RESEARCH REPORT
ANIMAL WELFARE

Title: Determining the Topical and Oral Pharmacokinetics of Flunixin Meglumine in Pre-wean Piglets and Developing Tools for Drug Study in Pre-Wean Piglets – **NPB #17-082**

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Industry Summary: Nearly all pigs experience tail-docking and/or castration in the United States and yet alternatives to castration and tail docking have not been adopted by US pork producers due to inherent challenges with alterntives. Public expectations to mitigate pain continues to build and influence producer decisions. Even in the absence of routine castration and tail-docking, disease and injury create conditions that are painful for piglets. Given that flunixin meglumine (FM) is already approved for IM use in pigs and extra-label use of drugs for pain is allowed, the main hurdle to potential application is confirmation that it reaches useful serum levels and to develop a dose regimen that is safe for the pre-wean piglet. An understanding of drug pharmacokinetics (PK) is a prerequisite to efficacy studies. Additionally, investigation of two additional routes of administration could lead to use of flunixin that does not require injection of every pig treated.

The primary objectives were as follows: *Objective 1*: Find the spot on a piglet where topical application of drug will most likely lead to transdermal absorption of drug and and quantify parameters of that area. The deliverable from this objective was to determine an anatomical target area on the piglet for testing and administration of topical drugs. Topical drugs are dosed differently than oral or injectable drugs and absorption can be affected by skin composition, thickness, and subcutaneous fat. Ten healthy pigs, 4-5 days of age were purchased and humanely euthanized. Full thickness skin biopsies were collected from 32 anatomical locations on each pig. The biopsies were fixed with formalin and examined microscopically for thickness of the skin and subcutaneous fat and density of hair follicles. The values were reported as an average, range and standard deviation (Table 1). *Objective 2*: Adapt a procedure to place

indwelling, dual lumen intravenous (IV) jugular catheters in pre-wean piglets. The deliverables from this objective were to describe the new procedure in a peer-reviewed, published report and to develop a video demonstrating the procedure. The dual-lumen IV jugular catheter in pre-wean piglets allows for IV dosing without contamination of blood samples and eliminates the need for two catheters. The catheter also provides an alternative to jugular venipuncture during serial serum sampling for pharmacokinetics studies in relatively fragile pre-wean piglets. Ten healthy piglets, 9 days of age, were randomly assigned to one of two groups: anesthesia or non-anesthesia. Isoflurane gas anesthesia was administered to the anesthesia group with the use of a pediatric gas mask. Anesthetic sedation was maintained throughout the duration of the catheterization procedure. Piglets were allowed to recover at the end of the catheterization procedure. The non-anesthetic group did not receive anesthesia. For jugular vein catheterization, each piglet was restrained in a V-tray. The jugular vein was catheterized with the use of a guide wire technique and a dual-lumen catheter. The catheter was sutured in place and bandaged (Fig. 1 and 2) to prevent self-mutilation by the piglets. Blood was collected from the catheter and the catheter flushed with heparinized saline twice daily, for three consecutive days. Following completion of the three days, the piglets were euthanized via anesthetic overdose with the administration of sodium pentobarbital through the jugular catheter. A necropsy was performed at the site of the jugular catheter, to the level of the cranial vena cava for verification of catheterization in the jugular vein (Figure 3). *Objective 3*: Measure and compare the pharmacokinetics of intramuscular (IM), oral (O) and topical (T) administration of flunixin meglumine in pre-wean piglets. The deliverables from this phase were to create an understanding of the fate of the drug in the pre-wean piglet including what concentrations are achieved in the serum and how long those are maintained using the oral, topical and intramuscular routes. Determining the fate of flunixin for pre-wean piglets by alternate routes, would provide the foundations necessary to develop effective treatment regimens. Twenty-four piglets, 9 days of age, weighing 5-10 pounds, were randomly allotted to 3 treatments (8 pigs / treatment): IM, O, or T administration of FM at 2.2mg/kg of body weight for oral and intramuscular administration and 3.3mg/kg of body weight for topical administration. Serum samples were collected at 0, 15, 30, 45, 60, 90, 120 minutes and 3, 6, 12, 24, 36, 48, 60, 72 hours post treatment, via jugular venipuncture. Samples were transferred to a sodium heparin blood collection tube and stored on ice for no longer than 130 minutes. Samples were centrifuged for 10 minutes at 1,500 g and collected plasma was placed in cryovials and frozen at -70°C until analysis. Plasma FM concentration were determined using ultrahigh performance liquid chromatography Q Exactive Focus Orbitrap mass spectrometry. Analysis produced plasma FM concentrations over time for each pig (Fig. 4). *Objective 4:* Precisely determine bioavailability of the topical route of administration. The deliverables from this objective, combined with parameters measured in objective 3, were to allow adjustment of the drug administration required to achieve target serum levels and develop dose regimens for topical application in pre-wean piglets. Sixteen piglets, 9 days of age, 5-10 pounds, were randomly allocated to two treatments in a cross-over study design with two rounds. In round one, eight pigs received FM via the T

route and eight pigs received FM via the IV route. Serum samples (1.0 mL volume) were collected at 0, 5, 15, 30, 45, 60, 90, 120 minutes and 3, 6, 12, 24, 36, 48, 60 hours post treatment for the IV route and 0, 15, 30, 45, 60, 90, 120 minutes and 3, 6, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204, and 216 hours for the topical route. Following the sampling, a washout period of nine days was used to ensure that the previous drug would not interfere with the next round of drug administration and sample collection. The groups then switched routes of administration and the sampling was repeated. The samples were prepared and submitted for analysis as previously outlined in Objective 3.

Objective 1: Statistical analysis of the data discovered significant differences ($p < 0.0092$) between the highest and lowest average sample site measurement for each skin characteristic evaluated (Table 1). These results reflect the importance of site selection for topical pharmaceutical application. Variations in skin thickness and hair follicle density across the body of a processing aged piglet reflect key factors that may influence transdermal absorption and pharmacokinetic principles of a drug. *Objective 2:* Jugular catheterization was successful in 3 of 10 piglets (all from the non-anesthesia group). Patency was maintained in the three catheters for three consecutive days (Table 2). If successful catheterization can be achieved in pre-wean piglets, the technique is useful and beneficial during pharmacology studies, however the ability to achieve and maintain catheterization was poor in this study. Due to the inconsistency of catheterization success, the blood collections for objective 3 and 4 were completed via serial jugular venipuncture. *Objective 3:* All three routes of administration produced measurable concentrations of FM (Fig. 4). Intramuscular and oral administration produced concentrations in a range that is consistent with pain mitigation in species where this has been investigated. These routes had short half-lives and clearance rates consistent with FM in older animals. The topical route produced much lower concentrations that persisted beyond 72 hours post administration. *Objective 4:* The bioavailability (F) of the topical route of administration was calculated using the area under the plasma concentration-time curve (AUC) for IV and topical (Table 3, Fig. 5) dosing and the bioavailability formula: $F = (AUC_{Topical} * Dose_{IV})/(AUC_{IV} * Dose_{Topical})$.

The bioavailability for the topical route of administration of FM was calculated to be approximately 6.3%.

Industry Takeaways

- Variation in skin characteristics show that topical application of pharmaceuticals should be researched further to determine if other site locations may lend better absorption of the NSAID. Much more variation was observed than expected and parameters did not trend in similar directions where a single spot optimized the factors expected to impact transdermal absorption.
- Percutaneous, jugular catheterization is possible but piglet anatomical variation at this age makes it an inconsistent technique for research that would require further refinement.

- Maximum serum concentration of the topical product was >100X lower than oral and intramuscular administration and is not consistent with levels demonstrated to mitigate pain. The high serum concentration of the oral route suggests that topical administration in a litter environment (versus in isolation as was done in this study) is warranted to include potential litter effects of licking and peer grooming on serum levels.
- In order to maintain consistency, only one location was used for topical application of FM. Future studies are warranted to determine if a more ideal location may lend better absorption of FM.

Keywords: swine, non-steroidal anti-inflammatory, flunixin meglumine, pain, topical

Scientific Abstract: Needle-free pain mitigation in pre-wean piglets is an area of need. In order to lay the foundation for future efficacy studies of flunixin meglumine (FM), scientific data of serum levels and the bioavailability of topical administration of FM are necessary. The objectives of this study were as follows: 1) Find the spot on a piglet where topical application of drug will most likely lead to transdermal absorption of drugs and systemic circulation and quantify that area. 2) Adapt a procedure to place indwelling, dual lumen intravenous (IV) jugular catheters in pre-wean piglets. 3) Measure and compare the pharmacokinetics of intramuscular (IM), oral (O) and topical (T) administration of FM in pre-wean piglets. 4) Precisely determine bioavailability of the topical route of administration. For each of the objectives, pre-wean piglets, 5-10 days of age, weighing 5-10 pounds were randomly allocated to groups, dependent upon the objective. Piglets for the first objective were humanely euthanized and skin samples collected from 32 anatomical location. Samples were prepared in formalin and submitted for histological evaluation. There were significant differences between sample sites for each skin characteristic evaluated. For objective 2, percutaneous jugular vein catheterization was attempted using dual lumen catheters in order to expand and refine pre-wean piglet catheterization techniques for pharmacokinetic studies. Catheterization was successful in 3 of 10 piglets and has potential as a useful technique for pharmacokinetics research. Piglets in objective 3 were assigned IM, O, or T route of administration of FM. Serum samples were collected from piglets post-administration of FM and submitted for analysis. Maximum plasma concentration (C_{max}) was 5,272 (ng/ml), 4,098 (ng/ml), and 31 (ng/ml) for IM, O, and T, respectively. Piglets in objective 4 were administered FM using both T and IV routes in a 2-way crossover design with a washout period of 9 days between routes. Preliminary calculations suggest the bioavailability for the topical route of administration is approximately 6.3%.

Introduction: Pain control for castration and/or tail-docking in pre-wean piglets as well as pain due to disease and injury is an increasing concern for consumers, as well as producers and veterinarians. Currently, FM is only approved for control of pyrexia, and as intramuscular injection in swine. Intramuscular injection can increase potential for infection and exist as a new source of pain for piglets. Recent approval of a topical, pour-on liquid FM for pain control in cattle would potentially allow extra-label drug use in other food animals, such as pigs. Evaluation of the serum concentrations and bioavailability of FM for oral, topical, and

intramuscular routes of administration would provide a foundation for determining dose in studies to assess the mitigation of pain using topical administration in piglets.

Objective: The project consisted for four objectives to collectively investigate the potential for topical administration of a non-steroidal anti-inflammatory (NSAID) drug to pre-wean piglets which may be useful for reducing the pain and inflammation associated with routine piglet processing procedures. *Objective 1*: Find the spot on a piglet where topical application of drug will most likely lead to transdermal absorption of drugs and systemic circulation and quantify that area. *Objective 2*: Adapt a procedure to place indwelling, dual lumen intravenous (IV) jugular catheters in pre-wean piglets. *Objective 3*: Measure and compare the pharmacokinetics of IM, O and T administration of FM in pre-wean piglets. *Objective 4:* Precisely determine bioavailability of the topical route of administration.

Materials and Methods: All live animal procedures were pre-approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC). *Objective 1:* Ten piglets, 4-5 days of age, were purchased and humanely euthanized. Using a sample site diagram and key, 32 anatomical locations were marked on each piglet by placing a dot via permanent marker on the skin for identification. Four methods were used to collect skin samples from the piglets. Method one involved tenting the skin with a pair of forceps and using a scalpel blade to remove the skin above the underlying subcutaneous fat and muscle. Method two utilized a 3-millimeter punch biopsy to remove two samples from each site including the snout and between the digits. Method three used tissue scissors to cut out a middle section of the ear. Orientation (dorsal and ventral surface) was determined upon microscopic evaluation of the histology slide. Method four included using a scalpel blade to incise above the lateral aspect of the coronary band and slice a sagittal section from the fore and hind limb, including the coronary band and hoof wall. The biopsies were fixed with formalin, and examined microscopically. The thickness of the skin and subcutaneous fat was measured and density of hair follicles were characterized. Statistical analysis was performed using a generalized linear mixed model using SAS® software, version 9.4 (SAS Foundation, Cary, NC). *Objective 2*: Ten healthy piglets, 9 days of age, weighing between 5 and 10 pounds, were randomly allocated into 2 groups. Five pigs were allocated to the anesthesia group and five pigs were allocated to the non-anesthesia group. Piglets were individually housed with ad lib water and received milk replacer three times daily, via orogastric tube. Piglets in the anesthesia group were administered isoflurane in oxygen with the use of a pediatric mask. A surgical anesthetic plane was maintained throughout the procedure. Upon completion of jugular catheterization, anesthesia was removed and the piglet was allowed to recover in sternal recumbency. Piglets assigned the non-anesthesia group did not receive anesthesia. For jugular vein catheterization, each pig was restrained in a V-shaped trough in dorsal recumbency. The front legs were abducted from the midline and the rear legs were manually restrained in a caudal position. The catheter site was cleaned using a three-step surgical scrub. The jugular vein was accessed with a 6.0 ml syringe and introducer needle. Once blood flow was obtained, the syringe was removed from the needle and the guide wire

introduced through the introducer needle. The guide wire was advanced until resistance is reached. With the guide wire in place, the introducer needle was removed over the wire. The dilator was threaded over the guide wire and advanced through the skin. The dilator was removed while maintaining the guide wire in place. The dual-lumen catheter was placed over the guide wire and advanced into the piglet and the jugular vein. The guide wire was removed. The catheter site was cleaned using high-pressure saline from a syringe. The catheter was sutured in place with 2-0 suture over the catheter and through the skin. The skin was then folded over the catheter and a cruciate pattern used to secure the skin over the catheter (Fig. 1). An elastic bandage was placed over the catheter so that the adhesive tape is not covering the catheter (Fig. 2). Blood was collected from the catheter twice daily for 3 days. Heparinized saline was used to flush catheters twice daily for 3 days. At the conclusion of 3 days of blood collection and catheter flushing, piglets were euthanized via anesthetic overdose with sodium pentobarbital through the jugular catheter. Following euthanasia, the piglets were necropsied at the area of the jugular vein to verify catheterization. *Objective 3:* Twenty-four piglets, 5-10 days of age, weighing 5-10 pounds, were randomly allotted to 3 treatments (8 pigs / treatment) IM, O, and T administration of FM at 2.2mg/kg of body weight (for oral and intramuscular administration) or 3.3mg/kg of body weight (for topical administration). Piglets were individually housed with ad lib water and received milk replacer three times daily, via orogastric tube until transitioned onto dry feed. Serum samples (1.0 mL volume) were collected at 0, 15, 30, 45, 60, 90, 120 minutes and 3, 6, 12, 24, 36, 48, 60, 72 hours post treatment. All blood samples were collected via jugular venipuncture with 18 gauge needle and 6mL syringe. During blood collection, piglets were manually restrained using a stainless steel "V-trough." Samples were immediately transferred to a sodium heparin blood collection tube and stored on ice before processing. Blood samples centrifuged for 10 minutes at 1,500 g. Collected plasma was placed in cryovials and frozen at −70°C until analysis. Plasma FM concentrations were determined using ultrahigh performance liquid chromatography Q Exactive Focus Orbitrap mass spectrometry (UHPLC-MS) (Thermo Scientific, San Jose, CA, USA). For the standards, QCs, blank and samples, an aliquot of 100 µL of serum was transferred into an Eppendorf tube, and 400 µL of the 100 ng/mL internal standard solution were added to each tube. The tubes were mixed using a vortex mixer and then centrifuged for 20 minutes at 600g. The supernatant was decanted into a test tube and the solvent was evaporated to dryness at room temperature. The samples were reconstituted with 200 µL of 25% acetonitrile in water. Each sample was transferred to an autosampler vial with insert and centrifuged at 600 g for 20 minutes prior to instrumental analysis. Two mobile phases were utilized as follows: A. 0.1% formic acid in water B. 0.1% formic acid in methanol. The mobile phase began at 10% B with a linear gradient to 95% B for 1.75 minutes, followed by a re-equilibration to 10% B. The flow rate was maintained at 0.3 mL/min. Separation was achieved with a Hypersil Gold Vanquish column (50 mm × 2.1 mm, 1.9 µm particles, Thermo Scientific, San Jose, CA, USA) maintained at 40°C. The following ions were used for identification and quantification: Flunixin (m/z 297.085) 264.050 and 279.074, Flunixin D3 (m/z 300.103) 267.050 and 279.074, 5-hydroxyflunixin (m/z 313.079) 280.045 and 295.069 and 5-hydroxyflunixin D3

(m/z 316.098) 280.045 and 298.088. The retention time for Flunixin and Flunixin D3 was 3.55, while the retention time for 5-hydroxyflunixin and 5-hydroxyflunixin D3 was 3.46. The limit of quantification was 1 and 2 ng/mL for 5-hydroxyflunixin and flunixin respectively. The limit of detection (LOD) was 0.06 and 0.3 ng/mL for 5-hydroxyflunixin and flunixin respectively. Results were viewed in the Quan Browser portion of the Xcalibur software. Analysis produced plasma FM concentrations over time for each pig. Analysis of these curves to determine pharmacokinetic parameters were performed with computer software (Monolix v4.3.2, Antony, France: Lixoft SAS, 2018) and standard goodness-of-fit (GOF) diagnostics, including population and individual predictions vs. observations, and the distributions of weighted residuals were used to evaluate the performances of the final model. Model selection was based on statistical significance between competing models using the objective function value and the Bayesian information criteria, together with the evaluation of GOFs and the precision of parameter estimates. *Objective 4:* Sixteen piglets, 9 days of age, 5-10 pounds, were randomly allocated to two treatments in a cross-over study design with two rounds. In round one, eight pigs received FM via the T route and eight pigs received FM via the IV route. Serum samples (1.0 mL volume) were collected at 0, 5, 15, 30, 45, 60, 90, 120 minutes and 3, 6, 12, 24, 36, 48, 60 hours post treatment for the IV route and 0, 15, 30, 45, 60, 90, 120 minutes and 3, 6, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192, 204, and 216 hours for the topical route. After a sufficient washout period of 9 days, the groups switched routes and the sampling was repeated. Sample analysis followed the procedures outlined in Objective 3 with modification. The topical route bioavailability was calculated by dividing the topical (AUC/Dose) by the IV (AUC/Dose) for each individual animal. Additional pharmacological parameter modeling is in process. The mean absorption time (MAT) will be calculated by subtracting the IV mean residence time (MRT) from the topical MRT.

Results: *Objective 1:* Statistical analysis of the data discovered significant differences ($p < 0.0092$) between the sample site with the highest and lowest average measurement for each skin characteristic evaluated (Table 1). *Objective 2:* Successful catheterization was achieved in 3 out of 10 piglets (Table 2) without the use of anesthesia. Catheterization was verified for 3 consecutive days through twice daily blood collections and heparinized saline flush. Necropsy was performed in the 3 piglets, from insertion of the catheter in the jugular vein to the level of the cranial vena cava (Fig. 3). Catheterization was unsuccessful in 7 out of 10 piglets. Catheterization was attempted up to 2 times per jugular vein (right or left), per piglet prior to catheterization being deemed unsuccessful. *Objective 3:* The average maximum serum concentration (C_{max}) for the IM, O, and T routes of administration were 5,272 (ng/ml), 4,098 (ng/ml), and 31 (ng/ml), respectively (Fig. 4). *Objective 4:* The area under the plasma concentration-time curve (AUC) for the IV and T routes of administration were 26810 (ng x hr/ml) and 2542 (ng x hr/ml), respectively (Fig 5). The bioavailability (F) of the topical route of administration was calculated using the (AUC), dose of drug administered, (Table 3) and the bioavailability formula: $F = (AUC_{Topical} * Dose_{IV})/(AUC_{IV} * Dose_{Topical})$. The

bioavailability for the topical route of administration of FM was calculate to be approximately 6.3%.

Discussion: *Objective 1:* These results suggest that there might be significant importance of site selection for topical pharmaceutical application. Variations in skin thickness and hair follicle density across the body of a processing aged piglet were measured in key factors that may influence transdermal absorption and pharmacokinetic principles of a drug. The scope and magnitude of variation in skin measurements implies that absorption could vary significantly between sites. However, in this study, the parameters measured varied in opposite ways between sites and no single site appeared to maximize the parameters tested to emerge as a clearly best choice for administration. Further work to weight the various skin parameters with respect to absorption of topical drugs in the pig may be warranted. *Objective 2:* The ability to successfully catheterize pre-wean piglets was inconsistent. The three piglets that were catheterized were in the non-anesthesia group. Successful catheterization seemed dependent upon several factors: ability to access the jugular vein, ability to maintain adequate blood flow and venous access throughout the catheterization procedure, the tortuous nature of the jugular vein, the relatively long length of the commercially available catheters for this size pig, and the ability to secure the catheter once inserted into the jugular vein. Dissection of the jugular vein in these piglets revealed that the jugular veins in pigs of this age were tortuous, lending to difficulty in advancing the guide wire, and therefore successful catheterization. Once the catheter was in place, suturing the catheter proved difficult due to movement of the piglet and securing the catheter properly to prevent movement and displacement of the catheter was challenging in smaller pigs. We hypothesize that the anesthesia group experienced some relative hypovolemia due to characteristic vasodilation attributed to the influence of the anesthesia and it was more difficult to initiate catheterization. *Objective 3:* All three routes of administration produced measurable concentrations of FM. Intramuscular and oral administration produced concentrations consistent with those expected to mitigate pain. These routes had short half-lives and clearance rates consistent with FM in older animals. The topical route produced much lower concentrations that persisted beyond 72 hours post administration. The lower C_{max} for the topical route suggests that it is not likely to achieve concentrations likely to mitigate pain in pre-wean piglets. Given the high C_{max} for the oral route of administration, future research measure serum concetrations of topical administration in a litter situation which would likely include both topical and oral exposure to the drug as opposed to the individual housing system separating the pigs in this study. *Objective 4:* The low bioavailability (approximately 6.3%) of the topical route of administration means that sufficient doses to mitigate pain will not likely be practical application on the pre-wean pig.

Table 1. Average sample site measurement for each skin characteristic evaluated

	Stratum corneum thickness (µm)	Epidermal thickness (µm)	Dermal thickness (µm)	Subcutaneous thickness (µm)	Average number of hair follicles*
Highest Value	154.96 Right, front coronary band of the lateral digit	466.99 Right, front coronary band of the lateral digit	824.90 Right, perianal region	1893.90 Left, ventral pinnae, cervical attachment	14.5017 Right, lateral carpus
Lowest Value	11.5730 Vulva	34.5640 Vulva	232.37 Vulva	306.92 Vulva	0.3 Vulva
Average	37.17	91.5	563.54	787.599	4.3767

*Average number of hair follicles reported as a count

Table 2. Jugular vein catheterization success

	Jugular Catheter *n*=10
Placement Success	
Patency time, days	3
Success rate, %	30 (3/10)

Table 3. Area under the curve for IV and Topical routes of administration were used to determine bioavailability of topical route of administration

	IV (2.2 mg/kg)	**Topical (3.3 mg/kg)**
Baseline	0	0
Total Area (ng x hr/ml)	26810	2542

Fig. 1: Jugular vein catheterization with suture to secure in place

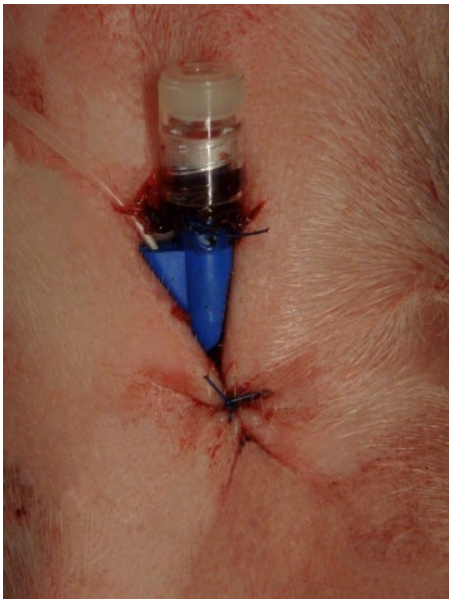


Fig. 2. Jugular vein catheter in place with bandage to maintain cleanliness

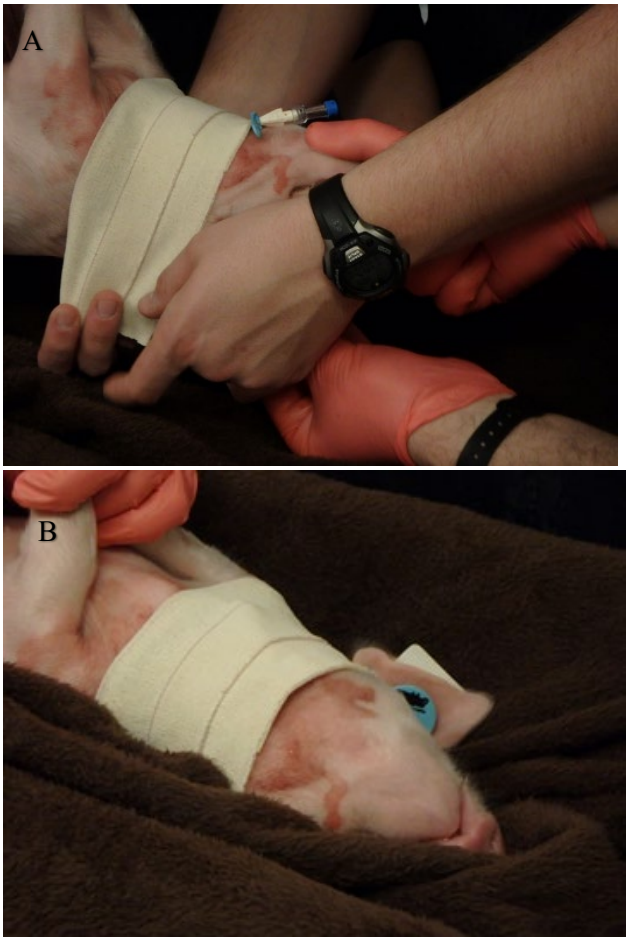


Fig. 3. Dissection of jugular vein (A) and cranial vena cava (B) to verify patency of catheter.

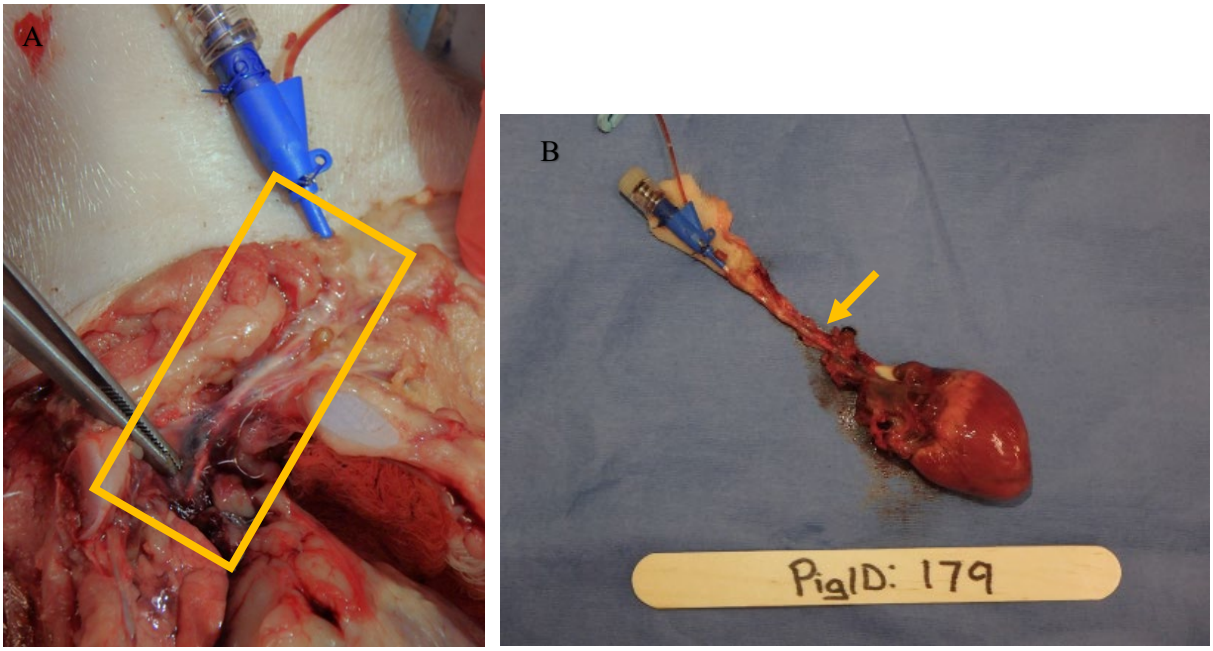


Fig. 4. Plasma flunixin meglumine concentration for each route of administration. Topical administration is on the right y-axis; oral and intramuscular administration is on the left y-axis.

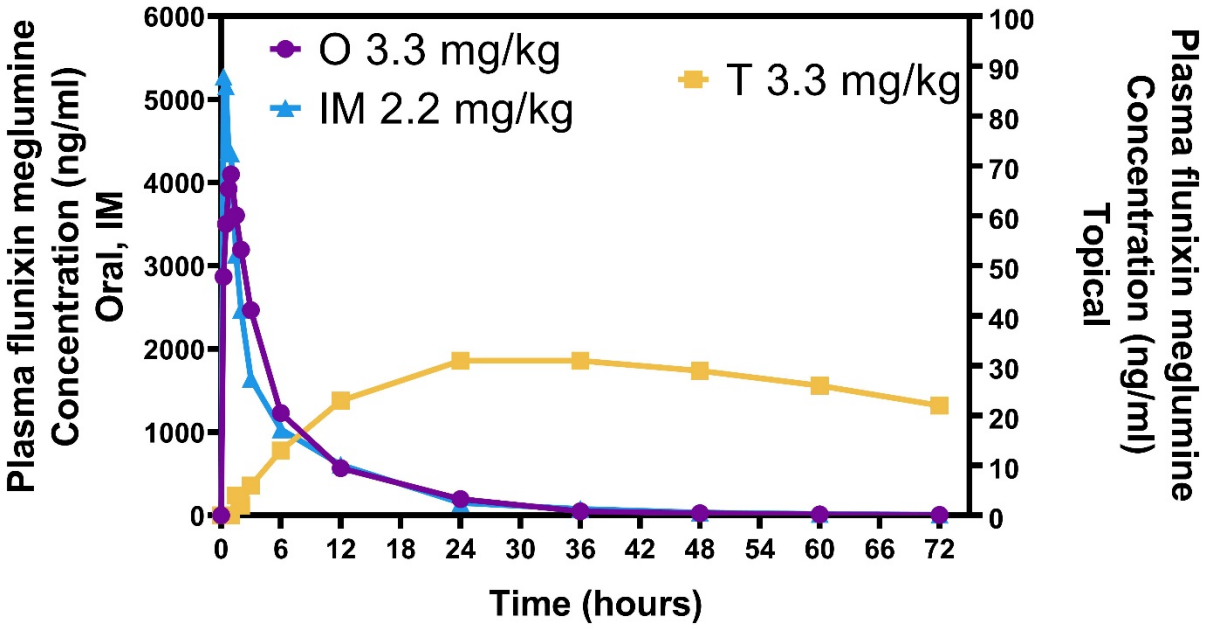
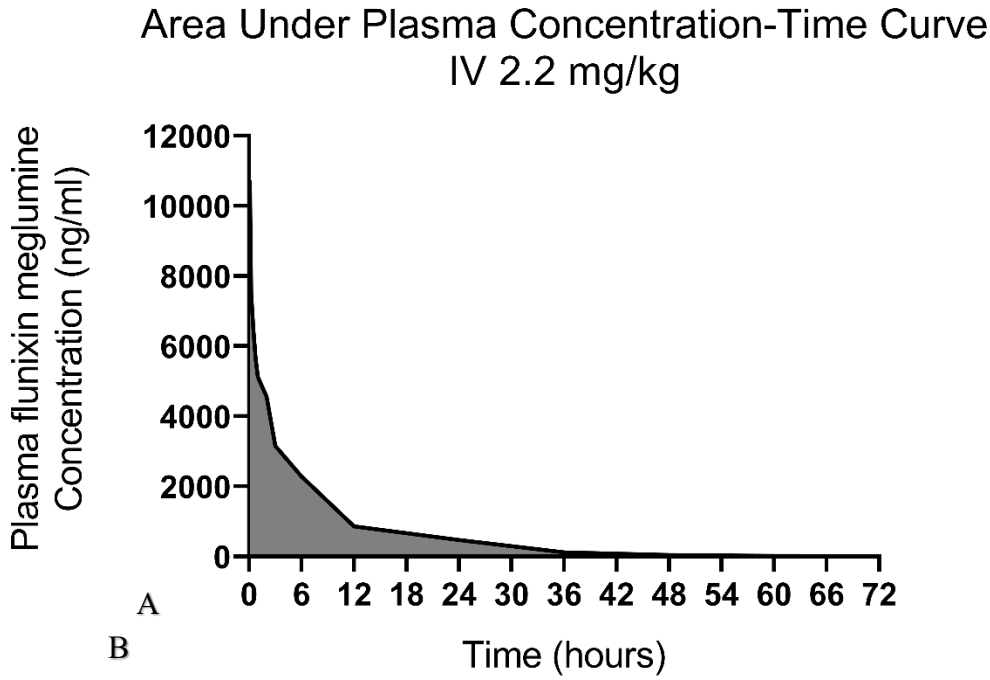


Fig. 5. Area under the plasma concentration-time curve for the IV (A) and topical (B) routes of administration obtained in Objective 4 of the study.



A

B