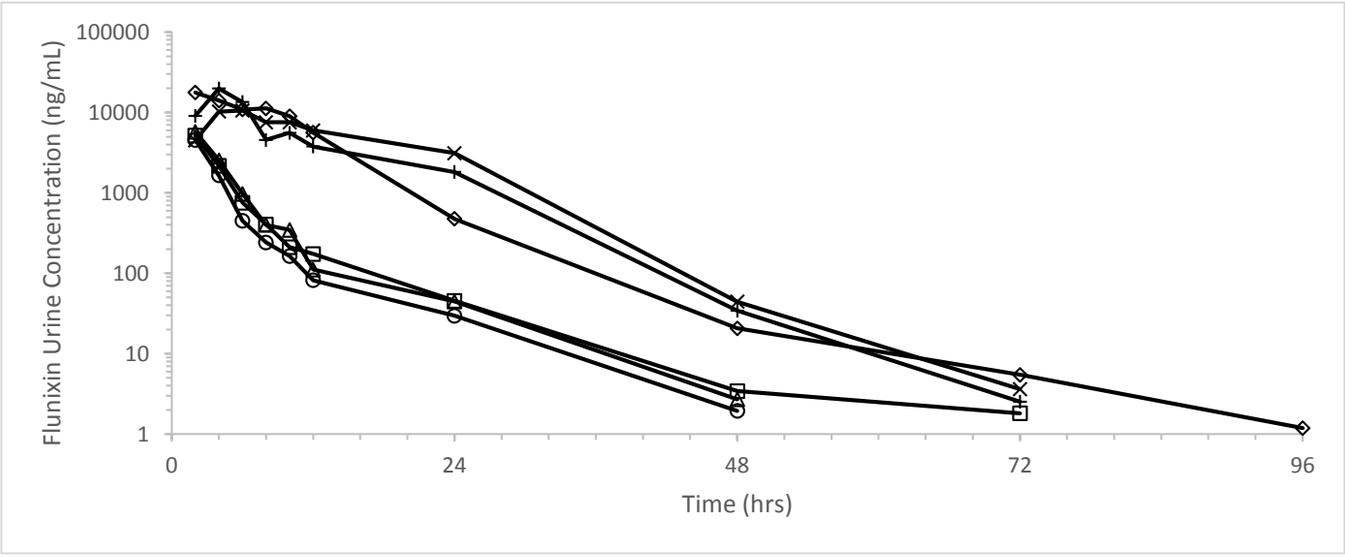
Animal	Weight (Kg)	V1 (L/Kg)	V2 (L/Kg)	alpha HL (hrs)	beta HL (hrs)	M R T (hrs)	Vss (L/Kg)	CL (mL/min/Kg)
Dog 1	35.2	0.095	0.148	0.58	5.57	2.84	0.24	1.43
Dog 2	30.7	0.076	0.111	0.58	4.74	3.07	0.19	1.01
Dog 3	39.4	0.087	0.159	0.57	5.22	4.11	0.25	1.00
Dog 4	33.8	0.098	0.289	0.61	9.05	5.21	0.39	1.24
Dog 5	31.4	0.059	0.182	0.32	5.10	2.68	0.24	1.50
Dog 6	28	0.094	0.162	0.68	6.03	4.29	0.26	1.00
Mean	**33.1**	**0.085**	**0.175**	**0.54***	**5.81***	**3.70**	**0.26**	**1.20**
Median	**32.6**	**0.091**	**0.161**	**0.58**	**5.39**	**3.59**	**0.25**	**1.13**

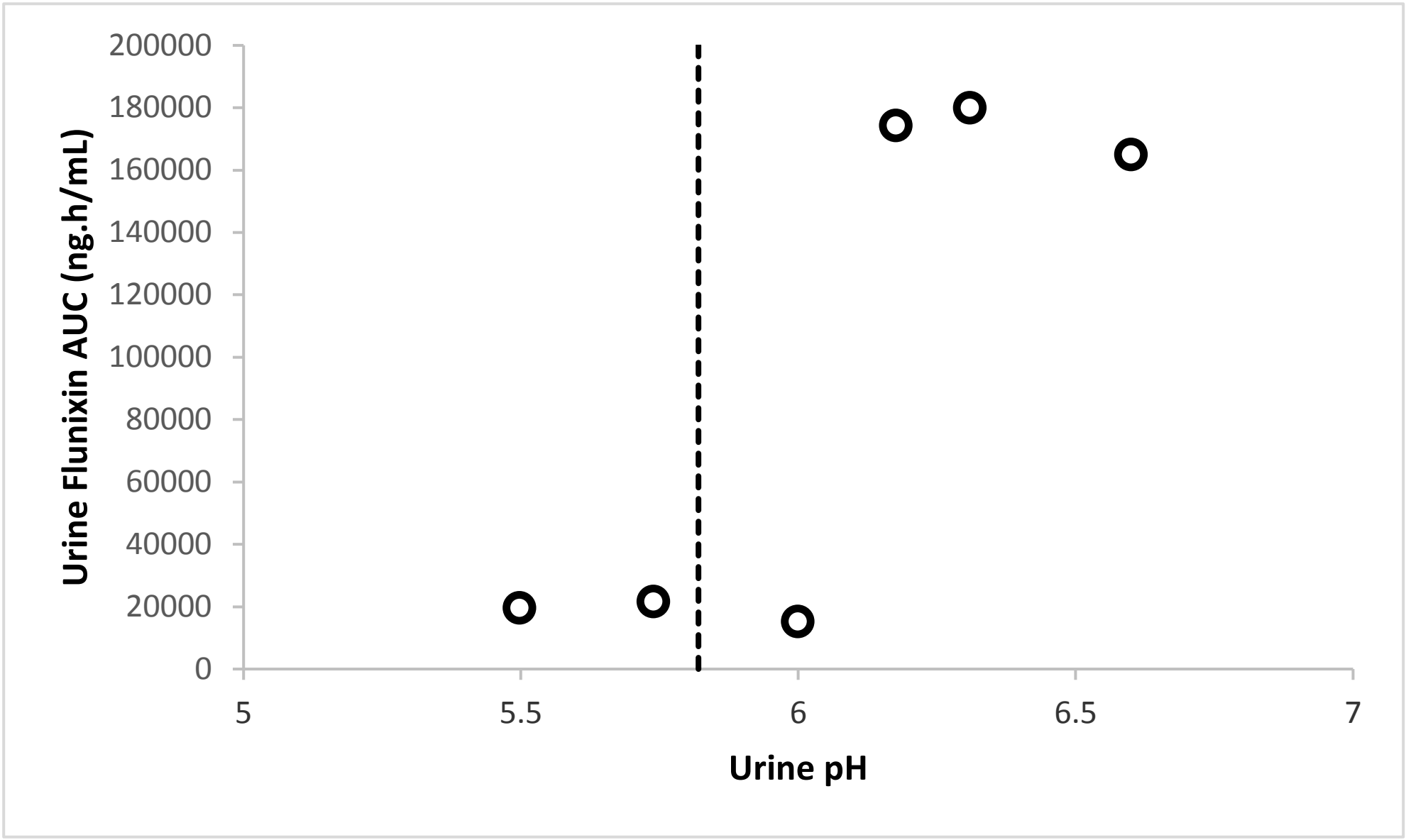
Figure 1a Plasma concentrations in ng/mL of flunixin following a single intravenous dose of 1mg/kg to 6 greyhounds. Symbols on each line, which are different between lines, indicate the data from individual animals.

Figure 1b Urine concentrations in ng/mL of flunixin following a single intravenous dose of 1mg/kg to 6 greyhounds. Symbols on each line, which are different between lines, indicate the data from individual animals.

Figure 2 Area under the urine flunixin concentration-time curve, a measure of flunixin exposure in the urine, compared to the time weighted average urine pH for each dog using a non-compartmental approach Closed circles indicate data from individual animals.


Flunixin Plasma Concentration (ng/mL)
10000
1000
100
10
1
0.1
0
12
24
36
48
Time (hrs)


Flunixin Urine Concentration (ng/mL)
100000
10000
1000
100
10
1
0
24
48
72
96
Time (hrs)


Urine Flunixin AUC (ng.h/mL)
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
5
5.5
6
6.5
7
Urine pH