RESEARCH REPORT
SWINE HEALTH

Title: A study to assess the correlation between plasma, oral fluid and urine concentrations of flunixin meglumine with the tissue residue depletion profile in finishing age swine – **NPB #13-210**

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Industry Summary:

The United States is the world's leading exporter of pork. In recent years, several of these pork export destination countries have implemented more stringent monitoring protocols for drug residues in meat. In order to maintain a safe, healthy food supply and preserve trade relations, the swine industry must assess the impact of these improved tissue residue testing methods on drug residue detection in meat. This study investigated the residue depletion of flunixin, a commonly used anti-inflammatory drug labeled for use as an adjunctive therapy for swine respiratory disease. This project explored the usefulness of plasma, oral fluid (OF) and urine concentrations of flunixin to predict the residue depletion profile of flunixin in edible tissues of finishing age swine. This project also assessed the potential for untreated pigs to acquire flunixin residues following comingled housing with flunixin treated pigs.

Twenty crossbred finishing pigs were housed in groups of three treated and one untreated control pig. Treated pigs were administered flunixin meglumine at 2.2 mg/kg IM according to product label. Plasma flunixin samples were obtained at 0, 1, 3, 6, 12, 24, 36 and 48 hours after treatment. Necropsy and collection of urine, OF, muscle, liver, kidney, and injection site were conducted at 1, 4, 8, 12, and 16 days post treatment.

A physiologically-based pharmacokinetic (PBPK) model was developed that correlated measured flunixin concentrations in the plasma, OF, urine, liver, kidneys, and muscle. The regression coefficient was $R^2 = 0.91$, suggesting high overall goodness-of-fit. This indicates that the PBPK model could be parameterized with flunixin concentrations in plasma, urine and OF and the results could assist with predicting tissue residues and withdrawal periods in pigs at earlier time points ($\leq$24 h) with a high confidence of accuracy. Thus, OF and urine together with this PBPK model could potentially be a less invasive and more easily administered ante mortem biological monitoring tools for assessing tissue residue potential especially if measured over the first 24 h following flunixin exposure. Although flunixin was not detected in pen-level OF on day 8 post-treatment, OF samples collected at 1, 4 and 12 days after administration were positive for parent flunixin. Furthermore, the 5-hydroxy metabolite of flunixin (5-OH) was present in OF on Day 1, 4, 12 and 16 but not those collected on Day 8. Further studies are needed to refine OF collection for drug analysis.

The potential for environmental residue contamination was also demonstrated in this study. Urine samples from untreated control pigs (no flunixin administration) tested positive for flunixin out to four days post-exposure to flunixin treated pigs. However, flunixin concentrations in the muscle, liver, kidney, and injection sites from these same untreated pigs were below the limit of detection of the assay at all sampling time points after exposure.

With is an increased focus on drug residue concentrations in export markets, often at levels below the tolerance accepted by the USDA FSIS, ante mortem drug monitoring options are needed to ensure pre-harvest food safety. Given the relationship between plasma, urine and oral fluid flunixin concentrations and drug concentrations in body tissue demonstrated in this study, the PBPK model developed as part of this research may be applied as an adjunct to current testing methods. Furthermore, comingling flunixin treated and untreated pigs could result in positive urine tests but does not appear to be a significant risk factor in positive tissue residue tests.

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Scientific Abstract:

The United States is the world's leading exporter of pork. In recent years, several of these pork export destination countries have heightened monitoring for meat drug residues. In order to maintain a safe, healthy food supply and preserve trade relations, the swine industry must assess the impact of these improved tissue residue testing methods on drug residue detection in meat. This study investigated the use of flunixin, a commonly used anti-inflammatory drug labeled for use as an adjunctive therapy for swine respiratory disease. This project explored the usefulness of plasma, oral fluid (OF) and urine concentrations of flunixin to predict the residue depletion profile of flunixin in edible tissues of finishing age swine. This project also assessed the potential for untreated pigs to acquire flunixin residues following comingled housing with flunixin treated pigs.

Twenty crossbred finishing pigs were housed in groups of three treated and one untreated control pig. Treated pigs were administered flunixin meglumine as a single dose on one occasion at 2.2 mg/kg intramuscularly according to product label. Samples for plasma flunixin determination were obtained at 0, 1, 3, 6, 12, 24, 36 and 48 hours after treatment. Necropsy and collection of urine, OF, muscle, liver, kidney, and injection site were conducted in groups at 1, 4, 8, 12 and 16 days post treatment. flunixin levels were analyzed by liquid chromatography/tandem mass spectrometry.

A physiologically-based pharmacokinetic (PBPK) model was developed that correlated measured flunixin concentrations in the plasma, OF, urine, liver, kidneys, and muscle. The regression coefficient was $R^2 = 0.91$, suggesting high overall goodness-of-fit. This indicates that the PBPK model could be parameterized with plasma, urine, and OF flunixin concentrations that could assist with predicting tissue residues and withdrawal periods in pigs especially when sampled at earlier time points (≤24 h) with a high confidence of accuracy. Thus, oral fluids and urine together with this PBPK model could potentially be a less invasive and more easily administered ante mortem biological monitoring tools for assessing tissue residue potential. Although flunixin was not detected in pen-level OF on day 8 post-treatment, OF samples collected at 1, 4 and 12 days after administration were positive for parent flunixin. Furthermore, the 5-hydroxy metabolite of flunixin (5-OH) was also present in all the OF samples except those collected on Day 8. Further studies are needed to refine OF collection for drug analysis.

The potential for environmental residue contamination was also demonstrated in this study. Urine samples in untreated control pigs (no flunixin administration) tested positive for flunixin out to four days post-exposure to flunixin treated pigs. However, flunixin concentrations in the muscle, liver, kidney, and injection sites from these same untreated pigs were below the limit of detection of the assay at every time point after exposure.

Given the relationship between plasma, urine and oral fluid flunixin concentrations and drug concentrations in body tissue demonstrated in this study, the PBPK model developed as part of this research may be applied as an adjunct to current testing methods. Furthermore, comingling flunixin treated and untreated pigs could result in positive urine tests but does not appear to be a significant risk factor in positive tissue residue tests.

Introduction:

Pork is the most widely consumed protein in the world, and the United States is its most prolific exporter. However, in recent years, many export destination countries have lower acceptable drug residue limits than those accepted by the United States Department of Agriculture Food Safety Inspection Service (USDA FSIS). In order to meet the stringent

demands of other nations, it is imperative that swine veterinarians assist the industry in providing responsible pre-harvest food safety with the treatments they prescribe and by developing and using monitoring tests for carcass drug residues.

Flunixin meglumine is approved as an intramuscular (IM) injection in swine labeled for controlling pyrexia associated with swine respiratory disease (Merck label, 2013). Flunixin meglumine is regularly used as an adjunctive treatment in respiratory disease of finishing swine, which is one of the most serious disease problems in modern swine production, causing substantial losses to the industry (Zimmerman et al., 2012). Therefore, tissue residue assessment methods need to be established in this class of animal.

Traditional assessment methods include urine and carcass sampling. Environmental contamination with drug residues must be considered in any population of animals in which treated and untreated animals are comingled, as is typically the case with flunixin therapy. This can be assessed using both oral fluids and urine. In a study by Popot et al. (2011), horses that were dosed with flunixin meglumine and allowed to remain in the same stall with no daily bedding change had flunixin levels in urine that were extended beyond the period of drug therapy. It was concluded this was likely from ingestion of urine contaminated bedding due renal clearance as the main route of flunixin excretion. This suggests that this route of exposure must be considered as a potential source of tissue residues in swine production systems. Furthermore, using oral fluids and urine provides and effective, less invasive, and more easily executed means of ante mortem drug monitoring.

The objectives of this study provide to further our understanding of tissue residues in swine and to provide novel testing methods needed to provide a safe, healthy, and high quality pork products to continue leading the global market.

Objectives:

- Correlate plasma, oral fluid and urine concentrations of flunixin meglumine with the residue depletion profile in edible tissues of finishing age swine
- Assess the potential for flunixin meglumine exposure of untreated pigs by treated pigs due to environmental contamination
- Determine the usefulness of determining plasma, urine and oral fluid flunixin meglumine concentrations as potential markers of tissue residue status and withdrawal period in pigs
- Determine the utility of using a Rapid One Step Assay test validated in milk to determine flunixin concentrations in oral fluids and urine

Materials and Methods:

Before the initiation of this experiment, all animal use, handling, and sampling techniques described were approved by the Iowa State University Animal Care and Use Committee (IACUC # 3-14-7768-S).

Animals

Twenty crossbred barrows with an average weight of 128.2 kg were obtained from a commercial swine finishing system. None of the 20 pigs had records of previous non-steroidal anti-inflammatory (NSAID) treatment. Upon arrival, each pig was confirmed healthy by physical examination by a veterinarian, received a numerical ear tag in the tag (Allflex Global Ear Tags, Allflex USA, Inc., DFW Airport, TX) in their right ear and were weighed. A one inch diameter, circular tattoo was placed in each pig on the left side in the post-auricular area, approximately 2 inches ventral to the dorsal midline and 2 inches caudal to the ear. This tattoo was applied on the skin above the trapezius muscle using a commercial tattoo applicator (Stone Mfg., Kansas City, MO) with a slap tattoo. Study animals were also confirmed free of NSAIDS in plasma with a standard screening ELISA method (Flunixin kit lot #FX-0269, Neogen Corp, Lexington, KY).

Pigs were housed in Building 29 of Laboratory Animal Resources Research Facilities at the Iowa State University College of Veterinary Medicine. The entry weights were used to randomly allocate the barrows into housing group based on anticipated necropsy date. Five housing groups of 4 pigs, corresponding with each necropsy date were determined and placed in separate rooms, based on anticipated necropsy date and treatment group. Housing conditions were in accordance with the recommendations outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Use and Research and Teaching 3rd Edition.

Pigs were hand fed once daily with an organic corn/soybean meal diet, confirmed free of any medications and compatible with the NRC nutrient requirements for finishing pigs. They had free access to water through a nipple waterer at all times.

Animal phase study design

Each pig was weighed the day before treatment administration to determine an accurate dosing weight. One pig of the each housing group was designated to be the negative control pig using a random number generator, while the other three pigs in the housing group were administered flunixin meglumine (**Table 1**). The first day of the study, each pig was restrained with a hog snare and had a blood sample collected immediately prior to treatment. Then, each treatment pig was administered 2.2 mg/kg flunixin meglumine (Banamine-S, Merck Animal Health, Lot# 3037102, Expiration Date:2/2015) and control pigs were administered and equivalent volume of sterile water (VetOne Sterile Water, Nova-Tech, Inc., Grand Island, NE, Lot # B131107-2, Expiration Date 11/2015).. This dose was administered IM with a 16 gauge, 1 inch needle inside circular tattoo placed on arrival in the post-auricular area to ensure accurate recovery of the injection site.

Pig blood samples were collected at 0, 1, 3, 6, 12, 24, 36 and 48 hours after treatment administration. Each sample (8 mL) was collected via the left or right jugular vein using a 25.4 mm 16 gauge hypodermic needle (Air-Tite Products, Virginia Beach, VA) and 12 mL Luer lock syringe (TycoHealth Care, Mansfield, MA). Additionally, a final blood sample was collected immediately prior to necropsy for each group. During blood collection, pigs were manually restrained using a pig snare. To collect oral fluids a ½" diameter cotton rope was suspended from a hook hanging to the pigs' shoulder height. After 20-30 minutes of pen-level sampling to allow each pig to chew on the ropes, the ropes were removed. Oral fluids were extracted by placing the wet portion of the rope into a clean plastic bad and squeezing the rope so the fluid accumulates in the bag, with a minimum of 5 mL being acceptable. The fluids were then poured into a Falcon tube (Becton Dickinson and Co., Franklin Lakes,NJ) and stored at -80 degrees Celcius prior to analysis.

Necropsy and tissue sample collection occurred at 1, 4, 8, 12, and 16 days after treatment administration. Immediately before euthanasia and necropsy, an 8 mL blood sample were collected at times corresponding to the initial treatment time on Day 1. Pigs were humanely euthanized by penetrating captive bolt followed by exsanguination according to AVMA guidelines. At necropsy, the injection site, liver, kidney, semitendinosus/semimembranosus muscle, and urine were collected for analysis.

Sample Collection, Processing, and Analysis

All drug concentrations in collected samples were analyzed at Iowa State University Veterinary Diagnostic Lab and the Iowa State University-Pharmacology Analytical Support Team (ISU-PhAST). Plasma, tissues, oral fluids and urine were quantified via liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, standards were prepared at a final concentration ranging between 5-5000 ng/mL flunixin and 5-hydroxyflunxin (5-OH). Internal standard, flunixin D-3, was added to samples and standards. Oral fluid samples were buffered to a pH of 2.9-3.0. The drugs were extracted using methyl tert-butyl ether (MTBE). Urine samples were prepared by diluting 0.5 mL urine with water. A base catalyzed hydrolysis was carried out at room temperature for 15 minutes. The samples were buffered to 2.9-3.0 and extracted using 10:1 dichloromethane: petroleum ether. For both the oral fluids and the urine the organic layer was transferred, dried down, reconstituted in 12.5% acetonitrile in water. Plasma samples were diluted with acetonitrile, centrifuged, and then the supernatant was transferred and dried down. Drugs from tissue samples (liver, kidney, muscle, and injection site) using an FSIS method (CLG-MRM 1.02) with several modifications. Briefly, drug extracted in 80% (v/v) acetonitrile in water and further purified using Bakerbond (C18). Both plasma and tissue were reconstituted in 25% acetonitrile in water. All samples were transferred to an autosampler vial (with glass insert) and centrifuged prior to LC-MS/MS analysis.

Concentrations of flunixin and 5-OH in oral fluids and urine were measured using LC-MS/MS utilizing a TSQ Quantum Discover Max triple quadrupole mass spectrometer. The three ions used in positive ion mode were (m/z) of 297→ 109/264/279 for flunixin and (m/z) 313 →109/280/295 for 5-hydroxyflunixin. Concentrations of flunixin and 5-OH in plasma samples were determined utilizing an ABSciex QTRAP 4500 mass spectrometer. The three ions measured in negative ion mode were (m/z) 295 → 251, 210, and 197 for flunixin and (m/z) 311 → 267, 227, and 247 for 5-OH. All curves had a coefficient of determination that exceeded 0.99. Quality control (QC) samples passed when calculated levels were within 20% of expected levels.

Additionally, urine samples were analyzed for flunixin using a drug detection enzyme-linked immunosorbent assay (ELISA) Kit (Lot # FX-0269 Neogen, Lansing, MI).

Physiologically-based pharmacokinetic modeling

A physiologically based pharmacokinetic (PBPK) model was constructed, as previously described in cattle (Leavens et al., 2014), to demonstrate the flunixin concentrations found with what is expected given the nature of the drug and its absorption, distribution, metabolism, and excretion in normal adult swine. This model was developed by Drs. Zhoumeng Lin, Ronette Gehring, Mengjie Li, Teresa L. Leavens and Jim E. Riviere affiliated with the Institute of Computational Comparative Medicine at Kansas State University, Manhattan, KS. Briefly, the model was composed of eight compartments (blood, liver, kidneys, muscle, fat, lungs, richly perfused tissues, and slowly perfused tissues) for flunixin and a one-compartment sub-model for pooled metabolites (**Figure 1**). Intramuscular injection of flunixin was modeled as a first-order absorption process for the non-ionized fraction with a two-compartment model. The distribution in all compartments was well-mixed and flow-limited. The elimination pathways included metabolism in the liver, biliary excretion via the liver, and urinary excretion by the kidney. In order to evaluate the correlation between plasma, oral fluid and urine concentrations of flunixin and the residue depletion profile in edible tissues, the model was modified to include the salivary excretion pathway. Oral fluid flunixin concentration was defined as the flunixin concentration in plasma multiplied by saliva/plasma flunixin partitioning coefficient (Smith et al., 2010). The saliva/plasma flunixin partitioning coefficient was determined experimentally using the mean saliva/plasma area under the concentration (AUC) ratio method. All physiological parameters were changed to be swine-specific (Buur et al., 2005; Upton, 2008). All chemical-specific parameters were kept the same except the absorption parameters, which were estimated by fitting to our newly generated pharmacokinetic data for flunixin in pigs (the current study) using the Nelder-Mead optimization method in the AcslXTM modeling software package.

Results and Discussion:

- **Correlate plasma, oral fluid and urine concentrations of flunixin meglumine with the residue depletion profile in edible tissues of finishing age swine**
  The PBPK model predictions of plasma, tissue, oral fluid, and urine concentrations of flunixin were compared to measured data in pigs intramuscularly exposed to 2.2 mg/kg flunixin meglumine (**Figure 2**). The model accurately predicted the measured flunixin concentrations in the plasma, especially within 24 h after exposure (**Figure 2A).** At later time points (≥48 h), measured data are not shown because most of the measured values in plasma were less than limit of quantification of the assay (LOQ = 5 ng/g) **(Table 2a-b**). Specifically, at 48 h, measured flunixin concentrations were <LOQ in 7 out of the 12 experimental animals, and at ≥96 h measured values were <LOQ in all animals. These measured results were also consistent with the predicted value, which were <LOQ (e.g., 1.85 ng/ml at 48 h) at all these later time points.

  After calibration with experimental plasma data, the pharmacokinetic model we developed was directly applied to simulate liver flunixin concentrations and it successfully predicted the concentration at 24 h (**Figure 2B**). At ≥96 h, flunixin was not found (NF) in the liver. These experimental data were consistent with the simulated results that were either close to or less than LOQ in the liver at these time points (≥96 h). In the present study, we estimated this parameter by visually fitting to measured kidney data at 24 h (**Figure 2C**). Notably, using this estimated PK parameter, the predicted flunixin kidney concentrations at ≥96 h were all much less than LOQ, which correlated with the experimental data that were all NF. Additionally, model-predicted muscle flunixin concentrations at all studied time points were <LOQ, which corresponded to the measured data that were either <LOQ or NF except at the day 16 time point in Animal 259 where the concentration in the muscle was 9.8 ng/g (**Table1**). It is noteworthy that the concentrations of flunixin detected in the tissues beyond day 12 (the labeled tissue withdrawal period for flunixin in swine) were below the established tolerance of flunixin in pigs (30 ng/g in the liver and 25 ng/g in the muscle of pigs) (CFR, 2014).

  To evaluate the potential for using this model to predict residue depletion profile in edible tissues based on less invasive and easily accessible biological matrices, such as urine, the model was applied to simulate urine flunixin concentrations, and the results were compared to measured data of spot urine samples collected at slaughter times (**Figure 2D**). Remarkably, the predicted concentrations at 24 and 96 h were both in the range of observed values. However, it should be noted that the measured concentrations at 24 h were highly variable. As a result, the

predicted value at 24 h was similar to the measured values in 2 of the 3 animals, but the model somewhat (by 2.7-fold) underestimated the concentration of the third animal. It should be noted that spot urine flunixin concentrations depend on many factors, including the urinary output, voiding intervals, last voiding time, postvoid residual urine volume, etc (Gans and Mercer, 1977). Therefore, the slight underestimation of the third animal may be due to lower urinary output, longer voiding interval, and/or higher postvoid residual urine volume than two other animals. Nevertheless, successful predictions of spot urine flunixin concentrations suggest a potential use of the model for reverse dosimetry analysis, i.e., to predict the residue depletion profile in edible tissues based on spot urine data. In order to refine this model, future studies should consider using metabolism cages to collect cumulative urine samples.

The present swine model was expanded to simulate oral fluid flunixin concentrations in order to evaluate the usefulness of oral fluids as a potential marker for predicting tissue residues and withdrawal periods. As shown in **Figure 2E**, the predicted values were in the range of the experimentally determined concentrations across all studied time points, with perfect correlation obtained at 24 h. However, the observed data at 36 and 48 h were highly variable, and the model predictions were at the lower end of the range (**Figure 2E**). These results suggest that oral fluids collected at 24 h after dosing could be a useful biological matrix for predicting plasma and tissue flunixin residues by utilizing the present swine PK model. Additional studies with oral fluid data of low variability are needed in order to assess the predictive capability of the model to predict oral fluid flunixin concentrations at 36 and 48 h.

- **Assess the potential for flunixin meglumine exposure of untreated pigs by treated pigs due to environmental contamination.**
  Untreated control pigs at days 1 and 4 post flunixin administration had no detectable plasma concentrations (**Table 2a-b**) or tissue residues (**Table 3**), however flunixin was detected in their urine by both LC-MS/MS and ELISA testing at days 1 and 4 post-treatment (**Table 5**). The detection of flunixin at higher concentrations in urine and not plasma was anticipated based on previous studies in horses (Gu et al., 1997; Popot et al., 2011). The poor oral bioavailability of flunixin in swine (Pairis-Garcia et al., 2013) may explain why environmental exposure to contaminated urine would be sufficient to result in detectable tissue residues. This was confirmed by the results of the present study. On all necropsy days after Day 4, none of the saline-treated control pig had detectable flunixin residues in tissue, plasma, or urine.

- **Examine the usefulness of determining plasma, urine and oral fluid flunixin meglumine concentrations as potential markers of tissue residue status and withdrawal period in pigs**
  The previously described PBPK model was expanded to simulate oral fluid flunixin concentrations in order to evaluate the usefulness of oral fluids as a potential marker for predicting tissue residues and withdrawal periods. As shown in **Figure 2E**, the predicted values were in the range of the experimentally determined concentrations across all studied time points, with perfect correlation obtained at 24 h. However, the observed data at 36 and 48 h were highly variable, and the model predictions were at the lower end of the range (**Figure 2E**). These results suggest that oral fluids collected at 24 h after dosing could be a useful biological matrix for predicting plasma and tissue flunixin residues by utilizing this model. Additional studies with individual animal oral fluid data of low variability are needed in order to assess the predictive capability of the model to predict oral fluid flunixin concentrations at 36 and 48 h.

  Beyond 24 hours post-treatment, flunixin concentrations in all studied tissues were below the limit of quantitation of the analytical instrument (LOQ = 5 ng/g) except in the injection site of 2 pigs at 4 and 12 days after treatment respectively and in the muscle of 1 pig at 16 days after treatment (**Table 3**). It is noteworthy that the concentrations of flunixin detected in the tissues beyond day 12 (the labeled tissue withdrawal period for flunixin in swine) were below the established tolerance of flunixin in pigs (30 ng/g in the liver and 25 ng/g in the muscle of pigs) (CFR, 2014) and therefore present no food safety concerns. Furthermore, the 5-hydroxyflunixin metabolite of flunixin was also not detected in any of the tissues at any of the time points beyond 24 h (**Table 4**).

  Parent flunixin was detected in the urine of all pigs on Day 4 and in the pen-level oral fluids out to Day 12 (**Table 5**). However, flunixin was not detected in pen-level OF on day 8 post-treatment. Furthermore, the 5-hydroxy metabolite of flunixin (5-OH) was also present in all the pen-level OF samples out to Day 16 except those

collected on Day 8 (**Table 6**). The absence of parent and metabolite flunixin concentrations on day 8 could not be explained. However this may relate to sample collecting methods or the manner in which treated and control pigs interact with the rope at pen level. Although OF analysis may be useful in demonstrating drug exposure, especially in regards to the assessment of drug metabolites, further studies in individual and group-housed pigs are needed to refine OF collection for drug analysis.

- **Determine the utility of using a Rapid One Step Assay test validated in milk to determine flunixin concentrations in oral fluids and urine**
  At the time of this report, the group was unsuccessful in validating the Rapid One Step Assay test to work with urine and oral fluids. Instead, the Neogen ELISA test and LC-MS/MS were used for urine and oral fluids, respectively. Flunixin was detected in all urine samples in at least one treated pig out to 16 days post administration (**Table 7**).

In summary, the PBPK model predictions developed in this study correlated with measured flunixin concentrations in the plasma, oral fluids, urine, liver, kidneys, and muscle very well, with an overall regression coefficient (R^2) of 0.91. The model apparently correlated plasma, oral fluid and urine concentrations of flunixin with the residue depletion profile in the liver, kidney, and muscle of finishing age swine very well, especially within 24 h after dosing. These results suggest that plasma, urine, and oral fluid flunixin concentrations can be useful biomarkers for predicting tissue residues and withdrawal periods in pigs at earlier time points ($\leq$24 h) with a high confidence of accuracy ($R^2 = 0.91$) by using the present PBPK model. Thus, oral fluids and urine together with this PBPK model can be less invasive and more easily administered ante mortem biological monitoring tools for assessing tissue exposure to drugs especially during the first 24 h after drug exposure. However, this would limit the utility of this system in swine production systems where samples are seldom collected prior to the end of the label tissue withhold period. This system may have more applicability to drugs with longer plasma elimination half-lives that could be detected beyond 24 h post-administration.

Beyond 24 hours post-treatment, flunixin concentrations in all studied tissues were below the limit of quantitation of the analytical instrument (LOQ = 5 ng/g) except in the injection site of 2 pigs at 4 and 12 days after treatment respectively and in the muscle of 1 pig at 16 days after treatment. It is noteworthy that the concentrations of flunixin detected in the tissues beyond day 12 (the labeled tissue withdrawal period for flunixin in swine) were below the established tolerance of flunixin in pigs (30 ng/g in the liver and 25 ng/g in the muscle of pigs) (CFR, 2014) and therefore present no food safety concerns. Furthermore, the 5-hydroxyflunixin metabolite of flunixin was also not detected in any of the tissues at any of the time points beyond 24 h.

Parent flunixin was detected in the urine of all pigs on Day 4 and in the pen-level oral fluids out to Day 12. However, flunixin was not detected in pen-level OF on day 8 post-treatment. Furthermore, the 5-hydroxy metabolite of flunixin (5-OH) was also present in all the pen-level OF samples out to Day 16 except those collected on Day 8. Although OF analysis may be useful in demonstrating drug exposure, especially in regards to the assessment of drug metabolites, further studies in individual and group-housed pigs are needed to refine OF collection for drug analysis.

The potential for environmental residue contamination was also demonstrated in this study. Urine samples from untreated control pigs (no flunixin administration) tested positive for flunixin out to four days post-exposure to flunixin treated pigs. However, flunixin concentrations in the muscle, liver, kidney, and injection sites from these same untreated pigs were below the limit of detection of the assay at all sampling time points after exposure.

Figure 1. A schematic diagram for a physiologically based pharmacokinetic (PBPK) model for flunixin in pigs. A_{site1} and A_{site2} [μg] represent the amounts of flunixin in the absorption site 1 and site 2, respectively. K_{site12} and K_{site21} [h^{-1}] are first order transport rate constants from absorption site 1 to site 2 and from site 2 to site 1, respectively. R_{bile} and R_{urine} [μg/h] stand for biliary and urinary excretion rates, respectively. C_{saliva} [ng/ml], flunixin concentration in the oral fluids; $P_{saliva/plasma}$ [unitless], saliva/plasma flunixin partitioning coefficient; R_{IM} [μg/h], intramuscular absorption rate; $R_{metabolism}$ [μg/h], hepatic metabolic rate of flunixin; $R_{regeneration}$ [μg/h], regeneration rate of flunixin from metabolites due to both enterohepatic recirculation and hydrolysis of conjugates.

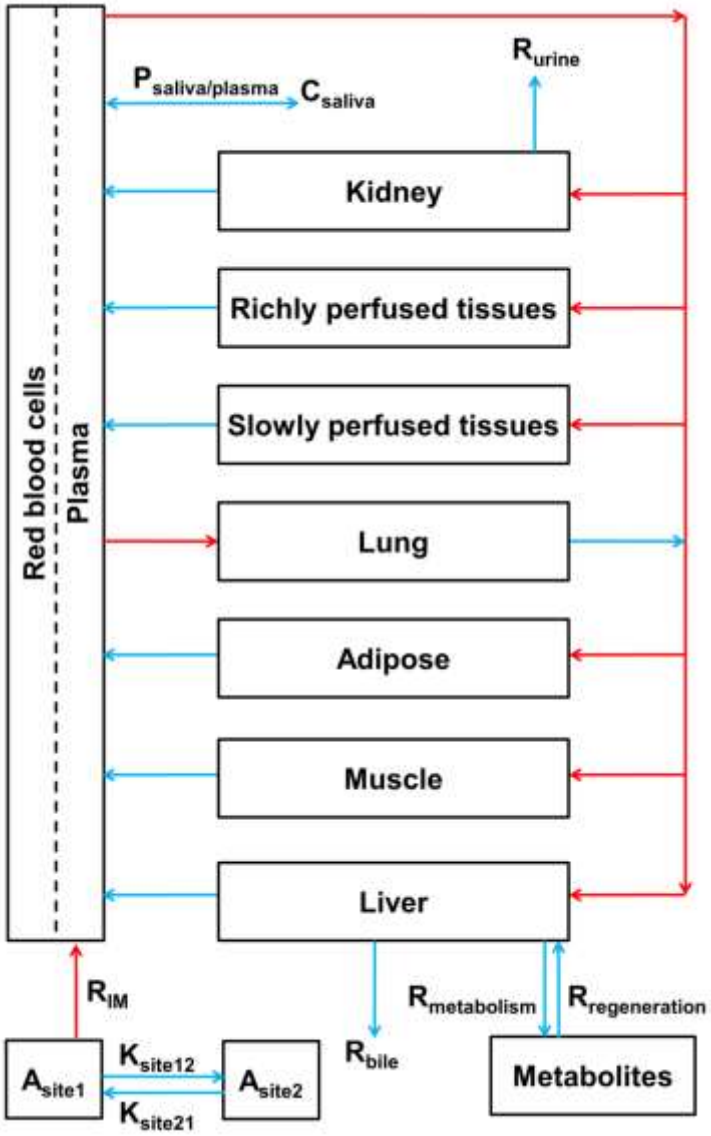

Figure 2. Model calibration results. Comparisons of model predictions (solid lines) and observed data (squares) for flunixin concentrations in the plasma (A), liver (B), kidneys (C), urine (D), and oral fluids (E) of pigs following intramuscular injection with 2.2 mg/kg flunixin meglumine. Only observed data that were higher than limit of quantification (LOQ = 5 ng/ml) are shown. Measured flunixin concentrations in the muscle at 24 h were <LOQ in two animals and not found (NF) in one animal, which is consistent with the simulated data (4.70 ng/g) that was <LOQ. At 96 h after injection, observed flunixin concentrations in liver, kidneys, or muscle were all NF, which also corresponds to simulated results (5.03, 2.26, 0.08 ng/g in liver, kidneys, and muscle, respectively). Panel F represent regression analysis result between measured and simulated data. The regression coefficient was $R^2 = 0.91$, suggesting high overall goodness-of-fit.

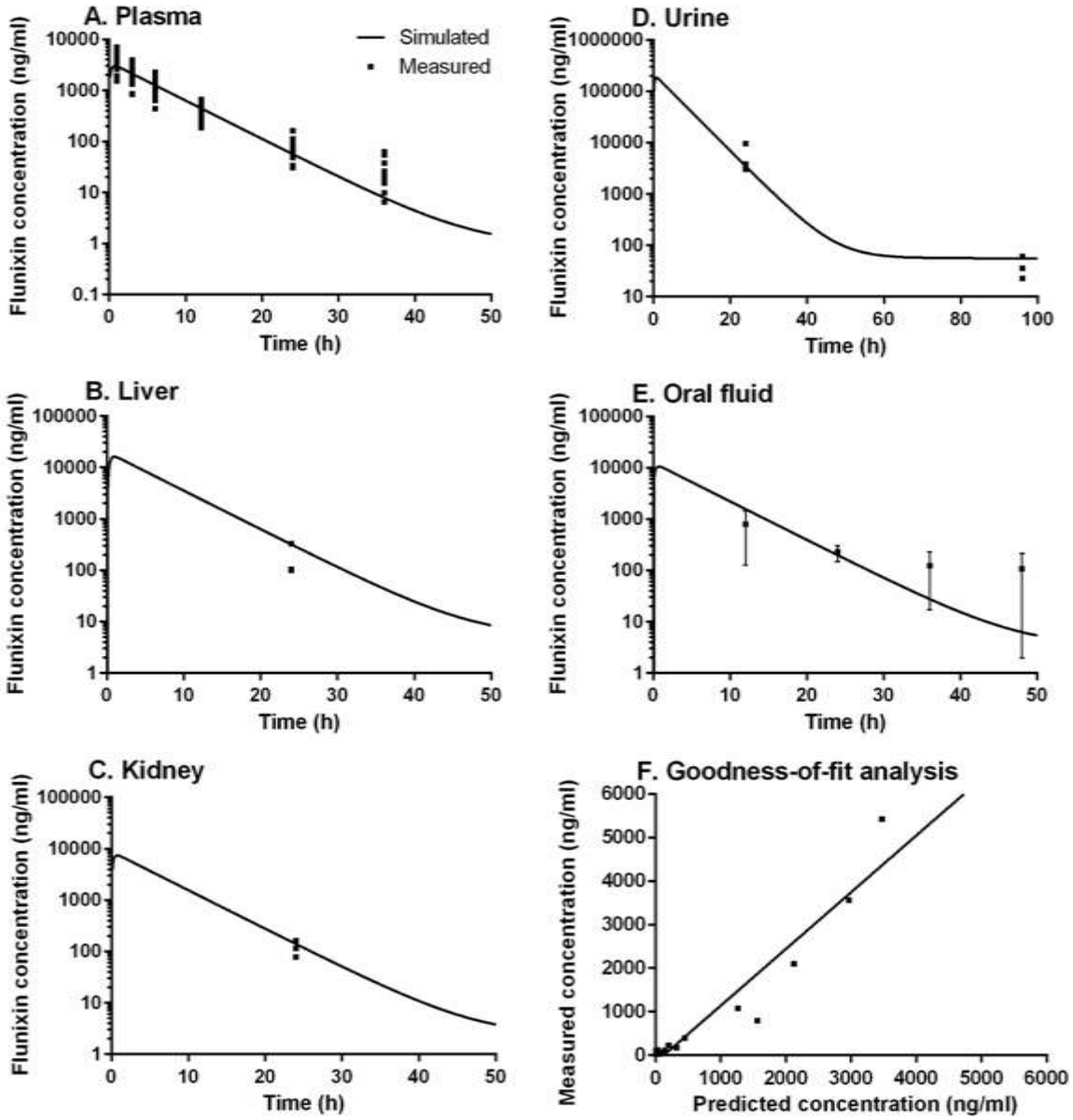

Table 1. Study animal weights, necropsy group allocations and treatment information. Treated sows received a single dose of 2.2 mg/kg flunixin meglumine (Banamine S, Merck Animal Health) IM on Day 0. Untreated, control pigs used to assess the potential for environmental contamination are designated (C) and received an equivalent volume of physiological saline.

Group	Pig ID	Weight (kg)	Injection Volume
G1 (Day 1)	**260**	125.00	5.5
	261	133.64	5.9
	270 (C)	123.64	5.4
	273	136.36	6.0
G2 (Day 4)	**262**	138.64	6.1
	264	128.18	5.6
	269	133.64	5.9
	274 (C)	136.36	6.0
G3 (Day 8)	**258**	134.09	5.9
	266 (C)	130.45	5.7
	268	134.55	5.9
	272	139.09	6.1
G4 (Day 12)	**263**	143.18	6.3
	267	132.73	5.8
	271	128.18	5.6
	275 (C)	131.82	5.8
G5 (Day 16)	**257**	130.00	5.7
	259	134.09	5.9
	265 (C)	126.82	5.6
	276	138.18	6.1

Table 2a. Plasma flunixin concentrations (ng/mL) after IM administration of flunixin meglumine at 2.2 mg/kg. Concentrations that were below the level of quantification (LOQ) (5 ng/mL) was designated "LOQ". Untreated, control pigs used to assess the potential for environmental contamination are designated (C).

Group	G1 (Day 1)				G2 (Day 4)				G3 (Day 8)				G4 (Day 12)				G5 (Day 16)			
Sow/ h	**260**	**261**	**270(C)**	**273**	**262**	**264**	**269**	**274(C)**	**258**	**266(C)**	**268**	**272**	**263**	**267**	**271**	**275(C)**	**257**	**259**	**265(C)**	**276**
0 h	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1 h	1534	4797	<LOQ	7040	4645	3365	2677	<LOQ	4093	<LOQ	1927	3791	2973	1857	5781	<LOQ	3293	2593	<LOQ	2969
3 h	863	2337	<LOQ	2955	1925	2049	1524	<LOQ	2278	<LOQ	1314	3262	3926	844	3149	<LOQ	1512	1423	<LOQ	2121
6 h	667	1015	<LOQ	1839	739	901	625	<LOQ	1217	<LOQ	633	1145	1352	439	1651	<LOQ	753	931	<LOQ	2262
12 h	495	442	<LOQ	321	325	352	238	<LOQ	492	<LOQ	314	563	403	187	490	<LOQ	284	327	<LOQ	664
24 h	113	51	<LOQ	53	63	88	31	<LOQ	55	<LOQ	73	59	33	49	83	<LOQ	71	49	<LOQ	163
36 h					21	21	7	<LOQ	15	<LOQ	54	19	10	26	19	<LOQ	38	18	<LOQ	63
Day 2					21	8	<LOQ	<LOQ	<LOQ	<LOQ	25	<LOQ	<LOQ	11	6	<LOQ	9	<LOQ	<LOQ	<LOQ
Day 4					<LOQ	<LOQ	<LOQ	<LOQ												
Day 8									<LOQ	<LOQ	<LOQ	<LOQ								
Day 12													<LOQ	<LOQ	<LOQ	<LOQ				
Day 16																	<LOQ	<LOQ	<LOQ	<LOQ

Table 2b. Plasma 5-hydroxyflunixin concentrations (ng/mL) after IM administration of flunixin meglumine at 2.2 mg/kg. Concentrations that were below the level of quantification (LOQ) (5 ng/mL) was designated "LOQ". Untreated, control pigs used to assess the potential for environmental contamination are designated (C).

Group	G1 (Day 1)				G2 (Day 4)				G3 (Day 8)				G4 (Day 12)				G5 (Day 16)			
Sow/ h	**260**	**261**	**270(C)**	**273**	**262**	**264**	**269**	**274(C)**	**258**	**266(C)**	**268**	**272**	**263**	**267**	**271**	**275(C)**	**257**	**259**	**265(C)**	**276**
0 h	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1 h	61	<LOQ	<LOQ	101	<LOQ	207	124	<LOQ	131	<LOQ	127	135	<LOQ	45	127	<LOQ	105	108	<LOQ	321
3 h	<LOQ	<LOQ	<LOQ	102	11	132	96	<LOQ	163	<LOQ	113	174	570	46	105	<LOQ	84	82	<LOQ	284
6 h	<LOQ	<LOQ	<LOQ	83	<LOQ	<LOQ	52	<LOQ	100	<LOQ	55	86	<LOQ	27	89	<LOQ	46	19	<LOQ	205
12 h	7	<LOQ	<LOQ	51	<LOQ	17	24	<LOQ	53	<LOQ	31	64	<LOQ	21	29	<LOQ	22	35	<LOQ	69
24 h	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	6	6	<LOQ	12
36 h					<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	8
Day 2					<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Day 4					<LOQ	<LOQ	<LOQ	<LOQ												
Day 8									<LOQ	<LOQ	<LOQ	<LOQ								
Day 12													<LOQ	<LOQ	<LOQ	<LOQ				
Day 16																	<LOQ	<LOQ	<LOQ	<LOQ

Table 3. Tissue flunixin concentrations (ng/g) in liver, kidney, semitendinosus/semimembranosus muscle, and injection site. Concentrations that were below the level of quantification (LOQ) was designated "LOQ". For this assay the LOQ was 5 ng/g. If no flunixin levels were detected, the samples was designated "NF". Untreated, control pigs used to assess the potential for environmental contamination are designated (C).

Tissue Flunixin Concentrations (ng/g)

		Liver	**Kidney**	**Muscle**	**Injection Site**
Group	Pig ID	Flunixin	Flunixin	Flunixin	Flunixin
G1 (Day 1)	**260**	326.8	160.1	NF	40,143.4
	261	105.8	77.5	<LOQ	7.5
	270 (C)	NF	NF	NF	NF
	273	98.6	115.2	<LOQ	887.7
G2 (Day 4)	**262**	NF	NF	NF	13.4
	264	NF	NF	NF	NF
	269	NF	NF	NF	<LOQ
	274 (C)	NF	NF	NF	NF
G3 (Day 8)	**258**	NF	NF	NF	NF
	266 (C)	NF	NF	NF	NF
	268	NF	NF	NF	<LOQ
	272	NF	NF	NF	NF
G4 (Day 12)	**263**	NF	NF	NF	NF
	267	NF	NF	NF	28.9
	271	NF	NF	NF	NF
	275 (C)	NF	NF	NF	NF
G5 (Day 16)	**257**	NF	NF	NF	<LOQ
	259	NF	NF	9.8	NF
	265 (C)	NF	NF	NF	<LOQ
	276	NF	NF	NF	NF

Table 4. Tissue 5-OH flunixin concentrations (ng/g) in liver, kidney, semitendinosus/semimembranosus muscle, and injection site. Concentrations that were below the level of quantification (LOQ) was designated "LOQ". For this assay the LOQ was 5 ng/g. If no 5-OH flunixin levels were detected, the sample was designated "NF". Untreated, control pigs used to assess the potential for environmental contamination are designated (C).

		Tissue 5-OH Flunixin Concentrations (ng/g)			
		Liver	**Kidney**	**Muscle**	**Injection Site**
Group	Pig ID	5-OH flunixin	5-OH flunixin	5-OH flunixin	5-OH flunixin
G1 (Day 1)	**260**	303.5	76.2	NF	<LOQ
	261	94.1	106.1	NF	NF
	270 (C)	NF	NF	NF	NF
	273	87.8	47.2	NF	<LOQ
G2 (Day 4)	**262**	NF	NF	NF	NF
	264	NF	NF	NF	NF
	269	NF	NF	NF	NF
	274 (C)	NF	NF	NF	NF
G3 (Day 8)	**258**	NF	NF	NF	<LOQ
	266 (C)	NF	NF	NF	NF
	268	NF	NF	NF	NF
	272	NF	NF	NF	NF
G4 (Day 12)	**263**	NF	NF	NF	NF
	267	NF	NF	NF	NF
	271	NF	NF	NF	NF
	275 (C)	NF	NF	NF	NF
G5 (Day 16)	**257**	NF	NF	NF	<LOQ
	259	NF	NF	NF	NF
	265 (C)	NF	NF	NF	NF
	276	NF	NF	NF	NF

Table 5. Flunixin concentrations (ng/mL) in plasma, urine, and oral fluids. Oral fluids were a group sample all four pigs in the group at day of necropsy. Concentrations that were below the level of quantification (LOQ) was designated "LOQ". For this assay the LOQ was 5 ng/mL. If no flunixin levels were detected, the sample was designated "NF". Untreated, control pigs used to assess the potential for environmental contamination are designated (C).

Plasma, Urine, and Oral Fluids Concentrations of Flunixin (ng/mL)

		Plasma	**Urine**	**Pen- Level Oral Fluids**
Group	Pig ID	Flunixin	Flunixin	Flunixin
G1 (Day 1)	**260**	112.5	9459.1	168.6
	261	50.6	3762.5	
	270 (C)	<LOQ	231.4	
	273	52.6	3032.0	
G2 (Day 4)	**262**	<LOQ	60.3	16.8
	264	<LOQ	22.7	
	269	<LOQ	35.5	
	274 (C)	<LOQ	11.6	
G3 (Day 8)	**258**	<LOQ	<LOQ	<LOQ
	266 (C)	<LOQ	<LOQ	
	268	<LOQ	<LOQ	
	272	<LOQ	<LOQ	
G4 (Day 12)	**263**	<LOQ	NF	12.3
	267	<LOQ	<LOQ	
	271	<LOQ	<LOQ	
	275 (C)	<LOQ	<LOQ	
G5 (Day 16)	**257**	<LOQ	NF	<LOQ
	259	<LOQ	NF	
	265 (C)	<LOQ	NF	
	276	<LOQ	NF	

Table 6. 5-OH flunixin concentrations (ng/mL) in plasma, urine, and oral fluids. Oral fluids were a group sample all four pigs in the group at day of necropsy. Concentrations that were below the level of quantification (LOQ) was designated "LOQ". For this assay the LOQ was 5 ng/mL. If no 5-OH flunixin levels were detected, the sample was designated "NF". Untreated, control pigs used to assess the potential for environmental contamination are designated (C).

Plasma, Urine, and Oral Fluids Concentrations of 5-OH flunixin (ng/mL)

		Plasma	**Urine**	**Pen-level Oral Fluids**
Group	Pig ID	5-OH flunixin	5-OH flunixin	5-OH flunixin
G1 (Day 1)	**260**	<LOQ	1294.9	21.0
	261	<LOQ	972.0	
	270 (C)	<LOQ	16.5	
	273	<LOQ	724.4	
G2 (Day 4)	**262**	<LOQ	<LOQ	10.9
	264	<LOQ	<LOQ	
	269	<LOQ	<LOQ	
	274 (C)	<LOQ	<LOQ	
G3 (Day 8)	**258**	<LOQ	<LOQ	<LOQ
	266 (C)	<LOQ	NF	
	268	<LOQ	NF	
	272	<LOQ	NF	
G4 (Day 12)	**263**	<LOQ	NF	11.3
	267	<LOQ	NF	
	271	<LOQ	**NF**	
	275 (C)	<LOQ	NF	
G5 (Day 16)	**257**	<LOQ	NF	10.6
	259	<LOQ	NF	
	265 (C)	<LOQ	NF	
	276	<LOQ	NF	

Table 7. Urine flunixin concentrations (ng/mL) as determined by urine ELISA. Concentrations that were below the level of quantification (LOQ) was designated "LOQ". For this assay the LOQ was 5 ng/mL. Untreated, control pigs used to assess the potential for environmental contamination are designated (C).

Urine Flunixin ELISA Concentrations (ng/mL)

Group	Pig ID	Urine ELISA
G1 (Day 1)	**260**	37851.1
	261	4808.9
	270 (C)	4.2
	273	7114.3
G2 (Day 4)	**262**	2.3
	264	1.0
	269	1.7
	274 (C)	0.5
G3 (Day 8)	**258**	0.1
	266 (C)	<LOQ
	268	<LOQ
	272	0.1
G4 (Day 12)	**263**	<LOQ
	267	<LOQ
	271	0.1
	275 (C)	<LOQ
G5 (Day 16)	**257**	<LOQ
	259	0.1
	265 (C)	<LOQ
	276	<LOQ

LITERATURE CITED:

Buur JL, Baynes RE, Craigmill AL, Riviere JE. Development of a physiologic-based pharmacokinetic model for estimating sulfamethazine concentrations in swine and application to prediction of violative residues in edible tissues. Am J Vet Res 2005;66:1686-93.

CFR. 21 CFR 556.600 - Oxytetracycline. (2014) Code of Federal Regulations. Title 21 - Food and Drugs. U.S. Government. Washington, DC. p. 383.

CLG-MRM1.02 Screening and Confirmation of Animal Drug Residues by UHPLC-MS-MS. (2013) United States Department of Agriculture, Food Safety and Inspection Service, Office of Public Health Science.

Gans JH, Mercer PF. Chapter 37 The Kidneys. In: M. J. Swenson, editor. Dukes' Physiology of Domestic Animals, 9th edition. Cornell University Press, Ithaca, New York1977. p. 463-92.

Guide for the Care and Use of Agricultural Animals Used in Research and Teaching. (2010) Third Edition ed. pp. 142-156. http://www.fass.org/docs/agguide3rd/Ag_Guide_3rd_ed.pdf. Accessed 28 January 2015.

Gu X, Meleka-Boules M, Chen CL, Ceska DM, Tiffany DM. 1997. Determination of flunixin in equine urine and serum by capillary electrophoresis. J Chromatogr B Biomed Sci Appl. Apr 25;692(1):187-98.

Intervet/Merck, Inc. (2012) Banamine®-S Injectable Solution, North American Compendiums, March,2005.http://sploughus.naccvp.com/index.php?u=country&p=msds&prodnum=1047251&id=1047251&m=product_basic_view. Accessed 28 January 2015.

National Research Council. (2012) National Reasearch Council Models for Estimating Nutrient Requirements of Pigs. Eleventh Edition. The National Academies Press, Washington D.C pp. 208-238.

Leary S, et al. (2013_AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. pp. 58-62. https://www.avma.org/kb/policies/documents/euthanasia.pdf Accessed 28 January 2015.

Leavens TL, Tell LA, Kissell LW, Smith GW, Smith DJ, Wagner SA, et al. Development of a physiologically based pharmacokinetic model for flunixin in cattle (Bos taurus). Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2014;31:1506-21.

Pairis-Garcia MD, Karriker LA, Johnson AK, Kukanich B, Wulf L, Sander S, Millman ST, Stalder KJ, Coetzee JF. 2013. Pharmacokinetics of flunixin meglumine in mature swine after intravenous, intramuscular and oral administration. BMC Vet Res. Aug 13;9:165. doi: 10.1186/1746-6148-9-165.

Popot MA, Garcia P, Bonnaire Y. (2011). Doping control in horses: housing conditions and oral recycling of flunixin by ingestion of contaminated straw. J Vet Pharmacol Ther. 34(6):612-4. doi: 10.1111/j.1365-2885.2011.01276.x. Epub 2011 Feb 16.

Smith JN, Wang J, Lin Y, Timchalk C. Pharmacokinetics of the chlorpyrifos metabolite 3,5,6-trichloro-2-pyridinol (TCPy) in rat saliva. Toxicol Sci 2010;113:315-25.

Upton RN. Organ weights and blood flows of sheep and pig for physiological pharmacokinetic modelling. J Pharmacol Toxicol Methods 2008;58:198-205.

Zimmerman JJ, et al. (2012) Diseases of Swine. 10th edition. Wiley-Blackwell. Chichester, West Sussex, U.K.