JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Comparison of ELISA and LC−MS/MS for the Measurement of Flunixin Plasma Concentrations in Beef Cattle after Intravenous and Subcutaneous Administration

Weilin L. Shelver,*[,†] Lisa A. Tell,[‡] Sarah Wagner,[§] Scott E. Wetzlich,[‡] Ronald E. Baynes,[⊥] Jim E. Riviere,[⊥,¶] and David J. Smith*[,†]

[†]USDA-ARS Biosciences Research Laboratory, 1605 Albrecht Boulevard, Fargo, North Dakota 58102, United States

[‡]FARAD, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, California 95616, United States

[§]Department of Animal Sciences, North Dakota State University, Fargo, North Dakota 58108, United States

[⊥]FARAD, Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606, United States

ABSTRACT: Eight cattle (288 ± 22 kg) were treated with 2.2 mg/kg of body weight of flunixin free acid in a crossover design by subcutaneous (SC) and intravenous (IV) administration. After a minimum 1:10 dilution with 50 mM phosphate buffer, a commercial immunoassay was adapted to determine plasma concentrations of flunixin. The limit of detection was 0.42 ng/mL and the working range was 0.76−66.4 ng/mL when adjusted with the dilution factor. Plasma samples were extracted using mixed-mode cation exchange solid phase extraction prior to the LC−MS/MS analyses. The linear calibration curve for LC−MS/MS was 0.5−2000 ng/mL with a limit of detection of 0.1 ng/mL for flunixin and 0.3 ng/mL for 5-hydroxy flunixin. Flunixin concentrations determined using the ELISAs were compared to concentrations derived from the same samples using LC−MS/MS analyses. Pharmacokinetic parameters of time versus concentration data from each analysis were estimated and compared. Differences ($P < 0.05$) in estimates of area under the curve, volume of distribution, and clearance were apparent between ELISA and LC−MS/MS analyses after IV dosing; after SC dosing, however, there were no differences among the estimated parameters between the two methods. Quantitative immunoassay was a satisfactory method of flunixin analysis and that it would be difficult to differentiate routes of administration in healthy beef cattle based on the plasma elimination profile of flunixin after IV or SC administration.

KEYWORDS: *cattle, flunixin, ELISA, pharmacokinetics, elimination, administration, concentration*

INTRODUCTION

Flunixin is a nonsteroidal anti-inflammatory drug approved by the United States Food and Drug Administration (FDA) for use in cattle to control pyrexia associated with respiratory disease, endotoxemia, and mastitis, and for the control of inflammation in endotoxemia. Its rapid onset, potent anti-inflammatory action, and compatibility with coadministered antibiotics make flunixin a commonly used medication for livestock. The FDA approved route of administration for cattle is intravenous (IV), with a preslaughter withdrawal period of 4 days (Federal Register 38749). For swine, the FDA approved route of flunixin administration is via intramuscular injection (IM); however, in swine, a 12-day preslaughter withdrawal time (Federal Register 70998) must be observed. For food animals, observance of the preslaughter withdrawal period is critical for ensuring that flunixin concentrations in tissues intended for human consumption are below tolerance concentrations [(125 ppb in cattle liver, 25 ppb in cattle and swine muscle, 30 ppb in swine liver for flunixin free acid), and 2 ppb in milk with 5-OH-flunixin as the marker residue (Federal Register 2011-6793)].

In animals having violative tissue concentrations of drug residues in the United States, flunixin is one of the most commonly found, especially in cull dairy cattle.[1] For example, in 2008, the US Food Safety and Inspection Service (FSIS) used the fast antibiotic screening test (FAST) to investigate a number of animals suspected of having violative antimicrobial residues.[2] Those animals that tested positive for antibiotic residues were also screened for the presence of flunixin. A total of 327 flunixin violations occurred in FAST positive cattle, indicating that nearly 20% of animals that tested positive for antibiotics also tested positive for flunixin. Although the number of flunixin violations decreased to 242 in production cattle in 2009,[3] problems with flunixin violative residues continue.[4−6] Several factors could contribute to the incidence of violative flunixin residues in cattle including excessive dosage, slaughter with insufficient preslaughter withdrawal periods, lack of proper animal identification methods, disease status that negatively impacts normal organ function and drug clearance, or flunixin administration through a nonapproved route in conjunction with an insufficient preslaughter withdrawal. For flunixin in cattle, IV administration is not as convenient as subcutaneous (SC) or IM administration. Flunixin violative

Received: November 9, 2012
Revised: February 12, 2013
Accepted: February 21, 2013
Published: March 7, 2013

residues were investigated by the FDA and most instances were attributed to flunixin administration by extralabel routes.[7]

The pharmacokinetic parameters for flunixin plasma/serum concentrations versus time subsequent to dosing have been reported for many species including camels,[8] cattle,[9−12] horses,[13] donkeys,[14] goats,[15] sheep,[16] cats,[17] pigs,[18] rats,[19] mice,[20] and various birds.[21] Most of these studies estimated pharmacokinetic parameters after IV administration, but some studies compared pharmacokinetic estimates after IM or oral flunixin administration. For example, in heifers, the oral bioavailability of flunixin granules was 60% with mean appearance (absorption) (MAT, oral) and mean residence times (MRT, IV) of 6.3 and 12.7 h, respectively. Prolonged oral absorption after granule administration was confirmed by a moderately long t_{max} of 3.5 h.[10] In a study in lactating cattle,[12] flunixin concentrations were fit by a 2 compartment model; a shorter elimination phase occurred after IV administration than after IM administration. In pigs,[18] the bioavailability of flunixin after IM administration (2.2 mg/kg of body weight (bw)) was approximately 70% with rapid absorption (K_a = 8.5 h^{-1}) that was confirmed by a short t_{max} of 0.3 h. The terminal half-life after IM administration increased, relative to the terminal half-life after IV administration, from 6.3 to 8.8 h.[18] Other studies have compared the effect of disease on the pharmacokinetics of flunixin. For example, in rabbits, the half-life of flunixin elimination increased, and its clearance decreased, in endotoxaemic individuals compared to healthy animals.[22] While studies in investigating the effect of disease state and flunixin pharmacokinetics have not been investigated in cattle, other studies with nonsteroidal anti-inflammatory drugs suggest that infection has profound effects on drug elimination. Specifically, carprofen pharmacokinetics were altered significantly by disease state with a lower clearance and an increased half-life in endotoxin induced mastitic dairy cows.[23] To our knowledge, only a few plasma pharmacokinetic studies of flunixin after SC administration in cattle have been reported, but in these studies flunixin was coadministered with oxytetracycline[24] or florfenicol;[25,26] an additional study compared flunixin pharmacokinetics after IV, IM, or SC administration in dairy cattle.[27] Metabolism studies have indicated that the marker residue is flunixin for tissues, but for milk the marker residue is 5-OH-flunixin.[28]

A variety of analytical methods have been used to determine flunixin concentrations in animal matrices, including an ELISA with a limit of detection of 1 ng/mL in dog urine.[29] An HPLC method with UV detection at 330 nm had a limit of quantitation of 50 ng/mL[22] in rabbit plasma. A similar HPLC procedure[9] using a light diode array (no peak specified) had a limit of quantitation of 20 ng/mL for plasma obtained from cows post flunixin administration (2.2 mg/kg). An HPLC/UV method was used to quantify flunixin and 5-OH flunixin in heifers with a limit of detection at 30 ng/mL for both compounds.[30] The use of LC-MS/MS coupling quadrupole time-of-flight tandem mass spectrometry with liquid chromatography[31] has resulted in increased sensitivity with a limit of quantification (1 ng/mL) for flunixin in equine plasma. Currently, the US FSIS uses a flunixin ELISA test kit to screen bovine liver and muscle samples[32] while HPLC/ESI-MS/MS has been utilized in determining and confirming the presence of flunixin in bovine and porcine liver and muscle tissue samples.[33] ELISA offers a format suitable for running the large number of samples necessary for pharmacokinetic evaluation without the complexity of sophisticated instrumental methods. However, ELISAs can be subject to matrix effects, have narrower assay dynamic range and can cross-react with metabolites. In this study, an ELISA test was used to measure plasma concentrations of flunixin free acid in cattle administered flunixin meglumine by IV or SC routes and ELISA data were compared to data obtained from the analyses of the same samples using LC−MS/MS. Estimates of pharmacokinetic parameters were calculated from the plasma flunixin free acid concentration vs time curves resulting from each analytical method. These parameters were compared to determine if the analytical method used influenced kinetic estimates.

MATERIALS AND METHODS

Chemicals and Supplies. Banamine (flunixin meglumine; Schering-Plough; Summit, NJ) solution for injection was obtained from Stockman's supply (West Fargo, ND). Flunixin ELISA kits were purchased from Neogen Corporation (Lexington, KY). Flunixin analytical grade standard was obtained from Sigma-Aldrich (St. Louis, MO). Deuterated forms of flunixin and 5-hydroxy flunixin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. All other reagents were obtained from common chemical suppliers.

Plasma Collection. An animal protocol was approved by the North Dakota State University Animal Care and Use Committee prior to the start of the study. Four healthy Angus heifers and four healthy Angus steers (*Bos taurus*), age 8−9 months, 258−340 kg bw, were housed in a fenced pasture with open access to a covered shelter. Prior to the beginning of study procedures, the animals were evaluated by a veterinarian and found to be healthy. Animals had ad libitum access to grass hay and water for the duration of the study. Cattle were dosed with 2.2 mg/kg bw of flunixin free acid by either the IV (mean bw 288.1 ± 21.9 kg) or SC (mean bw 288.0 ± 23.5 kg) route using a crossover design with a four week wash-out period. Subcutaneous administration was superficial to the Trapezius muscle of the neck. Blood samples (10 mL) were collected at 0, 5, 10, 15, 20, 30, 45, 60, and 90 min and at 2, 4, 8, 12, 24, 36, 48, 60, 72, 96, 120, 240 h post dosing via jugular venipuncture using 20 gauge × 1.5 in. needles into BD Vaccutainer tubes containing 143 units of sodium heparin (Franklin Lakes, NJ). After gentle inversion (10×), the tubes were centrifuged at 3210*g* for 15 min (Beckman GS-6R, Beckman Coulter, Inc.; Indianapolis, IN), approximately within 2 h after sample collection. Plasma aliquots were distributed into replicate, labeled, 2-mL polypropylene vials that were subsequently stored at −20 °C until flunixin analysis.

ELISA Procedure. The flunixin antibody coated plate, horseradish peroxidase conjugate, and substrate provided by the manufacturer (Neogen Corporation, Lexington, KY) were used for assay development. The ELISA utilized a matrix-matched plasma curve diluted 1:10 in 50 mM phosphate buffer, pH 7.4, with calibration points of 0, 0.1, 0.2, 0.5, 1, 3, 10, 30, and 300 ng/mL. Aliquots of plasma samples and controls were likewise diluted 1:10 in 50 mM phosphate buffer. If greater than 1:10 dilution was required for a given incurred plasma sample, diluted blank plasma (1:10) was used as a diluent. Briefly, in each well, 50 μL of diluted sample, control, or standard was co-incubated with 180 μL of enzyme-conjugate solution at room temperature. After 45 min, the plates were washed with 350 μL of phosphate buffered saline containing 0.05% Tween 20 (5×). Following the plate wash, 150 μL of the single component peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (K-blue), was added and incubated at room temperature for 30 min. After color development, 50 μL of 1 N hydrochloric acid was added. The plates were read at 450 nm (Bio-Rad model 550 ELISA plate reader, Bio-Rad Laboratories, Inc., Hercules, CA). Standard curve data were fitted to a four parameter logistic equation:

$$\text{Abs} = (A - D)/(1 + (\text{Conc}/\text{IC}_{50})^b) + D$$

where A is the absorbance from a standard with no analyte; D is the absorbance from a standard containing the maximum analyte concentration; b is the slope at the inflection point of the curve

(roughly at the IC_{50}); IC_{50} is the concentration at the inflection point of the curve; and where "Conc" and "Abs" are the X and Y variables, respectively, that were fit to derive the above parameters.

The unknown concentrations of flunixin free acid in test samples were computed from the standard curve and adjusted for the dilution factor. Each sample concentration was calculated from the mean of two wells. All samples were measured on two different days and the mean value of the two days was used for subsequent kinetic modeling. In cases in which across day replicates differed by >50% (over all 24% of the total assayed samples, particularly the 5, 10, 15, and 20 min plasma where the dilution factor was as high as 1:128,000), additional analyses were repeated. Plasma diluted at 1:5, 1:10, and 1:20 with 50 mM phosphate buffer were used to evaluate matrix interference, while diluted plasma (1:10) fortified at 0.2, 0.5, 2, and 5 ng/mL were used to evaluate intraday and interday assay variation for ELISA validation.

LC−MS/MS Analysis. Concentrations of flunixin free acid and 5-hydroxy flunixin were also quantified using ultra high performance liquid chromatography (UHPLC) with mass spectrometric (MS/MS) detection. Quantification was based on the method by Buur et al.[34] Briefly, 0.5 mL of plasma samples was fortified with the deuterated forms of flunixin free acid and 5-hydroxy flunixin as internal standards and then acidified with 20 μL of concentrated phosphoric acid (85% v/v). Internal standard-fortified samples were loaded onto Waters Oasis MCX (Waters Corp., Milford, MA) cartridges (1 mL, 30 mg) that had been conditioned with 1 mL of methanol followed by 1 mL of water. The loaded cartridges were washed with 1 mL of 0.1 N hydrochloric acid followed by 1 mL of methanol/0.1 N hydrochloric acid (90:10, v/v). Cartridges were subsequently dried under vacuum for 30 s before elution of flunixin free acid with 1 mL of methanol: ammonium hydroxide (90:10, v/v). The eluents were evaporated to dryness under nitrogen at 50 °C (N-Evap, Organomation, Berlin, MA), reconstituted with 250 μL of acetonitrile/water (40:60, v/v) and filtered with a 0.2 μm PVDF syringeless filter device (Mini-Uni Prep, Whatman, Maidstone, Kent, U.K.). Concentrations of flunixin and 5-hydroxy flunixin in unknowns were determined by regressing peak area ratios of flunixin, 5-hydroxy flunixin and their respective dueterated internal standard analogs with peak area ratios of the standards which were fortified into blank plasma samples and extracted along with unknown samples. Sample concentrations exceeding the highest calibration point were diluted with control plasma and re-extracted prior to being reanalyzed. Limit of detection (LOD) and limit of quantitation (LOQ) were based on baseline measurements of blank control matrix. LOD was the average plus 3 standard deviations while the LOQ was average plus 10 standard deviations.

Analysis was carried out on an ACQUITY UPLC (Waters Corp., Milford, MA) coupled with a Thermo TSQ Quantum Discovery Max (Thermo Electron, West Palm Beach, FL) tandem quadrupole mass spectrometer with a heated electrospray ionization source operated in the positive ion mode. The column was an ACQUITY UPLC HSS T3 (1.8 μm, 2.1 × 100 mm) maintained at 30 °C. The mobile phase was 0.1% acetic acid/acetonitrile (32:68) at 0.4 mL/min. Ions were monitored in the selected reaction monitoring mode with transitions at m/z 297 → 279 for flunixin, and 300 → 282 for flunixin-d_3; 313 → 295 for 5-hydroxy flunixin, and 316 → 298 for 5-hydroxy flunixin-d_3.

Pharmacokinetic Parameter Estimation. Plasma flunixin free acid concentrations versus time data were analyzed on an individual animal basis using PK Solutions 2.0 (Summit Research Services; Motrose, CO). Data points up to 60 h were used for the computations since data points beyond this were low concentrations showing high variance. The data were analyzed using noncompartmental techniques. To determine the area under the concentration versus time curve ($AUC_{0-60\ h}$), the trapezoid rule was used. The extrapolation of AUC to infinite time was not done because the change from $AUC_{0-60\ h}$ would be small and would suffer from uncertainty surrounding the validity of late time points. Curve stripping was used to estimate first exponential term (elimination) and then additional exponential terms as determined to be appropriate using visual graphics and coefficient constants of fits provided by the PK Solutions 2.0 software. The C_{max} and the associated time (t_{max}) were obtained directly from the raw data (i.e., were observed rather than calculated). The parameters from each animal were computed in this manner and the parameters were averaged for each of the treatments (IV and SC, n = 8).

Statistical Analyses. Data from each analytical method were treated separately. For example, within time point, mean plasma concentrations as determined after ELISA or LC−MS/MS analysis were compared using the student's t-test after testing for equal variance. Type-I statistical error, common with multiple comparisons, was minimized by comparing only means between analytical method within dose route and time point. A similar procedure was used for the comparison of within dose-route pharmacokinetic parameters calculated from ELISA or LC−MS/MS data. In some instances, data were transformed to ensure that the equal variance assumption was met. Specific transformations are noted in the appropriate tables.

RESULTS AND DISCUSSION

As an initial evaluation of the flunixin ELISA kit, the effect of matrix dilution on flunixin binding relative to 50 mM phosphate buffer was determined. Figure 1 shows that relative

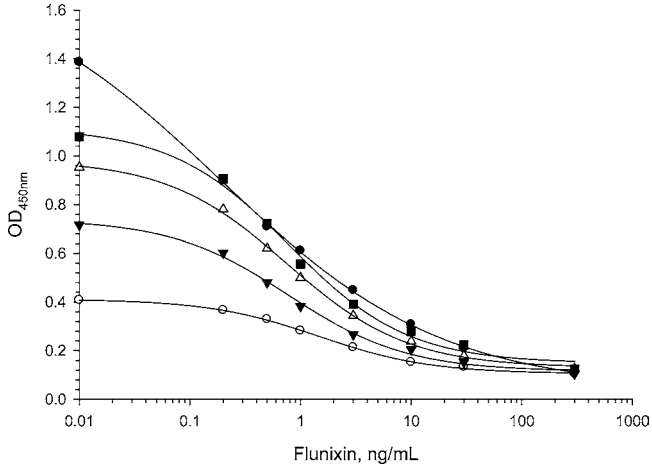

Figure 1. Effect of dilution of beef cattle plasma samples on ELISA response relative to 50 mM phosphate buffer (●). Plasma samples (n = 4 per point) were diluted 1:2 (○), 1:5 (▼), 1:10 (Δ), and 1:20 (■) in phosphate buffer. Even at the greatest level of dilution (1:20) some suppression of assay absorbance occurred.

to phosphate buffer, plasma interfered with flunixin binding, even at a 1:20 dilution. The best compromise for maintaining sensitivity, while minimizing interference with matrix, was 1:10 dilution of plasma with phosphate buffer. Figure 2 shows the average of 38 calibration curves generated in a 1:10 dilution of plasma with 50 mM phosphate buffer during the course of the study. The mean limit of detection, based on IC_{10}, was 0.042 ± 0.014 ng/mL of flunixin. Correspondingly, the mean working range, based on IC_{15} to IC_{85} was 0.076 ± 0.023 to 6.64 ± 1.51 ng/mL with a corresponding IC_{50} of 0.70 ± 0.16 ng/mL (n = 38). For LC−MS/MS, the LOD was 0.1 ng/mL for flunixin free acid and the LOQ was 0.4 ng/mL, whereas the LOD and LOQ for 5-hydroxy flunixin were 0.3 and 0.9 ng/mL, respectively. When adjusted for the dilution factor (10), the ELISA sensitivity was similar to the LC−MS/MS method used for analysis of equine plasma (1 ng/mL)[27] and somewhat less than the LOQ (0.4 ng/mL) of the LC−MS/MS used in our study.

Table 1 shows the interday and intraday variation of the plasma flunixin immunoassay and LC−MS/MS over a range of flunixin free acid fortification levels and the corresponding recoveries of flunixin in the fortified samples. At 0.2, 0.5, 2, and 5 ng/mL of spiked flunixin free acid, the intraday recoveries

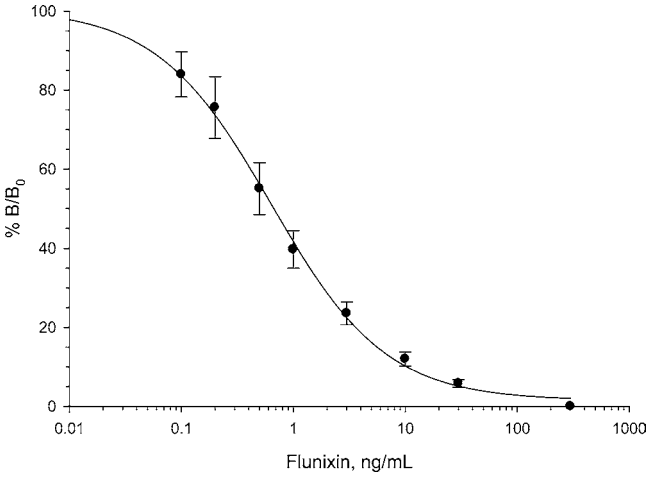

Figure 2. Calibration curve of the flunixin ELISA. Data are summarized from 38 independent curves generated over a 4 month period. The IC_{50} averaged 0.70 ± 0.16 ng/mL.

ranged from 92 to 106%, with corresponding RSDs of 12−24% for ELISA. At the same fortification levels, the interday analyses had RSDs of 14−34%. Because of the ELISA's "S" shaped calibration curve, the linear portion of the curve is most accurate and had intra- and interday variations of <20% with recoveries >90% for the 0.5, 2, and 5 ng/mL spiked samples. Because of the relatively limited linear range of the ELISA, samples containing high flunixin concentrations had to be diluted with subsequent correction of results for dilution. When plasma was fortified with 500−50 000 ng/mL of flunixin free acid and analyzed after dilution with 1:10 blank plasma, recoveries of 101−113% were obtained with intraday RSD of 13−16% and no apparent differences from the recoveries of plasma spiked with lower concentrations of flunixin.

At 1, 20, and 2000 ng/mL fortification levels, the recoveries ranged from 98 to 104% with RSDs of 3.6−14% for flunixin free acid and the recoveries ranged from 98 to 101% with RSDs of 3.8−8.8% for 5-hydroxy flunixin for the LC−MS/MS method. For LC−MS/MS, the 20 and 2000 ng/mL fortifications had intraday RSDs of <5%. The standard curve was linear between 0.5 and 2000 ng/mL for flunixin free acid and 1.0 and 2000 ng/mL for 5-hydroxy flunixin with mean R^2 values of 0.996 and 0.994, respectively.

Table 2 shows means and ranges of plasma flunixin free acid concentrations from 0 to 60 h after dosing, for both IV and SC dosed cattle, as determined by both ELISA and LC−MS/MS methods. Although we collected blood samples up to 240 h post administration, plasma concentrations from 72 to 240 h were often < LOQ, or nondetectable. As would be expected after IV dosing, average plasma flunixin concentrations were high at the earliest time points (exceeding 35 000 ng/mL at 0.08 h, via ELISA), whereas mean plasma flunixin free acid concentrations in SC dosed cattle were much lower even when mean concentrations peaked (3600 ng/mL at 0.33 h, via ELISA). Table 2 also shows that at 0.08, 0.25, 0.75, 1.5, and 8 h after IV dosing, the immunochemical analysis returned significantly ($P < 0.05$) greater values for plasma flunixin free acid relative to the LC−MS/MS measurement. In addition, the overall trend in IV dosed animals was for immunoassay values to be numerically greater, although not statistically greater, than plasma flunixin free acid concentrations measured by LC−MS/MS (the 24 h point, being an exception). In contrast, plasma flunixin free acid concentrations in SC dosed animals were not generally different ($P > 0.05$) after quantification by immunoassay or LC−MS/MS, with the exception of the point at 4 h.

Figure 3 shows the correlation between immunoassay and LC−MS/MS derived plasma values for both IV (panels A−C) and SC (panel D) dosed animals. Figure 3 clearly shows that as plasma flunixin free acid concentration increased, the correlation between the returned values of the LC−MS/MS and immunoassay decreased. For example, for the IV dosed animals the overall correlation (R^2) between results from the

Table 1. Interday and Intraday Variations of Flunixin ELISAs and LC−MS/MS at Various Fortification Concentrations (ng/mL) Using Bovine Plasma as the Matrix

	Interday			Intraday			
fortification (ng/mL)	measured ± SD (ng/mL)	mean recovery (%)	RSD	measured ± SD (ng/mL)	mean recovery (%)	RSD	dilution factor
			ELISA				
0.2[a]	0.20 ± 0.07[b]	98	35	0.18 ± 0.04[b]	92	24	NA[c]
0.5	0.51 ± 0.09	102	18	0.51 ± 0.07	102	14	NA
2.0	2.22 ± 0.31	111	14	2.12 ± 0.27	106	12	NA
5.0	4.66 ± 0.86	93	18	4.60 ± 0.65	92	14	NA
500[b]	504 ± 71	101	14	507 ± 65	101	13	500
5000	5630 ± 1120	112	20	5670 ± 833	113	16	5000
50,000	52100 ± 9190	104	18	52300 ± 7850	104	14	50,000
			LC−MS/MS/Flunixin				
1	1.02 ± 0.07[d]	106	14	1.0 ± 0.05[d]	104	5	NA
20	19.7 ± 0.7	100	4	20.0 ± 0.59	100	3	NA
2000	1953 ± 74	99	4	1956 ± 49	98	3	NA
			LC−MS/MS/5-Hydroxyl Flunixin				
1	1.0 ± 0.09[d]	98	9	1.0 ± 0.07[d]	97	7	NA
20	20.2 ± 0.77	101	4	20.0 ± 0.52	101	3	NA
2000	1994 ± 77	100	4	1976 ± 48	100	2	NA

[a]Flunixin free acid was fortified into 1:10 bovine plasma prior to performing the assay for concentrations of 0.2-5 ng/mL. Flunixin free acid was fortified into blank plasma with 1:10 cattle plasma used as diluent prior to performing the assay for concentrations of 500-50,000 ng/mL. [b]Interday and intraday parameters were calculated from 10 replicates from each assay with average from 5 independent assays from five different days. [c]NA, not applicable. [d]Average from 16 separate sets of samples.

Table 2. Mean (± Std Dev) Plasma Concentrations of Flunixin Free Acid as a Function of Time in Cattle Dosed (2.2 mg/kg bw of Flunixin Free Acid as Flunixin Meglumine) by Intravenous (n = 8) or Subcutaneous (n = 8) Injection

	intravenous administration					subcutaneous administration				
	immunochemical analysis		LC−MSMS analysis			immunochemical analysis		LC−MSMS analysis		
time	mean ± SD	range	mean ± SD	range	P^a	mean ± SD	range	mean ± SD	range	P^a
h	ng/mL	ng/mL	ng/mL	ng/mL		ng/mL	ng/mL	ng/mL	ng/mL	
0	0.0		0.0			0.0		0.0		
0.08	36000 ± 10000	25000−52000	26000 ± 4400	18000−32000	0.03[b]	1900 ± 1300	390−4100	1200 ± 940	180−2500	0.24
0.17	23000 ± 8900	11000−38000	17000 ± 2700	13000−22000	0.44[c]	3500 ± 2100	360−5100	2100 ± 1200	580−3400	0.14
0.25	18000 ± 3800	12000−22000	13000 ± 2500	10000−18000	0.01	2800 ± 1800	1100−6100	2400 ± 1500	780−4700	0.64
0.33	15000 ± 3500	6000−20000	11000 ± 3000	8400−16000	0.06	3600 ± 3400	420−11000	2500 ± 1600	870−5100	0.42
0.50	8500 ± 2900	5000−15000	7000 ± 1700	5600−11000	0.34	3400 ± 2300	1100−7300	2500 ± 1300	1100−4400	0.37
0.75	6000 ± 1900	3200−8900	4100 ± 720	3500−5800	0.03[b]	3400 ± 2300	1400−8400	2800 ± 1100	1400−4200	0.48
1	2900 ± 670	1800−4000	2600 ± 510	2200−3800	0.29	2900 ± 2000	1500−7400	2900 ± 920	1500−4000	0.98
1.5	1600 ± 440	890−2000	1200 ± 220	960−1500	0.03	2900 ± 1300	1900−5600	2700 ± 710	1800−3700	0.63
2	1200 ± 460	410−1900	950 ± 350	600−1600	0.26	2900 ± 1400	1200−4900	2400 ± 780	1500−3700	0.40
4	1300 ± 370	870−1800	1000 ± 270	690−1400	0.12	1700 ± 450	1200−2400	1200 ± 340	830−1600	0.03
8	730 ± 320	260−940	440 ± 92	340−630	0.01[b]	640 ± 180	320−840	630 ± 180	460−1000	0.85
12	220 ± 110	42−430	190 ± 76	110−360	0.65	350 ± 120	170−500	310 ± 87	160−440	0.47
24	21 ± 20	5.6−67	29 ± 28	7.2−86	0.50	31 ± 13	18−51	36 ± 15	17−56	0.52
36	3.9 ± 2.6	1.3−8.9	3.2 ± 2.5	0.5−7.8	0.62	3.6 ± 1.3	2.0−5.5	4.9 ± 2.3	2.6−9.6	0.18
48[d]	1.6 ± 0.9[e]	0.6−3.1	1.0 ± 0.9[e]	0.4−3.0	0.37	1.7 ± 1.2[f]	0.7−3.8	0.8 ± 0.3[g]	0.4−1.1	0.51
60[d]	3.6 ± 4.0[h]	0.6−10	4.8 ± 7.4[i]	0.5−13.3	0.76	1.6 ± 0.4[j]	1.3−2.0	1.0[k]	0.9−1.0	0.06

[a] Values of $p \leq 0.05$ were considered as significant. [b]Means were log transformed to ensure equal variance prior to t-test. [c]Means were sin transformed to ensure equal variance prior to t-test. [d]Only values greater than limit of quantitation (LOQ) were used to calculate means. [e]Means were calculated from 7 of 8 animals that had residue concentrations greater or equal to than the LOQ. [f]Means were calculated from 6 of 8 animals that had residue concentrations greater or equal to than the LOQ. [g]Means were transformed with $X = X$ + random (gaussian, SD = K) where K was set to 0.7 to ensure equal variance prior to t-test. [h]Means were calculated from 5 of 8 animals that had residue concentrations greater or equal to than the LOQ. [i]Means were calculated from 3 of 8 animals that had residue concentrations greater or equal to than the LOQ. [j]Means were calculated from 4 of 8 animals that had residue concentrations greater or equal to than the LOQ. [k]Means were calculated from 2 of 8 animals that had residue concentrations greater or equal to than the LOQ.

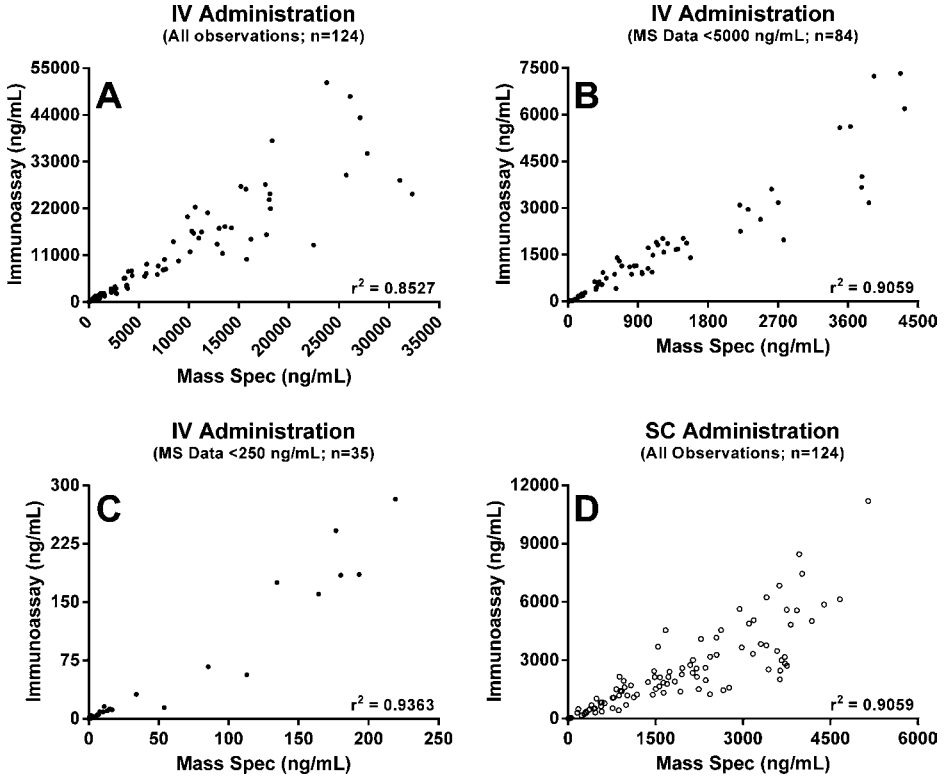

Figure 3. Correlations between plasma flunixin concentrations measured by flunixin immunoassay (Y) and LC−MS/MS (X) after intravenous (IV; panels A−C) or subcutaneous (SC, panel D) administration of 2.2 mg/kg of flunixin free acid as flunixin meglumine.

dx.doi.org/10.1021/jf304773p | J. Agric. Food Chem. 2013, 61, 2679−2686

immuno- and mass-spectral assay was 0.85; however, when data points greater than 5,000 and 250 ng/mL (based on the LC−MS/MS analysis) were eliminated, the correlation between the assays increased to 0.90 and 0.94, respectively. When only values greater than 5000 ng/mL (LC−MS/MS basis) were compared, the correlation dropped to 0.58 (correlation not shown). When data derived from SC dosed animals were analyzed (Figure 3, panel D) the overall correlation was 0.91, but flunixin free acid concentrations in SC dosed animals were much lower than those of IV dosed animals.

Some of the discrepancy between the immunoassay and LC−MS/MS results could result from the high dilution utilized in the ELISA analysis required by the limited linear range of this method. The tendency of the ELISA method to give higher values than LC−MS/MS could be partially due to the interaction with flunixin metabolites because the cross-reactivity of 5-hydroxy flunxin was 35% (data not shown). However, 5-hydroxy flunixin concentrations (Figure 4), represented a maximum of 7.1% of the total plasma residue (flunixin +5-hydroxy flunixin) in the SC dosed animals (12 h) and 8.1% of the total in the IV dosed animals (4 h). Hence, the presence of 5-hydroxy flunixin did not completely account for the difference observed between the LC−MS/MS and immunochemical methods since this metabolite accounted for less than 10% of the total flunixin free acid (Figure 4). Most likely, the differences arose from undefined matrix effects or other unknown causes.

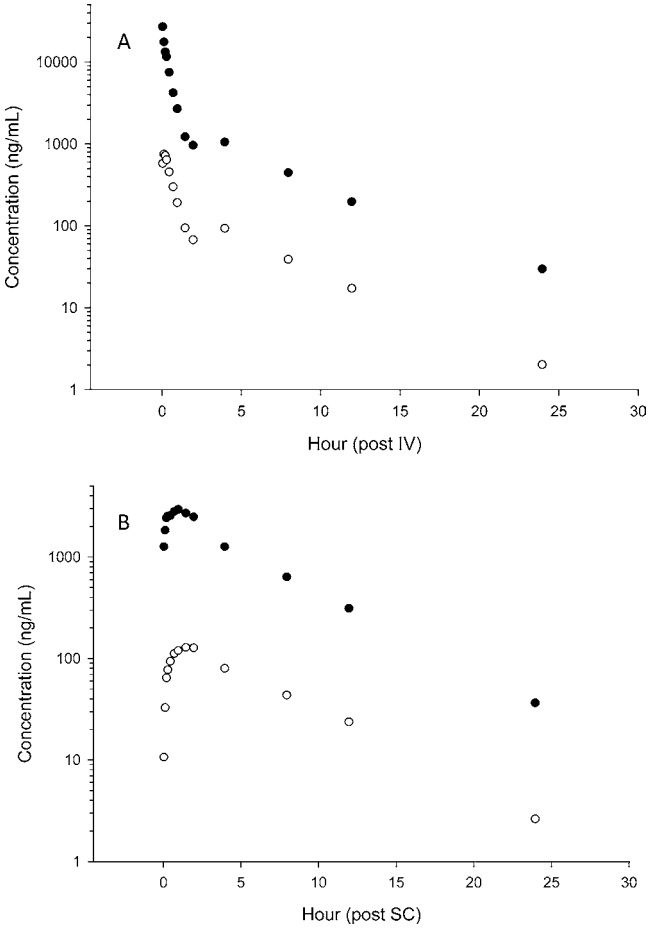

Figure 4. Mean plasma concentrations of flunixin (●) and 5-hydroxy flunixin (○) as a function of time after intravenous (A) or subcutaneous (B) administration of 2.2 mg/kg bw of flunixin free acid as flunixin meglumine.

Even though concentration differences existed, the use of the results from each analytical method for pharmacokinetic parameter computations was of interest. The use of the curve stripping process indicated the IV data required two exponential terms to obtain a satisfactory fit. Data were fit using typical noncompartmental techniques within the software. That is, visual observation of graphed data allowed the selection of points used for curve stripping in conjunction with the simultaneous computation of R^2 to indicate goodness of fit for the selection portion of the curve. After exploring the fit of the IV data, the fit of the subcutaneous data was explored by the addition of a third exponential term to describe the absorption process. Again, the software allowed observation of the goodness of fit by both visual and analytical computation. When the process of manually adjusting the regions of stripping was completed, the software was used to perform least-squared fits for to minimize parameter error. Finally, plots are made showing the fitted line and the actual data points. Means of kinetic parameters calculated for each route of administration and for each method of analysis are summarized in Table 3. Kinetic estimates of parameters derived from ELISA and LC−MS/MS data were essentially the same, except for clearance estimates which were significantly different ($P < 0.001$). However, the difference in clearance can ultimately be traced to the numerically higher $AUC_{0\text{-}t}$ returned by the ELISA data in combination with the lower bioavailability calculated with the ELISA data (also a function of $AUC_{0\text{-}t}$). $AUC_{0\text{-}t}$ computations are expected to be significantly influenced by the points at the highest flunixin concentration, and these points are the ones where the greatest differences between the two analytical methods occur. Differences ($P \leq 0.02$) also occurred when the $AUC_{0\text{-}t}$ was used in parameter computation such as volume of distribution and clearance. Thus, parameters derived from AUC were greatly influenced by the high ELISA values at early time points. In addition, differences in AUC-related parameters were exacerbated by variables affecting dose absorption (rate and extent) of the SC group, which has the overall effect of muting AUC values, relative to IV values, at early sampling times.

The terminal half-lives and rate constants within a dosing route were similar ($P > 0.20$) and were representative of previous estimates, with elimination half-lives averaging 4.8 and 5.4 h, for the IV and SC routes, respectively. This moderately rapid elimination was confirmed by the MRT of 3.2 h for flunixin free acid after IV administration. The MAT of flunixin free acid after SC administration was 2.0 h. Clearance calculated with the LC−MS/MS data set (114 $mL{\cdot}h^{-1}{\cdot}kg^{-1}$) was close to clearance reported in heifers[10] (115 $mL{\cdot}h^{-1}{\cdot}kg^{-1}$) and a little lower than in cows with mastitis[9] (140 $mL{\cdot}h^{-1}{\cdot}kg^{-1}$). Clearance values calculated from the ELISA data appeared to be lower than the estimates of Odensvick[10] and Rantala,[9] likely due to the high AUC obtained for the immunochemically derived data set. $AUC_{0\text{-}t}$, t_{max}, and terminal elimination half-life estimates (Table 3) after SC administration, compared reasonably well with those reported in NADA 141−299 (13.4 $\mu g{\cdot}h{\cdot}mL^{-1}$), 1.14 h, and 9.5 h, $n = 28$).[25] However, for this study, the C_{max}, 3.5−5.4 μg/mL, was much higher and the MRT of about 5 h, was much shorter than those reported in NADA 141−299 (1.9 μg/mL and 11.4 h).[24] The pharmacokinetic study reported in the NADA 141−299 utilized 28 crossbred commercial cattle weighing between 142 and 251 kg at the start of the study and the formulation included florfenicol and flunixin in 2-

dx.doi.org/10.1021/jf304773p | J. Agric. Food Chem. 2013, 61, 2679−2686

Table 3. The Effect of Analytical Method on the Pharmacokinetic Parameters Calculated after Non-compartmental Analyses of 8 Beef Cattle per Dose Route, Administered 2.2 mg/kg of Flunixin Free Acid as Flunixin Meglumine[a]

		route of administration					
		intravenous			subcutaneous		
parameter[b]	unit	ELISA	LC−MS/MS	P^c	ELISA	LC−MS/MS	P
Curve Stripping (Noncompartmental) Derived Parameters							
k_α	h^{-1}	3.32 ± 0.78	2.91 ± 0.24	0.77[d]	3.99 ± 1.78	3.4 ± 3.1	0.66
Half-life, α	h	0.22 ± 0.06	0.24 ± 0.02	0.97[e]	0.21 ± 0.10	0.34 ± 0.20	0.14
k_β	h^{-1}	NA[f]	NA	-	0.95 ± 0.85	0.88 ± 0.88	0.88
Half-life, β	h	NA	NA	-	2.13 ± 2.10	1.5 ± 1.0	0.44
k_γ	h^{-1}	0.15 ± 0.04	0.15 ± 0.03	0.90	0.13 ± 0.05	0.15 ± 0.01	0.70[e]
Half-life, γ	h	4.8 ± 1.0	4.7 ± 0.9	0.81	6.3 ± 3.2	4.5 ± 0.4	0.20[g]
Calculated Parameters Using AUC							
$AUC_{(0\rightarrow 60\ h)}$	$\mu g \cdot h \cdot mL^{-1}$	25.6 ± 3.5	19.5 ± 2.4	0.001	19.9 ± 3.8	16.6 ± 2.5	0.06
Bioavailability $(AUC_{sc}/AUC_{iv})*100$	%	NA	NA	-	76.9 ± 14.0	85.1 ± 11.3	0.19
Mean residence time (MRT_{iv} or MRT_{sc})	h	3.0 ± 1.0	3.3 ± 1.0	0.62	5.0 ± 0.9	5.3 ± 0.7	0.60
Mean appearance time (MAT)	h	NA	NA		2.0 ± 1.3	2.0 ± 1.4	0.96
Observed Parameters							
C_{max}	$\mu g/mL$	NA	NA	-	5.4 ± 3.1	3.5 ± 1.0	0.18[h]
t_{max}	h	NA	NA	-	1.0 ± 1.3	1.1 ± 0.6	0.28[h]
Calculated from Stripped Parameters and AUC							
V_d	mL kg^{-1}	596 ± 112	760 ± 130	0.02	773 ± 398	744 ± 86	0.87[i]
Clearance	$mL \cdot h^{-1} \cdot kg^{-1}$	87.5 ± 12.3	114 ± 14	0.001	NA	NA	

[a]Pharmacokinetic parameters were determined for each individual animal and then the means for each route of administration were calculated.[b] P-values represent the difference between the parameters determined for each analytical method. [b]α-, β-, and γ-half lives for data obtained after subcutaneous dose administration represent absorption, distribution, and elimination phases, respectively. For the IV dose, the α- and γ-rates and half-lives represent the distribution and elimination phases, respectively. [c]Values of $p \leq 0.05$ were considered as significant. [d]Values were transformed with $X = X$ + random (gaussian, SD = K) where K was set to 0.50 to ensure equal variance prior to t-test. [e]Values were transformed with $X = X$ + random (gaussian, SD = K) where K was set to 0.025 to ensure equal variance prior to t-test. [f]Not applicable. [g]Values were transformed with $X = X$ + random (gaussian, SD = K) where K was set to 1.8 to ensure equal variance prior to t-test. [h]Values were log transformed to ensure equal variance prior to t-test. [i]Values were transformed with $X = X$ + random (gaussian, SD = K) where K was set to 250 to ensure equal variance prior to t-test.

pyrrolidone/triacetin which was administered subcutaneously. The apparent differences observed could be due to a number of variables including coadministration of the antibiotic, the formulation of the dosage form, animal breed, or age. Most probably the formulation produced a prolonged absorption that would lower the peak and increase MRT.

Bioavailability of the SC administered flunixin free acid was 77% when calculated from the ELISA data set and 85% when calculated from the LC−MS/MS data set confirming that flunixin was well absorbed after SC administration. Flunixin is well absorbed with extravascular administration, with bioavailabilities in the 60−80% range[15,35] for variety of routes in different species.

The concentration of the major metabolite, 5-hydroxy flunixin, was monitored by LC−MS/MS and showed a mean C_{max} of 755 ng/mL and t_{max} of 0.19 h by the IV route and a mean C_{max} of 155 ng/mL and t_{max} of 1.75 h by the subcutaneous route (data not shown). The short t_{max} and high C_{max} for the IV route relative to the SC route is expected with a metabolism route that reaches equilibrium rather quickly with rapid elimination of the product. This was confirmed with the data displayed in Figure 4 where the parallel curves of parent and metabolite for both the IV (panel A) and SC (panel B) routes are clearly indicated. The parallel elimination curves are typical for metabolite elimination being more rapid than the parent drug elimination.

In conclusion, the ELISA assay performed well with good sensitivity and reproducibility, demonstrating that use of ELISA as a quantitative screening tool is feasible. Based on LC−MS/MS results, quantitative ELISA performed best when flunixin concentrations were at or below 250 ng/mL (R^2 = 0.94); nevertheless, relatively good agreement occurred with flunixin concentrations of 5000 ng/mL (R^2 = 0.91), suggesting broader utility for quantitative ELISA. The pharmacokinetic parameters calculated using data from either analytical method were similar with a few significant differences mostly attributable to the higher $AUC_{0\text{-}t}$ values calculated from the immunochemical method. Flunixin was rapidly and relatively completely absorbed after SC administration and, after initial distribution, did not appear to have terminal kinetics different than after IV administration. Collectively, the data suggest that quantitative ELISA could be a useful tool for conducting kinetic studies in circumstances in which LC−MS/MS is not available or is unaffordable.

AUTHOR INFORMATION

Corresponding Author

*(W.L.S.) E-mail, Weilin.Shelver@ars.usda.gov; phone, 1-701-239-1425; (D.J.S.) e-mail, David.J.Smith@ars.usda.gov, phone, 1-701-239-1238.

Present Address

¶J.E.R.: Department of Anatomy and Physiology, Kansas State University, Manhattan, KS

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Amy McGarvey and Grant Herges for skillful technical assistance, Santana Nez for blood collection

and plasma preparation, and Dr. Renee Larson, FSIS, for providing the information of Resflor gold and Hexasol. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. USDA is an equal opportunity provider and employer.

REFERENCES

(1) Deyrup, C. L.; Southern, K. J.; Cornett, J. A.; Shultz, C. E. E.; Cera, D. A. Examining the occurrence of residues of flunixin meglumine in cull dairy cows by use of the flunixin cull cow survey. *J. Am. Vet. Med. Assoc.* **2012**, *241*, 249−253.

(2) FSIS. *2008 FSIS National Residue Program Data*; USDA, FSIS: Washington, DC, 2008; p 90; http://www.fsis.usda.gov/PDF/2008_Red_Book.pdf.

(3) FSIS. *2009 FSIS National Residue Program Data*; USDA, FSIS: Washington, DC, 2009; p 110; http://www.fsis.usda.gov/PDF/2009_Red_Book.pdf

(4) FSIS. Residue repeat violator list for use by FSIS inspection program personnel part I. 2012; www.fsis.usda.gov/PDF/residue_IPP.PDF (accessed Oct 2012).

(5) U.S. FDA Warning letter W/L 42-11 http://www.fda.gov/ICECI/EnforcementActions/WarningLetters/ucm257579.htm

(6) U.S. FDA Warning letter 2012-DET-01 http://www.fda.gov/ICECI/EnforcementActions/WarningLetters/ucm276922.htm

(7) Smith, G. W.; Davis, J. L.; Tell, L. A.; Webb, A. I.; Riviere, J. E. Extralabel use of nonsteroidal anti-inflammatory drugs in cattle. *J. Am. Vet. Med. Assoc.* **2008**, *232*, 697−701.

(8) Wasfi, I. A.; Boni, N. S.; Abdel Hadi, A. A.; Elghazali, M.; Zorob, O.; Alkatheeri, N. A. Pharmacokinetics, metabolism and urinary detection time of flunixin after intravenous administration in camels. *J. Vet. Pharmacol. Ther.* **1998**, *21*, 203−208.

(9) Rantala, M.; Kaartinen, L.; Välimäki, E.; Stryman, M.; Hiekkaranta, M.; Niemi, A.; Saari, L.; Pyörälä, S. Efficacy and pharmacokinetics of enrofloxacin and flunixin meglumine for treatment of cows with experimentally induced *Escherichia coli* mastitis. *J. Vet. Pharmacol. Ther.* **2002**, *25*, 251−258.

(10) Odensvik, K. Pharmacokinetics of flunixin and its effect on prostaglandin F2 alpha metabolite concentrations after oral and intravenous administration in heifers. *J. Vet. Pharmacol. Ther.* **1995**, *18*, 254−259.

(11) Anderson, K. L.; Neff-Davis, C. A.; Davis, L. E.; Bass, F. D. Pharmacokinetics of flunixin meglumine in lactating cattle after single and multiple intramuxcular and intravenous administrations. *Am. J. Vet. Res.* **1990**, *51*, 1464−1467.

(12) Odensvik, K.; Johansson, M. High-performance liquid chromatography method for determination of flunixin in bovine plasma and pharmacokinetics after single and repeated doses of the drug. *Am. J. Vet. Res.* **1995**, *56*, 489−495.

(13) Soma, L. R.; Behrend, E.; Ruby, J.; Sweeney, R. W. Disposition and excretion of flunixin meglumine in horses. *Am. J. Vet. Res.* **1988**, *49*, 1894−1898.

(14) Cheng, Z.; McKeller, Q.; Nolan, A.; Lees, P. Preliminary pharmacokinetic and pharmacodynamic studies on flunixin meglumine in donkeys. *Vet. Res. Commun.* **1996**, *20*, 469−472.

(15) Königsson, K.; Törneke, K.; Engeland, I. V.; Odensvik, K.; Kindahl, H. Pharmacokinetics and pharmacodynamic effects of flunixin after intravenous, intramuscuclar and oral administration to dairy goats. *Acta Vet. Scand.* **2003**, *44*, 153−159.

(16) Cheng, Z.; McKeller, Q.; Nolan, A. Pharmacokinetics studies of flunixin meglumine and phenylbutazone in plasma, exudates and transudate in sheep. *J. Vet. Pharmacol. Ther.* **1998**, *21*, 315−321.

(17) Lees, P.; Taylor, P. M. Pharmacodynamics and pharmacokinetics of flunixin in the cat. *Br. Vet. J.* **1991**, *147*, 298−305.

(18) Yu, Z.-G.; Jiang, C.-M.; Guo, Y.-G.; Hu, Y.-Y.; Chen, D.-J. Pharmacokinetics of flunixin meglumine after intravenous and intramuscular administration in pigs. *Agric. Sci. China* **2007**, *6*, 1396−1401.

(19) Hwang, Y.; Yun, H. Effects of acute hepatic and renal failure on pharmacokinetics of flunixin meglumine in rats. *Exp. Anim.* **2011**, *60*, 187−191.

(20) Ogino, T.; Arai, T. Pharmacokinetic interactions of flunixin meglumine and enrofloxacin in icr mice. *Exp. Anim.* **2007**, *56*, 79−84.

(21) Baert, K.; De Backer, P. Comparative pharmacokinetics of three non-steroidal anti-inflammatory drugs in five bird species. *Comp. Biochem. Physiol., C* **2003**, *134*, 25−33.

(22) Elmas, M.; Yazar, E.; Uney, K.; Karabacak, A. Pharmacokinetics of flunixin after intravenous administration in healthy and endotoxaemic rabbits. *Vet. Res. Commun.* **2006**, *30*, 73−81.

(23) Lohuis, J. A.C.M.; Van Werven, T.; Brand, A.; Van Miert, A. S. J. P. A. M.; Rohde, E.; Ludwig, B.; Heizmann, P.; Rehm, W. F. Pharmacodynamics and pharmacokinetics of carprofen, a non-steroidal anti-inflammatory drug, in healthy cows and cows with *Escherichia coli* endotoxin-induced mastitis. *J. Vet. Pharmacol. Ther.* **1991**, *14*, 219−229.

(24) U.S. FDA. *HEXASOL Injection, Oxytetracycline and Flunixin Meglumine Injectable Solution, Beef and Non-Lactating Dairy Cattle*; Freedom of Information Summary: NADA141-312; U.S. FDA: Washington, DC, 2011.

(25) U.S. FDA. *RESFLOR GOLD, Florfenicol and Flunixin Meglumine (In 2-Pyrrolidone and Triacetin) Injectable Solution, Beef and Non-lactating Dairy Cattle*; Freedom of Information Summary: NADA141-299; U.S. FDA: Washington, DC, 2009.

(26) LaCroix, M. Z.; Gayrard, V.; Picard-Hagen, N.; Toutain, P. L. Comparative bioavailability between two routes of administration of florfenicol and flunixin in cattle. *Rev. Med. Vet.* **2011**, *162*, 321−324.

(27) Kissell, L. W.; Smith, G. W.; Leavens, T. L.; Baynes, R. E.; Wu, H.; Riviere, J. E. Plasma pharmacokinetics and milk residues of flunixin and 5-hydroxy flunixin following different routes of administration in dairy cattle. *J. Dairy Sci.* **2012**, *95*, 7151−7157.

(28) Feely, W. F.; Chester-Yansen, C.; Thompson, K.; Cambell, J. W; Boner, P. L.; Liu, D. d. W.; Crouch, L. S. Flunixin residues in milk after intravenous treatment of dairy cattle with ^{14}C-Flunixin. *J. Agric. Food Chem.* **2003**, *50*, 7308−7313.

(29) Brady, T. C.; Yang, T. J.; Hyde, W. G.; Kind, A. J.; Hill, D. W. Detection of flunixin in greyhound urine by a kinetic enzyme-linked immunosorbent assay. *J. Anal. Toxicol.* **1997**, *21*, 190−196.

(30) Jedziniak, P.; Szprengier-Juszkiewicz, T.; Olejnik, M.; Jaroszewski, J. Determination of flunixin and 5-hydroxyflunixin in bovine plasma with HPLC-UV- method development, validation, and verification. *Bull. Vet. Inst. Pulawy* **2007**, *51*, 261−266.

(31) Luo, Y.; Rudy, J. A.; Uboh, C. E.; Soma, L. R.; Guan, F.; Enright, J. M.; Tsang, D. S. Quantification and confirmation of flunixin in equine plasma by liquid chromatography-quadrupole time-of-flight tandem mass spectrometry. *J. Chromatogr., B* **2004**, *801*, 173−84.

(32) United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science. *Screening of Flunixin Residues by ELISA*; CLG-FLX 3.01; USDA: Washington, DC, 2011.

(33) United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science. *Determination and Confirmation of Flunixin by HPLC/ESI-MS/MS*; CLG-FLX 4.03; USDA: Washington, DC, 2012.

(34) Buur, J. L.; Baynes, R. E.; Smith, G.; Riviere, J. E. Pharmacokinetics of flunixin meglumine in swine after intervenous dosing. *J. Vet Pharmacol. Ther.* **2006**, *29*, 437−440.

(35) Pellegrini-Masini, A.; Poppenga, R. H.; Sweeney, R. W. Disposition of flunixin meglumine injectable preparation administered orally to healthy horses. *J. Vet. Pharmacol. Ther.* **2004**, *27*, 183−186.