ABSTRACT

NIXON, EMMA. Pharmacokinetics and Pharmacodynamics of Nonsteroidal Anti-Inflammatory Drugs at Piglet Processing. (Under the direction of Dr. Kristen M. Messenger and Dr. Ronald E. Baynes).

In the United States, piglets undergo painful husbandry procedures such as castration and tail-docking without analgesia. NSAIDs are commonly used in Europe and Canada to reduce pain associated with piglet castration and tail-docking; however, results are conflicting regarding analgesic efficacy. Therefore, this research aimed to describe the pharmacokinetic and pharmacodynamic relationships of several NSAIDs at piglet processing. To achieve this goal, it was critical to 1) describe the pharmacokinetics of NSAIDs in piglets at the age at which routine processing occurs; 2) assess effects of NSAIDs at piglet castration and tail-docking; 3) develop PK/PD models to determine key PD parameters helpful in determining an effective dose regimen.

The first two studies focused on the pharmacokinetics of NSAIDs in neonatal piglets and weaned pigs, as dose recommendations for NSAIDs are based on studies conducted in older pigs and not neonatal piglets, and there are potential differences in pharmacokinetics related to age. To determine protein-unbound, pharmacologically active concentrations of NSAIDs and assess distribution to interstitial fluid (ISF), an *in vivo* ultrafiltration technique was utilized. The ratio of ISF: plasma maximal concentration was higher for piglets than weaned pigs, particularly for meloxicam. In addition, meloxicam and flunixin plasma half-lives were longer for piglets than weaned pigs. There is substantial interplay between pharmacokinetics and the resultant pharmacodynamic effects, and these reported differences in pharmacokinetics between neonatal and weaned pigs highlight the importance of performing pharmacokinetic studies in the population of interest before making dosing recommendations.

Efficacy of intramuscular meloxicam (0.4 mg/kg), ketoprofen (3.0 mg/kg), and flunixin (2.2 mg/kg) were also assessed utilizing three different objective measures: plasma cortisol, ISF prostaglandin E2 (PGE2), and activity via accelerometry. Unsurprisingly, castrated and tail-docked piglets had elevated plasma cortisol and ISF PGE2 concentrations and activity levels and alterations in 24-hour activity patterns compared to sham-handled controls. All NSAIDs reduced PGE2, but only flunixin maintained this inhibition beyond 24 hours post-dose. Flunixin also achieved the most significant reduction in plasma cortisol. Both flunixin and ketoprofen mitigated the effect of castration and tail-docking on piglet activity levels and patterns, as these piglets were closer to sham-handled piglets. Finally, effects of handling piglets and administering an intramuscular injection were negligible, at least in a controlled setting with a single researcher administering all injections. Based on these results, flunixin (2.2 mg/kg) and ketoprofen (3.0 mg/kg) administered intramuscularly two hours before castration and tail docking were the most efficacious treatments when considering effects on plasma cortisol, ISF PGE2, and activity. Meloxicam (0.4 mg/kg) did not provide adequate analgesia, despite its current use in Europe and Canada at this dose.

These data were used in a population-PK/PD approach to ensure optimal doses are given to piglets for pain mitigation at castration and tail-docking. Currently marketed doses of ketoprofen and flunixin correspond to drug responses of 33.97% (ketoprofen-PGE2), 40.75% (ketoprofen-cortisol), and 81.05% (flunixin-cortisol) of maximal possible responses. Given this information, flunixin may be the best NSAID in mitigating castration and tail-docking pain at the current label dose.

These studies were the first to utilize in vivo ultrafiltration and accelerometry in piglets at castration and tail docking. No previous studies have reported IC50 values associated with PGE2

reduction for NSAIDs in pigs or IC50 values associated with cortisol reduction for NSAIDs in any species. The results suggest flunixin may be the best NSAID to use at the current label dose; however, further studies are necessary to assess efficacy of a predetermined volume dose of flunixin (rather than per body weight) on-farm in a larger population. This would allow determination of on-farm feasibility and effectiveness and improve quality of piglet welfare recommendations.

Pharmacokinetics and Pharmacodynamics of Nonsteroidal Anti-Inflammatory Drugs at Piglet Processing

by
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BIOGRAPHY

Emma Nixon is originally from Doncaster, UK. She earned a Bachelor of Science in Veterinary Biosciences from the University of Surrey, UK where she was awarded a scholarship to spend a summer conducting research at North Carolina State University, Raleigh, NC. This experience prompted her interest in pharmacology and food animal research. During the final year of her undergraduate degree, Emma performed her undergraduate dissertation research under the direction of Dr. Simon Graham at the School of Veterinary Medicine at the University of Surrey. This dissertation was titled “Evaluation of Antiviral Nanoparticles Against Porcine Reproductive and Respiratory Syndrome Virus”.

After graduating from the University of Surrey in 2017, Emma then entered the Comparative Biomedical Sciences program at the College of Veterinary Medicine, North Carolina State University to pursue a Ph.D. in the concentration of pharmacology. Under the mentorship of Dr. Kristen Messenger and Dr. Ronald Baynes, her research has focused on the pharmacokinetics, efficacy, and PK/PD relationships of nonsteroidal anti-inflammatory drugs in food animals, particularly piglets at processing.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Appendix: Supplemental Chapter 6

CHAPTER 1

Introduction

Piglets raised on commercial farms in the United States undergo several painful husbandry procedures, including surgical castration and tail docking, for which they are often not provided sufficient analgesia or anesthesia for pain relief.[1] While Europe and Canada require analgesia before castration and tail docking,[2,3] there are no such requirements in the United States. In addition, there are no FDA-approved analgesic drugs for swine, and there are conflicting data regarding effective pain management strategies, making recommendations difficult.[1,4] Ideally, an analgesic treatment for piglets will be safe and effective, easy to administer by farm personnel, low cost, and long-acting, which leaves few feasible therapeutic options. Nonsteroidal anti-inflammatory drugs (NSAIDs) are approved in Europe and Canada to reduce pain associated with castration. However, despite the use of these drugs in other countries, there is limited data on both the pharmacokinetic (PK) and pharmacodynamic (PD) relationships of these drugs in piglets at the age at which routine processing occurs in the United States.[5–8] Recommended or labeled doses in Europe and Canada (0.4 mg/kg meloxicam and 3.0 mg/kg ketoprofen) are not well supported by the existing literature. In the United States, flunixin is approved for control of pyrexia associated with respiratory disease (but not pain) and is the only NSAID approved for pigs. Few studies assess the efficacy of flunixin at piglet processing,[5,9,10] and the label dose recommendation (2.2 mg/kg) is based on studies in older pigs and a different indication (pyrexia vs. pain).[11] As changes in the pharmacokinetics can alter the pharmacodynamic effects; it is essential to consider possible age differences; thus, chapters 3 and 4 assess the pharmacokinetics of NSAIDs in neonatal piglets and juvenile weaned pigs, respectively. Studies have also suggested that plasma drug concentrations of NSAIDs do not always correlate well with efficacy, making it challenging to base dose regimen

decisions on plasma concentrations alone.[12–14] A subcutaneously placed ultrafiltration probe can be used *in vivo* for minimally invasive collection of interstitial fluid (ISF; cutoff <30 kDa). *In vivo* ultrafiltration allows measurement of the assumed pharmacologically active, protein-unbound drug concentrations directly at the tissue level,[15] with the hypothesis that ISF drug concentrations will more accurately predict clinical outcomes than plasma concentrations regarding anti-inflammatory and analgesic effects. Chapter 6 supports this hypothesis and presents a population-PK/PD modeling approach with integrated ISF concentration data. The pharmacokinetic and pharmacodynamic data used in the PK/PD modeling are presented in chapters 3 and 5, respectively.

Overall, this research aims to describe the pharmacokinetics and pharmacodynamics of three different NSAIDs (meloxicam, ketoprofen, and flunixin) in neonatal piglets and correlate plasma and tissue drug concentrations with anti-inflammatory and analgesic effects in piglets undergoing routine castration and tail docking.

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CHAPTER 2

Literature Review

2.1 Piglet processing procedures

In the United States, piglet husbandry procedures such as castration, tail-docking, teeth clipping, ear notching/tagging, and injections are collectively referred to as "processing" piglets. Commercial farms routinely perform processing procedures without anesthesia or analgesia, even though these procedures are painful and distressing to piglets.[1–7] This review will focus primarily on castration and tail-docking because these two procedures have the potential to cause the most detrimental health and welfare effects.

2.1.1 Surgical castration

Surgical castration refers to surgical removal of the testicles or destruction of testicular formation. Surgical castration of male piglets is common in many countries, and most male piglets in the United States are castrated. Piglet castration occurs before weaning, most commonly within the first three days of life.[8] There are two main techniques: either two vertical cuts (most common) or one horizontal cut to the skin of the scrotum, and the testes are removed by cutting the spermatic cord with a scalpel or pulling until the cord tears.[8] A new experimental approach proposes using a CO2 surgical laser rather than a scalpel to reduce pain and inflammation and improve healing time; however, this technique requires further optimization before being clinically applicable.[9] Castration is performed to avoid boar taint in the meat of sexually mature male pigs. Boar taint is the accumulation of two main lipophilic compounds, skatole and androstenone,[10] which cause an offensive smell and taste in meat from intact male pigs. Barrows can be raised beyond puberty without developing strong boar taint; however, they have less efficient feed conversion and more fat deposits than boars. In addition to reducing the risk of boar taint, barrows exhibit less sexual

and aggressive behavior, making them easier to handle and less likely to fight and injure each other in group pens.

Surgical castration of piglets is painful and distressing with potential adverse health impacts as demonstrated by changes in intensity and frequency of vocalizations,[11–14] increased pre-weaning mortality,[15] changes in behavior,[2,13,16,17] increased heart rate,[14,18] and increased cortisol levels.[11,19] While painful responses are seen up to 4 days post-castration,[2,4] there is limited information regarding possible chronic pain beyond that time frame. Prolonged pain negatively affects immune function and growth in pigs,[20] and early life pain and stress in other species has significant long-term effects on pain processing, health outcomes, and susceptibility to chronic pain.[21,22]

Currently, the only truly viable alternatives to surgical castration are raising intact pigs or immunocastration. As mentioned previously, downsides associated with raising intact pigs can include aggression between pigs and caretakers and the development of boar taint.[23] Immunocastration is an active immunization against gonadotropin-releasing hormone (GnRH).[24] The vaccine consists of a GnRH construct that elicits anti-GnRH antibody production and, therefore, prevents stimulation of luteinizing hormone and follicle-stimulating hormone.[25] This results in suppression of testicular development and steroid production in Leydig cells. The first of this series of two vaccines is administered around 8-12 weeks of age, and the second vaccination is around 4-6 weeks before slaughter. Within a few days of the second vaccination, the boars behave like castrates (reduced aggression and mounting behavior, increased feed intake), and the levels of androsterone/skatole are low.[26–30] Three vaccinations are required in production systems where the animals are slaughtered at a heavy weight.[31–33] The main disadvantage to immunocastration is the cost of labor and the vaccine itself, and particularly the second (or third)

vaccination can be more difficult in large animals. Monitoring for non-responders after the second vaccine is also necessary based on behavior and size of the testes, there is a health risk for the workers who administer the vaccine due to the risk of self-injection,[34] and consumer perception and acceptance is unclear.[35] Despite these difficulties, there may be some economic benefit associated with immunocastration. Before the second vaccination, immunocastrated pigs are biologically like intact males, exhibiting a more efficient feed conversion ratio and growth than surgically castrated pigs.[25,34,36,37] After the second vaccination, the feed intake of immunocastrated pigs increases, but they still exhibit more efficient growth than surgically castrated pigs.[34,36,38,39]

Finally, semen sorting to allow the production of only female piglets is an alternative approach suggested to replace surgical castration,[40] although current technology is not efficient enough for commercial use.[8]

2.1.2 Tail docking

Amputation of the distal portion of the tail is intended to reduce the prevalence of tail biting,[41] an abnormal behavior that may result in injury, inflammation, reduced weight gain, increased risk of infection, and even necessitating euthanasia.[41–48] Tail biting can lead to considerable economic loss to producers and has implications for poor animal welfare. Tail biting is a multifactorial syndrome, and many internal and external factors may affect the prevalence, such as weaning age, diet, genetics, gender, health status, climate, ventilation, stocking density, lack of stimuli, and other environmental factors.[47] To address tail-biting, there have been developments in environmental enrichment strategies, alternative housing systems, and precision livestock farming; however, there has been a failure in applying these findings on commercial farms.[49] Despite EU legislation stating that routine tail docking is forbidden and may only be performed when there is evidence that tail biting has occurred,[50] research suggests that 81%–100%

of EU pigs are tail-docked routinely.[51] Therefore, implementation of pain management at tail-docking is critical.

The tail is sensitive and innervated, and tail docking causes both physiological and behavioral responses. Acute responses include increased blood cortisol concentrations,[52,53] changes in white blood cell count,[52] increased intensity or duration of vocalizations,[52,54–56] changes in ear posture,[57] increased dog-sitting and scooting behavior,[58] increased tail-jamming,[56,59] increased time spent lying alone,[52,55] increased time spent away from the sow[60] and decreased mechanical nociceptive thresholds.[61] Tail docking is traditionally performed using side cutter pliers or with gas-heated cautery clippers. However, tail docking may also lead to the development of neuromas associated with increased sensitivity to pain regardless of method.[62–64] Other long-term changes include changes in tail posture, hesitancy to interact with an unknown immobile human (possibly fear of humans),[57] hypersensitivity determined via decreased mechanical nociceptive thresholds[61] as well as sustained transcriptomic expression changes in caudal dorsal root ganglia cells involved in inflammatory and neuropathic pain pathways.[65]

2.1.3 Other procedures

Individual piglet identification is becoming increasingly important as meat safety and traceability issues increase.[66,67] The most commonly used methods of identification include ear notching, ear tagging, and tattooing. Implantation of transponders is also an option; however, this is often cost-prohibitive. These methods have associated problems, such as loss of tags, difficulty reading tattoos, time and labor to apply notches or migration of transponders.[68,69] Both ear notching and ear tagging are painful;[70] notching elicits vocalizations with high peak frequency, while tagging elicits vocalizations with high mean frequency. Significant increases in head-shaking are

also associated with both ear tagging and notching.[56] There is limited research into the welfare effects of tattooing in pigs, but this method does cause increased cortisol and stress in pigs.[71,72]

Teeth clipping or grinding is another management tool that should be performed only when necessary to prevent trauma to littermates' faces and the sow's underline when piglets compete for a teat.[73] Regardless of technique, there is an injury to the teeth, and there are also conflicting data regarding which technique is better for piglet welfare.[74–76] There is no feasible alternative to this procedure except to raise piglets with intact teeth and avoid larger litter sizes to reduce competition for the teats.[77]

Finally, iron can be administered intramuscularly or orally, with the former being more commonly used. Hepatic iron levels decrease rapidly during the first week of life and milk from the sow does not provide adequate iron to meet the needs of piglets.[78] Supplemental iron is usually acquired by rooting in the earth; however, most US commercial farms house pigs indoors, and thus iron administration is necessary to prevent iron deficiency and anemia-associated poor health and growth. Few studies assess the welfare implications associated with iron administration, but stress, regardless of route of administration, is similar to piglets handled but not administered iron.[68] Therefore, the health benefits outweigh the minimal stress associated with this procedure.[42]

2.2 Pain identification in piglets at castration and tail-docking

Pain is a complex multi-dimensional experience involving both sensory and affective components. It is difficult to assess in veterinary species, and studies often require proxy or indirect measures to quantify pain. When taken in isolation, these measures may not be considered as definitive evidence of 'pain'.[79,80] However, when taken together, they may provide evidence of the underlying affective state.[79] To date, piglet pain is generally assessed using either one or multiple of three approaches:

1. Performance measures
2. Physiological measures
3. Behavioral measures

2.2.1 Performance measures

Several studies of piglet castration have assessed weight gain; however, there is conflicting evidence to support whether castration improves, reduces, or has no effect on short-term weight gain.[2,81–83] One study that reports temporary weight loss suggested that processing may occur during the time at which teat order is established; therefore, male castrates may obtain a less productive teat. Alternatively, activity levels may influence the efficiency of suckling,[84] and if piglets suckle less vigorously, the milk yield of the teat may be affected.[82] On the other hand, weight gain could occur as painful piglets are more likely to have reduced activity, reducing calorie expenditure and possibly increasing weight gain.[83] Given the conflicting data, and weight gain or loss is not specific to pain; this may not be a useful measure for determining pain in piglets. However, castration can also increase pre-weaning mortality in low body weight piglets, possibly related to post-procedural complications.[15] While not a measure of pain, increased pre-weaning mortality is a welfare issue of concern and leads to production losses for producers.

2.2.2 Physiological measures

Major pathways activated by stressors are the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS). Activation of these pathways triggers release of endogenous compounds, including glucocorticoids, catecholamines, and opiate neuropeptides, to promote recovery by increasing metabolism and reducing inflammation.[85] However, sample collection to obtain information regarding these physiological biomarkers typically requires handling or restraining the animal, which can become a source of stress, confounding the results.[86]

Despite this limitation, physiological measures are objective and commonly used research of pain associated with piglet castration and tail-docking studies.

2.2.2.1 Cortisol and ACTH

Both physical and psychological stressors activate the HPA axis, stimulating the release of corticotrophin-releasing hormone (CRH) from the hypothalamus, which promotes secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. ACTH then acts on the adrenal cortex to produce cortisol. However, this response is not pain-specific. Tissue trauma associated with surgery can lead to increased cortisol and ACTH even under general anesthesia.[87,88]

Many studies in pigs have examined cortisol or ACTH concentrations to measure HPA axis activity relating to processing pain.[2–4,7,11,58,68,83,89–93] A majority of studies show that castration increases cortisol and/or ACTH levels after castration,[3,7,11,68,89,93] however, the cortisol response to tail-docking seems variable.[52,58,68,93] There are also differences in the timing and degree of increase. First, there is a 40-fold increase in plasma ACTH which peaks within 5 minutes after castration, followed by a 3-fold increase in plasma cortisol which peaks 15-30 minutes after castration.[93,94] Release of cortisol from the adrenal cortex also promotes the mobilization of glycogen, leading to a transient increase in glucose and lactate.[93] While increased plasma lactate has been observed following piglet castration, no significant changes in blood glucose levels have been found.[94] The authors suggest that this is due to a lack of hepatic glycogen stores in neonatal piglets.

2.2.2.2 Autonomic responses

Catecholamines released from the adrenal medulla are essential neurotransmitters that play a role in the autonomic regulation of many homeostatic functions, including vascular tone, intestinal and bronchial smooth muscle tone, cardiac rate and contractility, and glucose

metabolism. Increased heart rate and blood pressure have been reported in piglets following castration,[95], and a transient increase in plasma epinephrine, followed by a longer-lasting increase in plasma norepinephrine.[96] Additionally, cutting the spermatic cord results in increased β-endorphin, which is secreted in parallel with ACTH,[97] whereas tearing the cord does not.[68] The authors hypothesize this may be due to cutting resulting in more significant blood loss than tearing, as hemorrhage is known to cause an increase in β-endorphin, and opiates may assist in regulating the hemodynamic response to blood loss.[12,98]

2.2.2.3 Infrared Thermography

As previously mentioned, activation of the sympathetic nervous system causes increases in heart rate, respiratory rate, blood pressure, and peripheral vasoconstriction. Vasoconstriction decreases blood flow to the skin, reducing the cutaneous temperature and resulting in loss of heat in the body's periphery.[99,100] Infrared thermography (IRT) non-invasively and objectively detects the skin surface temperature as a proxy measure of sympathetic nervous system responses and has been used to study procedural pain in several food animal species.[7,91,99–103] However, this method has low specificity as vasoconstriction is not only limited to painful events. For example, a cold environment may also trigger vasoconstriction.[104] Furthermore, temperature differences are also affected by circadian rhythm, with peak temperatures in the evening and trough temperatures in the morning.[91,105] Despite these difficulties, after surgical castration in piglets, increases in orbital and rectal temperature and decreases in cranial skin temperature have been observed.[7,91]

2.2.2.4 Inflammatory biomarkers

Inflammation is an immediate response to injury or infection, characterized by redness, swelling, heat, pain, and loss of function, and is associated with the acute-phase response, which causes changes in acute-phase proteins (APPs) such as haptoglobin (Hp), C-reactive protein

(CRP), and serum amyloid A (SAA). These changes are triggered by pro-inflammatory cytokines released by injured or infected cells. Cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin 1 beta (IL1-β), and the previously mentioned acute-phase proteins have been measured with tail docking (183) and castration (75, 78, 93). No differences in CRP, SAA, or Hp have been found between castrated piglets and piglets that are only handled.[4] However, there were increases in TNF-α and IL1-β in both groups, possibly associated with the sample collection, or because both groups were previously tail-docked, teeth-clipped, and ear-notched. Regardless of the method, no differences in CRP were detected between docked and non-docked piglets at three weeks post-tail-docking. However, at seven weeks, increased CRP levels were present in non-docked piglets due to injuries associated with tail biting.[106]

Cyclooxygenase enzymes are also upregulated by tissue damage and inflammatory stimuli and catalyze the conversion of arachidonic acid to prostaglandins. Prostaglandins contribute to pain signaling by activating and sensitizing nociceptors,[107] leading to an increase in the magnitude of response to noxious stimulation, and increases in prostaglandin E2 (PGE2) are detected following castration and tail docking in piglets.[83] However, it is important to note that the magnitude of the inflammatory response and the pain experienced are not necessarily proportional.[108]

2.2.2.5 Neurotransmission

Substance P (SP) is a neurotransmitter related to pain perception, and there is a significant increase in SP plasma concentration after castration in cattle.[109–111] SP is released from damaged nerve fibers when tissue damage occurs; however, piglet castration has not elicited an SP response at piglet castration or tail-docking in two separate studies.[91,112] Another relatively underexplored measure of the response to painful stimuli in pigs is the expression of c-fos and its protein product

(Fos).[113,114] Many types of physiological events induce expression of c-fos in neurons of the central nervous system, and following piglet castration, there are a greater number of Fos-positive dorsal horn neurons in untreated piglets than piglets treated with a local or general anesthetic.[115]

2.2.3 Behavioral measures

Behavioral measures are commonly used to quantify pain associated with castration and other processing procedures.[116] However, conflicting results have been reported across studies. There are inconsistencies in the methodology used (different ethograms, continuous vs. scan sampling, pain-related behaviors vs. maintenance behaviors) that could be responsible for these conflicts.

2.2.3.1 Pain-related behavior

Pain-related behaviors are behaviors that are specific to piglet castration (or other procedure). The duration of these behaviors is short, and the frequency of occurrence is variable.[117,118] A study comparing the effectiveness of scan sampling methodologies (in which behaviors are recorded at selected time-points) compared to continuous sampling determined that these types of behaviors are easily missed when scan sampling is used as the observation period is limited.[117] Piglets only spend less than 5 or 6% of their time expressing these short pain-related behaviors.[101,117,119] For these reasons, continuous sampling methodologies are ideal when monitoring pain-related behaviors in piglets. The most used pain-related behaviors measured in castration studies are shown in Table 1. In addition to these, one study also assessed abnormal walking (walking with a limp, back arch or hind leg stiffness; flopping down on the ground while walking), leg crossing or shaking (crossing or shaking hind legs while standing or sitting; scratching the body or ear with the legs is not included), and head shaking (shaking head vigorously from side to side).[120]

Although much research has been focused on pain behaviors associated with castration, some studies have observed pain behaviors after tail docking (tail wagging and jamming, and scooting), teeth clipping (teeth champing), and ear notching (head shaking).[56,58,59,121] In response to tail-docking, tail wagging and tail jamming are seen within the first minute after the procedure.[56]

Table 2.1. *Pain-related behaviors commonly included in piglet castration studies, not including typical or maintenance behaviors. Adapted from Park et al., 2020.*[117]

Behavior	**Definition**	**Studies**
Scratching	Rubbing the rump against the floor, pen walls or littermates	2,4,6,52,92,101,117,119,120,122–129
Spasms	Quick and involuntary contractions of the muscle	2,4,6,101,117,119,123,125–128
Stiffness	Lying with extended and tensed legs	2,6,101,117,119,123,125,126,130
Tail-wagging	Tail movement from side-to-side (or up and down)	2,5,90,117,119,120,122,123,125–129
Trembling	Shivering, as with cold	2,4,5,101,117,119,120,123,125–129,131–133
Huddled up	Lying with at least three legs tucked under the body	2,4,6,52,92,101,123,124,127,129
Prostrated	Awake, standing or sitting, motionless with head down lower than shoulder level	2,6,90,101,120,123,129,131,133

2.2.3.2 Non-specific behaviors

Maintenance or state behaviors such as lying, sitting, and standing occur over a longer period than pain-specific behaviors,[6,123–125,133] and changes in duration or frequency may be more easily detected by scan sampling methods.[117] For example, piglets spend more time sitting after tail-docking, and the authors of that study speculate that this may relieve pain at the tail while protecting the wound site.[58] Other typical behaviors that piglets perform, such as nursing, general activity, and inactivity are also not specific to pain, but deviations in the duration or frequency of

these behaviors can indicate a painful experience.[123,134,135] For example, piglets suckled for decreased periods with a greater incidence of missed nursing bouts after castration[82] but not after tail-docking or ear notching.[59] Studies have also assessed aggression/agonistic behavior (defined as forceful pushing or hitting with the head or biting behavior toward littermates)[9,92,119,120,123,125–128] and social cohesion (time spent isolated from other littermates, or desynchronized/activity different to other littermates).[4,9,90,101,120,122,123,125–128] Some studies show that castration and tail-docking lead to increased isolation and avoidance of social contact with littermates,[2,4,52,92] but other castration studies have not discovered these same differences in social cohesion.[6,101,127]

2.2.3.3 Sensor-based activity monitoring

A recent review identified several sensor-based methods used for activity and posture-related measurement: cameras, photoelectric sensors (e.g., passive infrared detectors [PIDs]), radio frequency identification (RFID), and accelerometers.[136] These technologies have been used to measure general activity (active vs. inactive state), walking (step count), location tracking, and postural state (lying, standing, or sitting).[137]

Camera monitoring and computer-based object detection methods have been used to determine pig behavior and activity,[138] and specific events such as tail-biting.[139] However, they have not previously been used to quantify changes in activity in response to piglet castration or tail-docking. There are some significant problems with video-based detection and tracking individual pigs that are largely unavoidable due to the complexity of group behavior, which increases the risk of losing the tracked individual or tracking the wrong individual.[139] Pigs are also similar in appearance, making individual tracking based on appearance difficult, and in a group setting, pigs may occlude each other from the view of the camera or move out of frame. Light fluctuations can also cause issues with tracking, and sudden light changes frequently occur in the

pig sheds, including day vs. night (lights-on and lights off). Finally, the lens itself may become occluded (e.g., an insect may temporarily occlude the lens). Developing a method that can manage these conditions remains a challenge.[140]

Due to these difficulties, research has also assessed other sensor-based activity monitoring of pigs. For example, there has been success using RFID ear tags in monitoring pig movement.[141] RFID tags transmit data via radio waves to an RFID reader, which converts the radio waves to a usable form of data. However, because many RFID readers and tags are needed, this is an expensive surveillance option, and commercial feasibility is limited. Alternatively, PIDs are convenient and inexpensive. PIDs work by triggering an output when the sensor detects activity. For example, an alarm condition could be set when the sensor computes a certain number of triggers over a defined time frame, and then the activity is quantified by computing the time during which the alarm was activated for a certain period.[142] These times are totaled and stored by a data logger at set intervals (e.g., 10-minute intervals). PIDs have been used to describe weaned piglet activity, but not piglet activity associated with castration and tail-docking. PID outputs are easily interpreted, and correlations between PIDs and human observation are strong;[143] however, the precision of PID measurements decreases with extremes in activity, with an underestimation of mild activity and an overestimation of active behavior.[142,143]

Accelerometry (also known as actigraphy in sleep research) has also been used to measure movement. Accelerometers are small, battery-operated devices that are attached to the animal and measure acceleration. In veterinary species, the most common application of accelerometry in pain research has been the study of spontaneous activity in dogs and cats with osteoarthritis or degenerative joint disease.[144–151] While accelerometry offers high accuracy in detecting pig activity,[136] associated issues prevent on-farm implementation. Attachment of a device to the pigs

can motivate other pigs' chewing behavior in response to a novel object,[152] leading to loss or damage of the devices, and relatively short battery life also limits their use.[136] Despite this, accelerometers have been used successfully in research related to sow lameness,[153–155] predictions of farrowing time,[156] and early disease detection.[141] Accelerometers have also shown great promise as a surrogate measure of the impact of acute surgical pain. For example, comparisons of laparoscopic vs. open surgical procedures in both humans and dogs have demonstrated differences in activity levels, with lower activity following open procedures.[157,158] For acute pain related to husbandry procedures in large animal species, accelerometers have been used to assess the impact of pain in calves following castration and dehorning.[159–163] However, accelerometers have not previously been investigated as a surrogate measure of pain in piglets following surgical husbandry procedures, and Chapter 5 describes their first use in describing activity related to piglet castration and tail-docking.

Sensor-based activity monitoring of pigs offers a range of potential tools for welfare assessment in pig production. Each tool demonstrates the potential for continuous activity assessment, overcoming some of the issues surrounding other subjective behavior measures. However, problems associated with each technology must be overcome to allow these methods to monitor pig welfare.

2.2.3.4 Escape behavior

Piglet castration without anesthesia provokes resistance and escape behavior during the procedure.[164] These behaviors can potentially be used to measure pain and are typically scored in terms of frequency, duration, or intensity of resistance movements.[6,16,68,101,164] Escape attempts are significantly increased in piglets undergoing castration compared to sham-handled controls,[68] and the duration and intensity of resistance behavior is increased from the pretreatment period in

castrated piglets, but not in sham castrated piglets.[16] These measures may be helpful to assess the initial pain associated with the acute pain induced during the procedure.[165]

2.2.3.5 Vocalizations

Vocalizations are commonly used in piglet castration research to quantify pain and stress associated with the procedure. Piglets typically vocalize when handled and especially when restrained; however, studies have shown changes in piglet vocalization during castration, which may indicate pain. Piglets undergoing castration vocalize more loudly, more frequently, and at a higher frequency than piglets handled but not castrated,[5,11–13,16,68,101,114,126,166–168] and severing the spermatic cords is the step of the procedure that leads to the greatest vocalization response.[12,167] However, it is difficult to compare the data between studies, as the metrics used are very diverse.[169,170] Parameters that can be utilized in quantifying vocalizations include duration, energy or highest energy, loudness, peak frequency or pitch, vocalization rate, and the percent of piglets that vocalize.[114] The most consistent results come from parameters that describe a single event, such as peak level or peak frequency, rather than parameters that describe an average.[167] In addition, many studies have conducted piglet castration studies utilizing vocalizations as an outcome in acoustically isolated rooms, which is quite different from the typical environment in which piglets are castrated.[114] There are differences in baseline sound level to consider and the isolation of piglets from littermates and sow during the procedure.

2.2.3.6 Chute navigation

Many behavioral methodologies are subjective and risk misclassification bias. To combat this, some studies have utilized chute navigation as an objective pain assessment tool.[131,171,172] The time taken for a piglet to navigate a chute is recorded and compared between treatment groups. Typically, piglets take longer to navigate the chute post-castration compared to non-castrated

controls.[171,172] However, these studies have indicated that chute navigation may be unable to quantify pain after the early time points post castration (>30 min);[171,172] therefore, different methods of pain quantification may be needed to assess beyond the immediate post-procedural pain.

2.2.3.7 Piglet grimace scale

A piglet grimace scale (PGS) has been developed as an alternative, non-invasive method to assess piglet processing pain.[125] This approach is similar to previously developed grimace scales for other large animal species.[173–176] The piglet grimace scale describes three facial action units (FAUs): ear position, cheek tightening/nose bulge, and orbital tightening. FAUs are scored based on whether it is absent (score of 0), moderately present (score of 1), or obviously present (score of 2), except for orbital tightening, which is scored on a 2-point scale of absent (score of 0) and present (score of 1).[125] While the pilot study correlated PGS score and behavior after castration,[125] the follow-up study could not detect differences between castrated and sham-handled piglets.[119] In fact, that study found that piglet body weight may be a confounding factor and that grimacing could be related to weakness or stress in low body weight piglets. Additionally, another study utilizing a similar scale found inconsistent inter-observer reliability.[122] Therefore, further optimization and validation of these scales are needed before they can be used more widely.

2.2.3.8 Quantitative sensory testing

Quantitative sensory testing (QST) utilizes a noxious stimulus (mechanical, thermal, or electrical) to elicit an avoidance response. Although these stimulus-evoked assays only determine the sensitivity at the local (wound) site or a remote site, and not necessarily pain, they do allow detection of abnormal thresholds caused by painful conditions. Post-surgical afferent nerve sensitization can cause hyperalgesia,[177] which results in alterations in the nociceptive thresholds,

potentially detectable by these QST methods. Several tools are available, but mechanical nociceptive thresholds are the most used in studies of piglet pain.[61,178,179]

Von Frey filaments are a calibrated filaments that bend under a certain amount of pressure, allowing the delivery of a reproducible, graduating mechanical stimulus. Electronic von Frey aesthesiometers are also available. These methods have been used to determine wound hyperalgesia following husbandry procedures in other large animal species.[180–183] In piglets, von Frey filaments have been successfully used to study the effectiveness of pain mitigation for surgical castration,[166,184] although no studies have compared castrated and sham-handled piglets. Von Frey filaments have also been used to determine the effects of prenatal stress on nociceptive thresholds after tail-docking.[178] No differences were found between tail-docked and non-tail-docked piglets; however, prenatally stressed piglets had higher thresholds. An alternative method, pressure algometry, applies a force and measures the pressure at which an avoidance response is elicited. Piglets that are only handled show lower sensitivity to pressure algometry applied to the scrotum than castrated piglets,134, consistent with behavioral observations. Conversely, another study found difficulties with pressure algometry in piglets due to the soft nature of scrotal tissues.[114]

QST methods are limited because they require a direct stimulus and do not provide information on the more complex perception of pain. Combining these methods with other measures of pain (for example, spontaneous pain-related behaviors in the absence of a direct stimulus) may provide better assessments of pain mitigation strategies.

2.3 Current status of pain management in piglets at processing

2.3.1 Foreign Countries

In 2010, European pork sector stakeholders voluntarily signed the European Declaration on alternatives to surgical castration of pigs. This agreement stated that from 2012, surgical castration of pigs, if carried out, shall be performed with prolonged analgesia and/or anesthesia with methods mutually recognized. Surgical castration of pigs should be abandoned by 2018.[185] To date, neither goal has been met, and only six countries have banned surgical castration without anesthesia or/or analgesia.[186] According to European legislation, castration and/or tail-docking may not be performed after seven days of age without anesthesia and additional prolonged analgesia.[50] This legislation also states that routine tail docking is forbidden, and tail docking should only be done if an assessment of tail biting risks and application of improvement measures have not led to a sufficient reduction of tail biting. Canadian legislation has taken a much more explicit stance. As of 2016, analgesics must be used for castration or tail-docking performed at any age. In addition, castration performed after ten days of age must be performed with both anesthesia and analgesia.[187]

Anesthetics and analgesics for piglets are not authorized in all EU countries, and, in many countries, their use is restricted exclusively to veterinary surgeons.[186] However, in some parts of Europe, injectable meloxicam and ketoprofen are approved to relieve postoperative pain associated with minor soft tissue surgery such as castration. Meloxicam is approved in Canada and certain parts of Europe at a single intramuscular dose of 0.4 mg/kg given before surgery, even though a group of swine experts gave weak recommendations to support the use of this class of drug.[169] There is also an approved oral suspension of meloxicam in some parts of Europe, but it is not indicated for postoperative pain. Ketoprofen is also approved in some parts of Europe at a single

intramuscular dose of 3.0 mg/kg before surgery. There are also ketoprofen medicated water products, but these are not indicated for postoperative pain.

In contrast to the legislative approach used in Europe and Canada,[185,187] the United States has allowed policy development by retailers in response to consumer opinions to drive piglet pain management.[188–190] There are no FDA-approved analgesics currently indicated to treat pain of any kind in pigs.

2.3.2 Consumer, producer, and veterinary opinions on piglet castration

Research regarding consumer, producer, and veterinary opinions towards piglet castration and other alternatives is primarily dominated by European studies and studies originating in Canada and Brazil. It is not easy to find information describing these opinions in the United States. Overall, there is a stark contrast in opinion between consumers and pig producers. Consumers generally see castration without pain relief as unacceptable,[191] whereas producers see it as necessary for management, sales, and prevention of worse welfare states,[192,193] and some even prefer castration without analgesia to castration with analgesia.[192] The following summary of consumer, producer, and veterinary opinions is not a comprehensive review but an overview of a few key findings.

2.3.2.1 Consumer opinions

A 2017 meta-analysis of willingness-to-pay studies showed that pigs rank the lowest compared to other food-producing species in willingness-to-pay for welfare-friendly products.[194] The authors argue that consumers may find other attributes more important, such as price, origin, or other product characteristics, as pork may be considered low-value meat. In addition, 75% of consumers find meat from boars objectionable compared to meat from castrated males.[195]

In 2013, focus groups were undertaken to explore participants' opinions, attitudes, and perceptions of piglet castration without pain relief and the three alternative methods (castration with pain relief, immunocastration, and fattening boars).[35] In a ranking of these methods, most participants preferred castration with pain relief. Fattening of boars was the second most preferred option, followed by immunocastration, and castration without pain relief was the least preferred. The authors commented that some participants were mainly concerned with food safety regarding immunocastration, despite evidence that immunocastration bears no risk to human health arising from residues in meat.[196] Another focus group conducted in 2011 found that some participants interpret immunocastration in a very negative way.[191] In fact, a 2012 survey of pig sector stakeholders in Germany showed that slaughterhouses did not accept immunocastrated pigs due to a lack of consumer acceptance.[197]

2.3.2.2 Producer Opinions

Surveyed Flemish pig producers perceive surgical castration without anesthesia as the most favorable strategy for farm profitability, animal performance, and effectiveness against boar taint, despite expecting the lowest consumer acceptance for this strategy.[192] Canadian producers recognize castration and tail-docking as painful but also accept these procedures as they are seen as necessary for sales, management and to prevent worse welfare issues such as injury or infection.[193] These producers also judged the pain as short-term enough to be considered unimportant. Following hands-on experience with several management strategies (castration with analgesia, castration with CO2 anesthesia, immunocastration, production of entire males, and castration without pain relief), many producers saw entire male production as a valid alternative to surgical castration due to reduced labor, and improvement in performance and profitability.

Immunocastration, however, was perceived negatively following hands-on experience with this method.[198]

2.3.2.3 Veterinary Opinions

Veterinarians in the United States believe that pain mitigation for piglet castration is essential; however, it is difficult for veterinarians to advocate for pain management protocols due to the lack of an approved product, economic challenges, and insufficient science-based guidelines.[188] Similarly, Canadian veterinarians also noted a lack of cost-effective drugs available for livestock pain management on-farm.[199]

2.3.3 Economic impact if implemented in the United States

The cost of providing NSAID analgesia at piglet castration (not including labor costs) is estimated at approximately $0.05 to $1.32 per piglet, depending on the treatment.[188] Orally administered meloxicam at 0.5 mg/kg was the cheapest option, followed by intramuscular flunixin at 2.2 mg/kg, and finally, intramuscular ketoprofen at 6.0 mg/kg was the most expensive.

2.4 Analgesia and anesthesia for pain management in pigs

2.4.1 Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs are extensively used in veterinary practice to manage both acute and chronic pain and are among the most studied treatments for processing-associated pain in piglets.[120,200] The primary mechanism of action of NSAIDs in inhibition of cyclooxygenase (COX) enzymes, blocking the conversion of arachidonic acid to prostaglandins and thereby preventing or reducing activation of the inflammatory cascade.[201] There are two known isoforms of COX; COX-1 and COX-2.[202] COX-1 is constitutive, and prostaglandins produced by this isoform are responsible for normal physiologic function. COX-2 is inducible and

upregulated by cytokines and other inflammatory mediators.[201,202] NSAIDs exert their analgesic effects via reduction of prostaglandins, as prostaglandins contribute to pain signaling by activating and sensitizing nociceptors,[107] leading to an increase in the magnitude of response to noxious stimulation. As COX-1 is involved in normal physiologic function, such as maintaining gastrointestinal barrier integrity, many recently developed NSAIDs are COX-2 preferential, meaning they target COX-2 to a greater extent than COX-1.[202] There are still possible side effects associated with COX-2 preferential NSAIDs, such as cardiovascular toxicity.[201] In addition, the description of the divide between COX-1 and COX-2 may be overly simplistic, as there is overlap in the function of these two isoforms.[203] Another COX isoform was previously described as COX-3,[204]. Newer research suggests that COX-3 is a variant of COX-1 rather than a distinct isomer.[202,205]

Despite different chemical classes and structures, most NSAIDS share some common pharmacokinetic characteristics. Most are weak acids with high lipophilicity at physiologic tissue pH (with some notable exceptions, e.g., mavacoxib is a weak base).[206] In addition, NSAIDs are typically highly plasma protein-bound (>90%). Despite high lipophilicity and protein binding, the volume of distribution and half-lives are variable across NSAIDs. Generally, NSAIDs are eliminated by hepatic biotransformation, with renal excretion of the parent compound contributing to a small amount of total excretion (< 5%).[207] NSAIDs typically display high extravascular bioavailability (with some exceptions dependent on species and the NSAID administered), and for some NSAIDs (notably, robenacoxib), oral bioavailability is significantly affected by concurrent feeding.[206]

2.4.1.1 Meloxicam

Meloxicam is a COX-2 preferential NSAID with favorable gastrointestinal tolerance,[208] and it is one of only few analgesic drugs approved for use in pigs in Europe and Canada to treat

lameness and inflammation, as well as postoperative pain associated with minor soft tissue surgery such as castration. Research has shown that meloxicam mitigates pain associated with husbandry procedures in other food animal species;[209,210] however, conflicting results are reported for piglet castration and tail-docking.[11,83,90,91,101,123,126,170,171,211]

Some of these conflicts may be due to differences in methodology. Several of the studies that conclude meloxicam effectively reduces pain-related behaviors utilized a scan sampling method at few time points.[90,123] As previously mentioned, scan sampling methods are ineffective when assessing pain-related behaviors, as many of these behaviors are short-lived and easily missed. Another difference between the conflicting results is whether piglets were castrated only or castrated and tail-docked together. Meloxicam given before castration alone reduces plasma cortisol concentrations.[90] Two studies have examined the effect of meloxicam on plasma cortisol after both castration and tail-docking together.[83,211] One of these studies reported significantly lower plasma cortisol but no impact on growth performance in piglets.[211] However, this study also included female piglets that were only tail-docked in the analysis. The other study that included only male piglets reported that plasma cortisol was not significantly different from untreated castrated controls. While mitigating pain for both sexes is essential, male piglets may be exposed to a more severe degree of pain when castration and tail-docking are performed simultaneously. The conflicting results may also be due to the dose of meloxicam administered. The current label dose of meloxicam in Europe and Canada is 0.4 mg/kg. However, some studies have assessed 1.0 mg/kg.[101,126] One study assessed transmammary delivery as a novel route of administering meloxicam in piglets,[91] meaning that meloxicam transfers via milk to the piglets following oral administration to the sow. The sows were administered 30 mg/kg of meloxicam orally via the feed for three days. This high dose is associated with duodenitis in sows and gastric ulcers and subacute

gastritis in the litters of these sows.[212] Even though there is demonstrated efficacy via pain biomarkers (PGE2 and cortisol) and IRT, safety issues surrounding the administration of such a high dose likely precludes the use of this route of administration as a viable form of on-farm pain mitigation. Other studies have assessed compounding meloxicam with iron dextran, as iron is administered to piglets intramuscularly to prevent anemia as previously described. When administered in this way, meloxicam reduces plasma cortisol concentrations while still maintaining serum iron concentrations above the reference value of 18.0 mmol/L and additionally reduces chute navigation time compared to castrated piglets not provided analgesics. Administered meloxicam compounded with iron may provide a way to reduce stress in piglets by administering fewer injections; however, this does not solve the issue of the conflicting evidence to support the use of meloxicam at piglet processing.

2.4.1.2 Ketoprofen

Ketoprofen is a slightly COX-1 selective NSAID[213,214] that exists as a racemic mixture of R-(-)- and S-(+)-ketoprofen. Research has suggested that the S-(+)-enantiomer is a much more potent COX inhibitor than the R-(-)-enantiomer,[215] and the S-(+)-enantiomer has therefore been considered the pharmacologically active enantiomer.[216] In addition, S-(+)-ketoprofen predominates over R-(-)-ketoprofen in terms of plasma exposure following intramuscular administration of racemic ketoprofen in piglets.[217,218]

Despite being approved for use in pigs in Europe for inflammation, pain, and fever associated with postpartum dysgalactia, mastitis metritis agalactia syndrome, respiratory tract infections, and minor soft tissue surgery such as castration, there is limited research on the analgesic efficacy of ketoprofen for piglet processing.[83,122,172,219] However, the evidence for ketoprofen's analgesic efficacy at piglet castration is much more transparent than other NSAIDs.

Only one study showed that ketoprofen (6.0 mg/kg) did not significantly reduce pain-related behaviors or grimacing,[126] although the authors discussed that the piglet grimace scale does require further validation. Plasma cortisol concentrations are reduced when ketoprofen (3.0 mg/kg or 0.1 mL per piglet) is administered before castration in piglets,[83,122,219] but daily weight gain is not affected. In addition, Chapter 3 shows that ketoprofen reduces PGE2 concentrations and improves activity levels following administration before castration and tail-docking.[83] Ketoprofen also reduces sensitivity as measured by pressure algometry. Similar to meloxicam, the effects of ketoprofen have been examined when compounded with iron dextran.[172] When administered in this manner, ketoprofen reduces chute navigation time compared to castrated piglets not provided analgesics.

2.4.1.3 Flunixin

Flunixin is the only NSAID with FDA approval for pigs in the United States for control of pyrexia associated with swine respiratory disease, although not for the indication of pain. Despite this, relatively few studies have assessed the effects of flunixin at piglet processing[112,220,221]. One study shows that flunixin (2.2 mg/kg) only mitigates castration pain during the first 30 minutes when comparing behavior to untreated castrated controls and only marginally reduces the percentage of stress vocalizations during the procedure.[112] In addition, cortisol concentrations are not improved. However, this may be due to the sample collection methods used. Blood samples were collected via venipuncture, which requires additional restraint, and the pain and stress associated with the needle puncture likely influenced the results. In addition, the behavioral observations included scan sampling, which often leads to painful behaviors being missed, as previously discussed. However, another study that also used venipuncture and scan sampling found the opposite.[220] They found that flunixin (2.2 mg/kg) reduced plasma cortisol and reduced

the frequency of pain-related behaviors. Another study that reported limited efficacy following administration of flunixin gave the drug post-castration.[221] Preemptive analgesia may be more appropriate to reduce sensitization of the peripheral and central pain pathways.[222] Finally, Chapter 3 shows that flunixin reduces plasma cortisol, reduces PGE2, and improves activity levels following castration and tail-docking compared to untreated castrated controls when administered two hours before processing.[83]

2.4.2 Other analgesics

2.4.2.1 Opioids

Opioids produce their effects by acting on three major types of receptors: mu (μ), delta (δ), and kappa (κ) receptors. This class of drugs typically provides much more potent analgesic effects than NSAIDs; however, their practical applicability on-farm is limited as opioids are controlled substances which only a veterinarian may administer. There are also no FDA-approved opioids for use in food animals. Despite this, some limited research has been conducted to assess the effects of opioid analgesics at piglet processing. Buprenorphine is widely used in veterinary medicine.[223–225] It acts as an agonist against μ-opioid receptors and as an antagonist at δ- and κ-opioid receptors, and is more potent than morphine,[226] and is associated with a low risk of side effects such as respiratory depression.[227] While studies have demonstrated analgesic efficacy for postoperative pain relief in other species,[228–232] and pain and lameness in pigs,[233–235] it is relatively understudied for pain associated with processing.[119,128] Piglets administered buprenorphine demonstrate fewer pain-related behaviors and lower grimace scores than piglets that do not receive an analgesic, but no differences in vocalizations have been detected.[119,128]

Butorphanol acts as an agonist at κ-opioid receptors and as an antagonist at μ-opioid receptors. However, studies of butorphanol at piglet castration reported unexpected side effects

(cyanosis, dyspnea, vomiting, excitatory behavior, and anxiety[236]) even after dose reduction, and therefore discontinued use.[119,132] Given these challenges, butorphanol is likely not a good choice in pain mitigation for piglet processing.

2.4.3 Local and general anesthetics

2.4.3.1 General anesthetics

Unlike analgesics, general anesthetics induce a general insensibility to pain while the animal is unconscious. However, general anesthesia is difficult to implement on-farm, and it may contribute to increased piglet mortality due to respiratory depression, hypothermia, or being crushed by the sow.[164] Despite these limitations, studies have assessed the effectiveness of general anesthetics to mitigate processing-associated pain in piglets, using agents such as carbon dioxide, nitrous oxide, or a combination of xylazine, ketamine hydrochloride, and glyceryl guaiacolate.[6,52,112,127,237] None of these agents reduced pain associated with processing, and some had detrimental effects. Nitrous oxide was associated with reduced growth,[6] and the combination of xylazine, ketamine hydrochloride, and glyceryl guaiacolate was associated with suppressed nursing behavior and increased mortality.[237] Ketamine via intramuscular and intranasal administration has been investigated, but both routes resulted in a long recovery time and had no effect on cortisol concentrations.[238] Finally, isoflurane anesthesia has been investigated for piglet castration; however, this study focused on escape behaviors and vocal response during the procedure and did not follow up after recovery from anesthesia.[239]

2.4.3.2 Local anesthetics

Local anesthetics can be administered to the castration site, either topically or via intratesticular injection, and they act by inhibiting excitation and blocking the conduction of action potentials in peripheral nerves.[240] Lidocaine is the most commonly used local anesthetic in food

animals in the US, although it is not FDA approved for pigs. At piglet castration, lidocaine is administered via intratesticular injection, allowing diffusion into the spermatic cord[14,101] (although one study did assess lidocaine as a topical spray).[241] It is uncertain whether the administration of lidocaine itself is painful, effective, or ineffective. Increased vocalizations and escape behaviors have been reported in response to lidocaine administration;[16,242] however, other studies report decreased pain responses,[11,14,101,167,242]or no difference in pain responses following lidocaine administration before castration.[89,123,241] Lidocaine is also ineffective when administered subcutaneously at the base of the tail before tail-docking.[52] Regardless of the effectiveness, lidocaine administration on-farm is not entirely practical, as it requires a ten-minute delay between administration and castration to take effect. In addition, the pain mitigation provided is short-lived and becomes ineffective 1-2 hours after administration.[95,237]. Mepivacaine has only been investigated in one study, and the authors suggest both mepivacaine and lidocaine may provide significant perioperative pain relief, despite escape behaviors being the only outcome of the study. Procaine and bupivacaine administered via intratesticular administration result in similar outcomes to lidocaine and faces the same challenges with on-farm practicality.[16,242,243]

Non-injectable local anesthetics are also being investigated. Cetacaine (active ingredients include benzocaine, butamben, and tetracaine hydrochloride) and Tri-Solfen (active ingredients include lignocaine hydrochloride and bupivacaine hydrochloride) are applied topically; however, neither has been shown to provide effective pain mitigation at piglet tail-docking.[52] For castration, the initial skin incisions are made before applying either Cetacaine or Tri-Solfen, allowing application to the exposed spermatic cords. Tri-Solfen significantly reduces escape behaviors, vocalizations, and wound sensitivity up to 2-4 hours after castration.[166,184] Severing the spermatic cords are reported as the most painful part of the procedure,[12,167] so direct application to the cords

may help in reducing pain. Another study reports that Tri-Solfen and Cetacaine were not effective in reducing pain associated with piglet castration; however, the first plasma cortisol sample was taken 30 minutes after the procedure, and scan sampling was used to observe behavior. In summary, Tri-Solfen may effectively mitigate perioperative castration pain in piglets, but it is still short-acting and does not address the inflammatory response to castration. Therefore, a multimodal approach is still required to mitigate postoperative pain following castration.

2.4.4 Summary

In summary, analgesics or anesthetics for use on-farm must be effective, easy to administer, low cost, and ideally provide both immediate procedural pain relief as well as post-procedural pain relief. Both general and local anesthetics are limited by their short duration of action, resulting in post-procedural pain not being prevented.[95] Both methods also require additional training of farm personnel or administration by a veterinarian. Sedation following general anesthetics could also increase the risk of being crushed by the sow,[95,101] and local anesthesia requires careful timing of the injection and procedure to ensure pain prevention.[25] The duration of action for NSAIDs is longer, and no additional training is required for administration. Therefore, NSAIDs may have the greatest potential to provide long-term analgesia with practical on-farm application. However, there is limited information linking plasma or tissue concentrations with the efficacy of these NSAIDs.

2.5 Tissue drug concentrations and inflammation

Many pharmacokinetic and pharmacodynamic studies utilize plasma drug concentration data, as plasma samples are convenient to obtain and analyze. However, plasma samples include both plasma protein-bound and unbound drug, which is not representative of the pharmacologically active drug fraction, particularly for NSAIDs that are extensively bound to

plasma proteins.[206] Research has also indicated that plasma drug concentrations do not always correlate well with efficacy, particularly for NSAIDs which can accumulate at sites of inflammation.[244–246] Tissue concentrations may more accurately reflect drug concentrations at the site of action.

Studies of NSAID distribution under inflammatory conditions in veterinary species have primarily used tissue cage models (see Table 2.2 for examples). In brief, tissue cages are implanted subcutaneously while the animal is anesthetized. For models of inflammation, a pro-inflammatory substance, such as carrageenan solution, is injected into the cage. Following a recovery period, the tissue cages become sealed within a thin layer of connective tissue and are filled with exudate (injected cages) or transudate (non-injected cages).[247] Multiple cages may be inserted into one individual, allowing them to act as their own control. However, sampling of tissue fluid by this method still collects both protein-bound and unbound drug and does not represent only the pharmacologically active drug.[246] In addition, tissue cage insertion requires anesthesia, prolonged healing times, and either surgical removal or euthanasia, meaning it is not a very convenient method of tissue fluid collection. Other studies have used a polyester sponge model, in which a polyester sponge soaked in a pro-inflammatory substance, such as carrageenan solution, is implanted subcutaneously.[248] For exudate collection, the sponge is mixed with heparinized saline, and the diluted exudate is removed by squeezing the sponge to express any remaining fluid.[249] Similar to the tissue cage model, the collection of exudate still includes protein-bound drug.

A recently emerging technique known as *in vivo* ultrafiltration has been used to collect interstitial fluid in veterinary species.[246,250] This method involves the subcutaneous insertion of an ultrafiltration probe using a local anesthetic and an introducer needle, allowing for minimally invasive placement. Placement of the probe causes minimal inflammatory response and can be

maintained in the animal for several days without adverse effects.[251] The probe consists of three semi-permeable loops (Figure 2.1) that only allow the collection of protein-unbound (cutoff ~30 kDa) interstitial fluid, and the probe tubing is connected to a vacutainer which allows for continuous sampling with minimal disturbance to the animal. The aim of ultrafiltration probes is to quantify only the protein-unbound portion of the drug, as it is anticipated that it will be different from protein-bound and plasma concentrations. Previously, no data was available describing the protein-unbound tissue pharmacokinetics of meloxicam, flunixin, or ketoprofen in neonatal piglets. Chapters 3 and 4 present the first studies utilizing ultrafiltration probes in pigs to quantify NSAID concentrations in interstitial fluid.

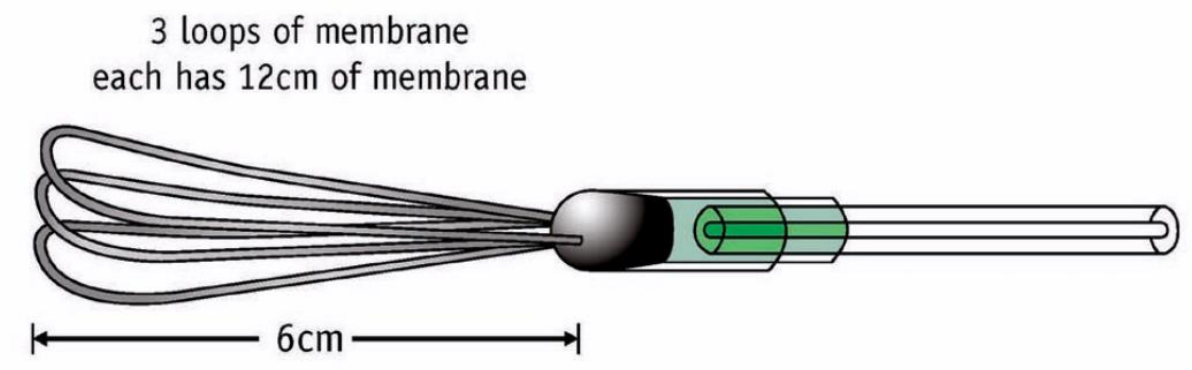

Figure 2.1. Picture representation of an in vivo ultrafiltration probe. *From https://www.basinc.com/assets/file_uploads/RUF%20Microdialysis%20Probe%20Manual.pdf.*

2.6 Pharmacokinetic/pharmacodynamic modeling

Pharmacometric drug models describe the relationship between exposure (pharmacokinetics [PK]), response (pharmacodynamics [PD]) for both efficacious and toxic effects. These models include concentration-effect, dose-response, and PK/PD relationships.

2.6.1 Pharmacokinetic modeling methods

Pharmacokinetics (PK) is defined as the use of mathematical models to quantitate the time course of drug absorption and disposition,[252] describing how drugs are absorbed, distributed, metabolized, and excreted from the body, and applying the physiochemical properties of the drug such as lipophilicity, charge, pH, molecular weight, and stereochemistry.

The significant differences between pharmacokinetic modeling approaches are the mathematical assumptions and techniques used. For example, compartmental modeling assumes the body is composed of several theoretical equilibrium compartments, representing nonspecific organs with similar rates of drug disappearance, allowing the formulation of mathematical models (e.g., nonlinear regression analysis) that describe drug movement within and between compartments.[252] Alternatively, a non-compartmental approach makes no assumptions about the underlying structure of the model and relies almost exclusively on algebraic equations to estimate PK parameters. PK parameters can elucidate and predict therapeutic plasma concentrations of drugs and determine an effective dosing regimen. However, it is important to note that PK parameters generated via modeling are only estimates of the true values, as an infinitely complicated model would be required to account for every possible outcome.[253]

Compartmental models usually involve one to three compartments (Figure 2.2), depending on the features of the drug concentration curve. A one-compartment model approximates the entire body as a single, well-mixed, homogenous compartment and describes PK showing a monoexponential decline (Figure 2.2A). In contrast, a three-compartment model accounts for a central plasma compartment, a rapidly equilibrating tissue compartment, and a slowly equilibrating tissue compartment (Figure 2.2C). Physiologically based pharmacokinetic (PBPK) models are typically used when the number of compartments exceeds three, and this approach uses knowledge

of the anatomy and physiology of the studied species to accurately describe plasma and tissue concentrations following drug administration.

Population-PK modeling investigates sources of variability in drug concentrations in the population of interest. While population-PK can utilize rich sampling data, one of the advantages of this approach is analyzing sparse sampling data, particularly where rich data sets are difficult to collect (e.g., on-farm animal studies where repeated sample collection may interfere with outcome measures). Mixed-effects modeling is the most used population-PK approach and incorporates fixed effects (parameters that do not vary across individuals) and random effects (parameters that vary across individuals).[254] There are three main parts of the population-PK model: the structural, statistical, and covariate models. The structural model describes the pharmacokinetic profile and utilizes compartmental pharmacokinetic methods, and the statistical model accounts for the random variability in the pharmacokinetics within a population, including inter-individual, inter-occasion, and residual variability. Covariate models utilize subject characteristics (e.g., age, sex, weight, breed, health status, or concomitant medications) to elucidate sources of PK variability within the population, allowing for the determination of appropriate dosages for a given population or subgroup. A population-PK approach is a robust methodology for describing drug disposition across a population and quantifying sources of variability. Clinically, it can be used to determine the optimal dose for individuals based on specific covariates.

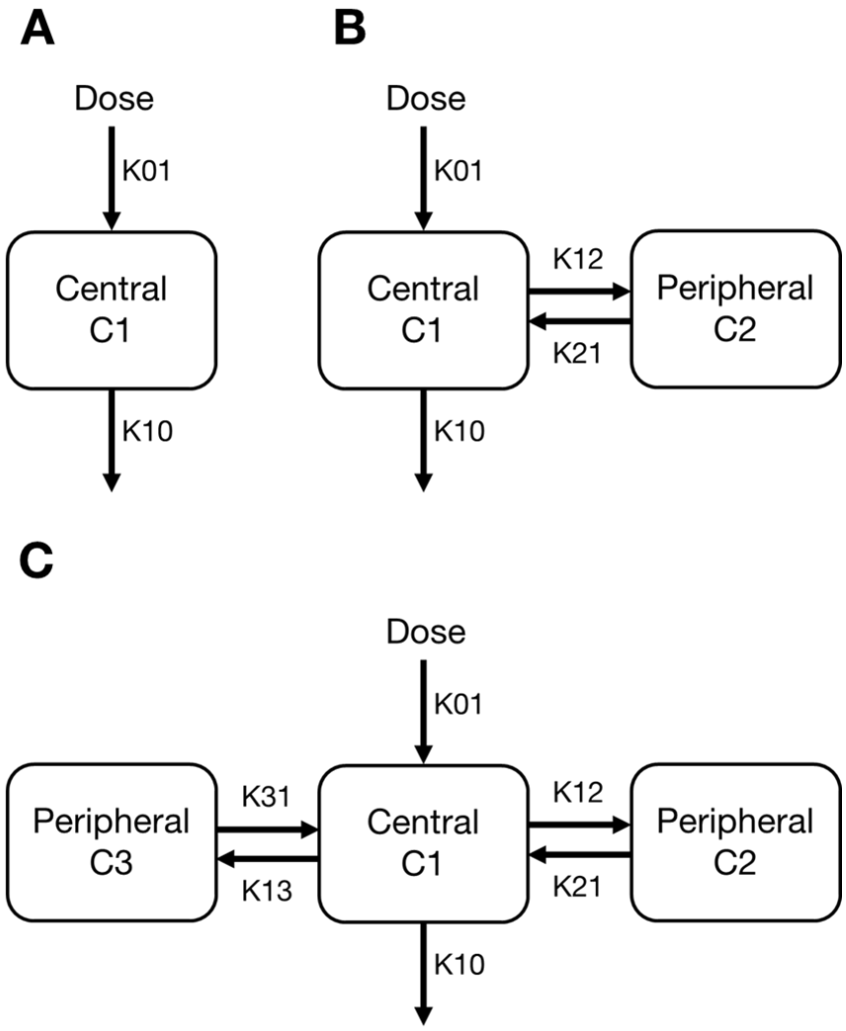

Figure 2.2. Schematic diagram of compartmental models representing drug movement to and from the central (plasma) compartment. *Part A shows a one-compartment model, part B shows a two-compartment model, and part C shows a three-compartment model. C1, central (plasma) compartment concentration; C2 and C3, peripheral (tissue) compartment concentrations, where in the three-compartment model, C2 represents rapidly equilibrating tissues, and C3 represents slowly equilibrating tissues; K01 rate constant for drug absorption for extravascular doses; K01, elimination rate constant; K12 and K13, transfer rate constants from the central to the peripheral compartments; K21 and K31, transfer rate constants from the peripheral compartments to the central compartment.*

2.6.2 Pharmacodynamic modeling methods

Pharmacodynamic (PD) models describe the relationship between drug concentration and therapeutic effect. The PD models that describe the main mechanisms of action are direct effect, biophase, and indirect response models.[255]

The basis of PD models can be traced back to studies of the biochemical, physiologic, and molecular effects of drugs on the body, involving receptor binding, post-receptor effects, and chemical interactions.[256] Many drug receptors are proteins that bind to endogenous ligands to perform normal physiological functions.[257] Drugs that bind to receptors and mimic physiological functions are known as agonists, and drugs that block or reduce the action of an agonist are known as antagonists. For example, classical non-aspirin NSAIDs are prostaglandin antagonists, exerting their mechanism of action by competitively and reversibly binding the COX enzymes and sterically preventing endogenous arachidonic acid binding.[258] Regarding receptor-drug interactions, there are four crucial parameters to consider: 1) Affinity: the extent of binding of a drug to a receptor; 2) Specificity: the ability of a drug to produce an action at a specific site; 3) Efficacy: the ability of the drug to produce an effect at the receptor; and 4) Potency: the relative amount of drug required to produce the desired effect. Many factors can affect the drug response, including but not limited to age, sex, bodyweight, basal metabolic rate, genetic factors, placebo effect, tolerance, and health status.

2.6.2.1 Direct effect models

The drug is directly responsible for the generated response in a direct effect model, and there is no time delay between the drug concentration and response profiles. An Emax model may be used to describe the direct concentration-effect relationship as follows:

$$E = \frac{Emax\ x\ Cp}{EC50 + Cp} \qquad \textit{Equation 1}$$

E is the effect, Emax is the maximum possible effect, Cp is the plasma drug concentration, and EC50 represents the drug concentration that produces a half-maximal effect. This Emax model is based on classic receptor occupancy theory, assuming drug effect is directly proportional to the fraction of occupied receptors, and can be derived from the following two equations:[255]

$$E = \gamma\ x\ RC \qquad \textit{Equation 2}$$

$$E = \frac{R0\ x\ Cp}{Kd + Cp} \qquad \textit{Equation 3}$$

RC is the concentration of drug-receptor complexes, γ is a proportionality factor, R0 is the total receptor concentration, and Kd is the dissociation constant for the drug-receptor complex. While direct effect models are helpful when there is no time delay between plasma drug concentration and therapeutic response, a lag between these is often observed. When a temporal delay is present, the plot of response vs. concentration will display a counterclockwise loop known as hysteresis. Depending on the mechanism, either a biophase model or an indirect response model can be used.

2.6.2.2 Biophase model

A biophase distribution model attributes the temporal delay between plasma drug concentration and therapeutic response to the time it takes for the drug to distribute to the target site. The plasma concentration is linked to a theoretical concentration in a proposed biophase compartment via the equation:

$$\frac{dCe}{dt} = Keo\ x\ (Cp - Ce) \qquad \textit{Equation 4}$$

Ce is the concentration in the biophase, and Keo is the first-order distribution rate constant.

2.6.2.3 Indirect response models

In an indirect response model, the drug acts as a precursor, which influences the response. The model describes the delayed response from an indirect drug mechanism, including inhibitory or stimulatory mechanisms that produce or dissipate a response, as described by the following equations:[255]

Model I $$\frac{dR}{dt} = Kin\left(1 - \frac{Imax\ x\ Cp}{IC50 + Cp}\right) - Kout\ x\ R$$ *Equation 5*

Model II $$\frac{dR}{dt} = Kin - Kout\left(1 - \frac{Imax\ x\ Cp}{IC50 + Cp}\right) x\ R$$ *Equation 6*

Model III $$\frac{dR}{dt} = Kin\left(1 + \frac{Smax\ x\ Cp}{SC50 + Cp}\right) - Kout\ x\ R$$ *Equation 7*

Model IV $$\frac{dR}{dt} = Kin - Kout\left(1 + \frac{Smax\ x\ Cp}{SC50 + Cp}\right) x\ R$$ *Equation 8*

R is the response, Kin is the first-order production constant of the response, Kout is the first-order dissipation rate constant of the response, is the Imax is the maximal effect of inhibition, IC50 is the drug concentration that triggers a half-maximal effect of inhibition, Smax is the maximal effect of stimulation, and SC50 is the drug concentration that triggers the half-maximal effect stimulation. A visual representation of these models is shown in Figure 2.3.

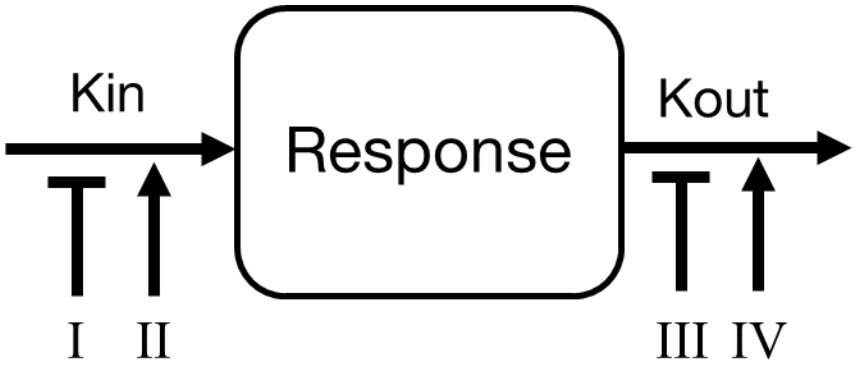

Figure 2.3. Indirect response model structure. *Kin and Kout represent the first-order production and dissipation constants of the response, respectively. Model I represents the inhibitory effect on the input of the response (Equation 5), model II represents the stimulatory effect on the input of the response (Equation 6), model III shows represents the inhibitory effect on the dissipation of the response (Equation 7), and model IV represents the stimulatory effect on the dissipation of the response (Equation 8).*

2.6.3 Pharmacokinetic/pharmacodynamic modeling of NSAIDs

Pharmacokinetic/pharmacodynamic (PK/PD) modeling examines the drug concentration-effect relationship over time and allows computation of key PD parameters important for describing the pharmacological profile and determining an optimal dosage regimen.[259] Indicators of potency (e.g., IC50) and effective dose (e.g., ED50) can be generated from PK/PD models, allowing informed decisions on the optimal dose given to a specific population. However, the numerical values of efficacy and potency should be interpreted with caution, as they depend on the model used (e.g., induced inflammation vs. naturally occurring), the outcomes measured (e.g., measurement of inflammatory mediators, gait analysis, mechanical nociceptive thresholds, or skin temperature) and species.[259] The PD parameters generated from a PK/PD model will vary between

species, in part due to differences in pharmacokinetics, but for NSAIDs specifically, potency and COX-inhibition ratios (COX-1:COX-2) vary between species.[244]

While several PK/PD models describe the effect of NSAIDs on pain and inflammation in other large animal species (Table 2.2), there are relatively few PK/PD models for pigs.[179,260] All PK/PD studies of NSAIDs in large animal species utilize an induced inflammation or arthritis model, and none have used naturally occurring conditions or routine husbandry procedures.

Table 2.2. PK/PD studies of NSAIDs in large animal species.

NSAID	Species	Model	Source
Carprofen	Sheep	Tissue cage model	[261]
Flunixin	Horse	Tissue cage model	[262]
		Induced arthritis model	[263]
	Calf	Tissue cage model	[264]
	Pig	Kaolin-induced inflammation model	[179]
Ketoprofen	Horse	Tissue cage model	[262,265]
	Calf	Tissue cage model	[266]
	Sheep	Tissue cage model	[267]
	Goat	Tissue cage model	[268]
	Pig	Kaolin-induced inflammation model	[260]
Meloxicam	Horse	Induced arthritis model	[269]
Phenylbutazone	Horse	Induced arthritis model	[263]
Tepoxalin	Horse	*Ex vivo* model	[270]
Tolfenamic acid	Horse	Tissue cage model	[271]
	Calf	Tissue cage model	[272]
Vedaprofen	Horse	Carrageenan sponge model	[248]

In pigs, both PK/PD models utilized a kaolin-induced inflammatory model.[179,260] Kaolin-induced hyperalgesia likely induces somatic pain, while surgical castration likely induces other

types of pain, such as visceral pain.[179] Therefore, caution should be taken when applying the results of these studies to clinical situations such as piglet processing.

The first of these piglet PK/PD studies administered either intramuscular saline or 6 mg/kg racemic ketoprofen 16 hours after subcutaneous injection of kaolin, determining body temperature, skin temperature, and mechanical nociceptive thresholds (MNTs) via pressure algometry.[260] The PK/PD model was based on an inhibitory indirect response model with a one-compartment PK model and utilized only the MNT data. This model generated an IC50 value of 26.7 and 1.6 μg/mL for S-(+)- and R-(-)-ketoprofen, respectively, indicating that R-(-)-ketoprofen is a more potent analgesic than S-(+)-ketoprofen in piglets. There was a biphasic analgesic effect in these piglets, and the authors hypothesize that R-(-)-ketoprofen was responsible for the initial comprehensive but short-lasting analgesia, and S-(+)-ketoprofen was responsible for the moderate but more sustained analgesia that followed. However, other studies have shown the S-(+)-enantiomer as a much more potent COX inhibitor and is considered the pharmacologically active enantiomer.[215,216] R-(-)-ketoprofen-mediated analgesia may work via a different mechanism,[260] but more research is required to support this hypothesis. There are no IC50 values derived from MNTs in other large animal species that can be used to compare to this ketoprofen piglet study; however, IC50 values derived from PGE2 inhibition in other large animal species tend to be much lower (0.007-0.042 μg/mL for S-(+)-ketoprofen in calves, goats, horses, and sheep).[265–268] Based on this kaolin model in piglets, an ED50 value of 2.5 mg/kg was estimated for racemic ketoprofen, close to the labeled dose of 3.0 mg/kg in Europe.

The only other PK/PD study for NSAIDs in piglets administered intravenous saline, 2.2 mg/kg flunixin or 4.4 mg/kg flunixin 10 hours after subcutaneous injection of kaolin and determined MNTs via pressure algometry.[179] The PK/PD model was based on an inhibitory

indirect response model with a two-compartment PK model. This generated IC50 values of 6.78 and 2.63 μg/mL for the 2.2 mg/kg and 4.4 mg/kg dose, respectively. The authors discussed the dose-dependent IC50 values, suggesting that the 4.4 mg/kg dose promoted analgesia by reducing inflammation to a larger degree than the 2.2 mg/kg dose by evoking a more rapid reversal of the kaolin-induced hyperalgesia. Again, there are no IC50 values derived from MNTs in other large animal species that can be used to compare to this study; however, similarly to ketoprofen, IC50 values derived from PGE2 inhibition in other large animal species tend to be much lower (0.009-0.063 μg/mL for flunixin in calves and horses).[262,273–276] The ED50 value was 6.6 mg/kg, markedly higher than the current label dose of 2.2 mg/kg. The ED50s reported in these studies should be used with caution as many reasons could explain differences to the label dose, including:

1. **Age**: The flunixin label dose is based on studies in older pigs, and age may influence the pharmacokinetics, potency, COX-selectivity, pain sensitivity, etc.
2. **Indication**: The label indication of flunixin is control of pyrexia associated with respiratory disease, and therefore the dose required to mediate kaolin-induced inflammation (or castration pain) may be different.
3. **Route**: Flunixin is labeled for intramuscular administration to pigs, and the study gave the drug via the intravenous route.
4. **Measured outcomes**: The label dose for flunixin is based on outcomes related to pyrexia (rectal temperature),[277] and this study utilized an outcome related to nociception (MNTs).

The authors of the flunixin PK/PD study in piglets also state that there was limited inter-individual variability in the plasma PK profile, but the analgesic efficacy was much more variable[179] (although a population model would be required to effectively estimate the inter-individual variability).[278] Some piglets showed minimal analgesia, and one piglet from the 4.4

mg/kg group was removed from the IC50 calculation due to a lack of response to flunixin administration. There is also marked individual variability in response to anti-inflammatory drugs in other species.[279,280]

In summary, several studies have assessed the effectiveness of NSAIDs to reduce pain associated with castration; however, there are conflicting results regarding their analgesic efficacy, and pharmacodynamic effects are highly variable.[90,91,112,123,126,170,211] In addition, there is limited knowledge regarding the optimal dose required to provide effective analgesic and anti-inflammatory effects. IC50 values specifically describing PGE2 suppression of NSAIDs are available for other large animal species, including cattle, sheep, goats, horses, and alpaca,[262,265–268,273–276,281–283] but not for pigs. In addition, only two studies have utilized PK/PD modeling for NSAIDs in pigs;[179,260] however, these studies utilize an induced-inflammation model, which may not translate well to pain mitigation associated with piglet processing. Finally, a panel of experts provided a weak recommendation for the use of NSAIDs for pain mitigation during piglet husbandry procedures.[169] Most of the panel decisions relied primarily on cortisol as an outcome. To date, there are no PK/PD models that assess the impact of NSAIDs on cortisol in any species.

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CHAPTER 3

Comparative Plasma and Interstitial Fluid Pharmacokinetics of Meloxicam, Flunixin, and Ketoprofen in Neonatal Piglets

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An erratum is being submitted to the journal regarding interstitial fluid lag times.

3.1 Abstract

Piglet castration and tail-docking are routinely performed in the United States without analgesia. Pain medications, predominately non-steroidal anti-inflammatory drugs, are used in the EU/Canada to decrease pain associated with processing and improve piglet welfare, however, past studies have shown the efficacy and required dose remain controversial, particularly for meloxicam. This study assessed the pharmacokinetics of three NSAIDs (meloxicam, flunixin, and ketoprofen) in piglets prior to undergoing routine castration and tail-docking. Five-day-old male piglets (8/group) received one of 3 randomized treatments; meloxicam (0.4 mg/kg), flunixin (2.2 mg/kg), ketoprofen (3.0 mg/kg). Two hours post-dose, piglets underwent processing. Drug concentrations were quantified in plasma and interstitial fluid (ISF) and pharmacokinetic parameters were generated by non-compartmental analysis. Time to peak concentration (Tmax) of meloxicam, flunixin, and S(+)-ketoprofen in plasma were 1.21, 0.85, and 0.59 h, compared to 4.19, 5.02, and 4.36 h in the ISF, respectively. The apparent terminal half-life of meloxicam, flunixin and S(+)-ketoprofen were 4.39, 7.69, and 3.50 h, compared to 11.26, 16.34, and 5.54 h, respectively in the ISF. If drug concentrations in the ISF are more closely related to efficacy than the plasma, then the delay between the Tmax in plasma and ISF may be relevant to the timing of castration in order to provide the greatest analgesic effect.

3.2 Introduction

Consumers are becoming increasingly concerned with welfare issues associated with castration and tail-docking of piglets, and this has prompted an increase in the investigation of pain medications to provide analgesia during these procedures. In the United States, husbandry procedures such as castration, tail-docking, teeth clipping, ear notching/tagging, and injections are collectively referred to as "processing" of piglets. Commercial farms in the United States routinely perform processing procedures (including castration and tail docking) without anesthesia or analgesia, despite the fact that these procedures are painful and distressing to piglets (1–7). The intent of surgical castration is to reduce aggression among male pigs as well as reduce the incidence of boar taint, an offensive odor or taste detected in pork obtained from intact male pigs following puberty. Tail docking preemptively aims to prevent tail biting. While currently there is no requirement for the provision of analgesics for piglets in the US, legislation in the EU and Canada requires that surgical castration of piglets should be performed with anesthesia and/or analgesia (8, 9). Additionally, routine tail docking is forbidden in the EU and may only be performed when there is evidence that tail biting has occurred (10).

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly studied treatments for processing-associated pain (11, 12), however, there are conflicting data supporting their use at castration or tail-docking. Particularly, there is conflicting data supporting the use of meloxicam at the European label dose of 0.4 mg/kg (13–16). There are no pharmacokinetic studies of meloxicam in piglets <1 week-of-age, which is surprising given the larger number of studies assessing the efficacy of meloxicam for pain-alleviation following castration, compared to other NSAIDs. Studies have assessed the plasma pharmacokinetics of meloxicam administered intramuscularly at 1.0 mg/kg in 8-day-old piglets (17), at 0.6 mg/kg in 2-week-old piglets (18),

and at 0.4 mg/kg intravenously in pigs 16–23 days old (19). None of these studies assessed the EU label dose of 0.4 mg/kg administered intramuscularly, and none assessed meloxicam in piglets <1 week old. Piglets are typically castrated between 3 and 7 days of age, and age may affect the pharmacokinetics of these NSAIDs (19). Flunixin plasma pharmacokinetics were previously described following an intravenous dose of 2.2 or 4.4 mg/kg in piglets 6–8 days old (20), but have not been described in neonatal piglets following intramuscular administration. Ketoprofen plasma pharmacokinetics parameters were reported following an intramuscular dose of 6.6 mg/kg in pigs 11 days-of-age (21), as well as piglets at 6 and 21 days-of-age receiving an intravenous dose of 6.6 mg/kg (22). However, there have been no pharmacokinetic studies of the EU label dose of 3.0 mg/kg in piglets.

Studies have suggested that plasma drug concentrations do not always reflect tissue drug concentrations, particularly for NSAIDs, which may become "trapped" at sites of inflammation (23–25). A minimally invasive technique (*in vivo* ultrafiltration) collects tissue interstitial fluid (ISF) over time (25, 26). ISF allows the measurement of only the pharmacologically active, protein-unbound drug concentrations, critical to assess drug concentrations directly at the tissue level. It is likely that drug concentrations measured in interstitial fluid will more accurately predict clinical outcome and can be used to make predictions regarding provision of analgesia. The tissue pharmacokinetics of meloxicam were described following an intravenous dose of 0.4 mg/kg in 16–23 day old piglets using a carrageenan-sponge model of acute inflammation (19). However, sampling of tissue fluid using this method collects both protein-bound and unbound drug, which is not representative of the pharmacologically active drug fraction (25). NSAIDs are bound extensively to plasma proteins (generally >95%) (23). As they are weakly acidic drugs, they primarily bind to albumin, which is found in the interstitial space and in sites of inflammation.

Protein-bound NSAIDs will become unbound, at which point they can exert their anti-inflammatory activity. The aim of the ultrafiltration probes is to quantify only the protein-unbound portion of each NSAID, as it is anticipated that it will be different from protein-bound, as well as plasma, concentrations. A transudate fluid is generally lower in protein and inflammation, when compared with an exudate, so ultrafiltration probes are likely describing pharmacokinetics in uninflamed transudate, which is likely different to inflamed exudate (27, 28). To date, there are no data available describing the protein-unbound tissue pharmacokinetics of meloxicam, flunixin, or ketoprofen in neonatal piglets, or plasma pharmacokinetic data of these drugs at EU labeled doses in the target age piglet.

This study aimed to assess the plasma and tissue pharmacokinetics of three NSAIDs; meloxicam, flunixin and ketoprofen, and utilized a novel LC-MS/MS method for the enantioselective quantification of ketoprofen in a small sample volume with no derivatization required.

3.3 Materials and Methods

3.3.1 Animals

Twenty-four Yorkshire/Landrace cross, uncastrated, male piglets from 12 different litters, of 6 ± 1 days of age and weighing 1.92–3.22 kg at the time of dosing, were enrolled as part of a larger study. The piglets were sourced from the North Carolina State University Swine Education Unit and transferred to the North Carolina State University College of Veterinary Medicine where they were housed individually, but able to see one another. Lighting consisted of 12/12 h light/dark, and heat lamps were positioned above the piglets on one end of the individual pens. Ambient room temperature was maintained at 26–30 degrees Celsius. Once removed from the sow, piglets were

fed non-medicated swine milk replacer (Milk Specialties Global, Eden Prairie, MN, USA) and offered fresh water every 4 h from 7 a.m. to 12 a.m.

3.3.2 Catheter and Interstitial Probe Placement

At 4 days-of-age (±1 day), piglets were removed from the sow and moved to individual housing to prevent damage to sampling apparatus. Piglets were anesthetized using sevoflurane (SevoFlo®, Zoetis, Parsippany, NJ) administered in 100% oxygen via face mask. An indwelling jugular catheter (22 Ga, 10 cm small animal long term venous catheterization kit, MILA International, Inc., Florence, KY, USA) was used for collection of blood samples. The catheter was placed percutaneously in the jugular vein using a Seldinger technique similar to previously described Flournoy and Mani (29). The catheter was sutured to the skin near the entry point and covered with a small piece of foam to protect the catheter. An extension set was attached to the catheter and then the neck was wrapped with Ioban to secure it. A small, handmade “pouch” was created using bandage tape and attached to the Ioban at the back of the piglet's neck to store the end of the catheter and allow easy access for sample collection. At the time of IV catheter placement, an ultrafiltration probe (RUF-3-12 Reinforced *In Vivo* Ultrafiltration Sampling Probes, BASi systems, W. LaFayette, IN, USA) was placed subcutaneously along the epaxial muscles using a previously described technique (26, 30). The interstitial probe allowed for continuous collection of interstitial fluid (ISF). Piglets were able to recover for 36–48 h following the placement of instrumentation. During this recovery period, patency of the catheter was maintained by removing the heparin lock, flushing the catheter with saline and replacing the heparin lock every 12 h.

3.3.3 Drug Administration

At 6 days of age (±1 day) piglets were injected intramuscularly with one of 3 treatments; 0.4 mg/kg meloxicam (Meloxicam solution for injection 5 mg/mL, Putney, Inc., Portland, ME, USA), 2.2 mg/kg flunixin meglumine (Banamine-S®, Merck Animal Health, Summit, NJ, USA) or 3 mg/kg ketoprofen (Ketofen®, Zoetis, Inc., Kalamazoo, MI, USA). Treatment groups were assigned using a random number generator (Microsoft Excel 2016, Microsoft Corporation). The doses were chosen based on existing EU labels for piglets at castration (meloxicam and ketoprofen) or existing USA label dose for other indications in pigs (flunixin). Two hours after drug administration, the piglets were processed (defined in this study as only castrated and tail-docked). Piglets were restrained to expose the anogenital region of the piglet, while a second person performed the procedure. An incision was made on each side of the scrotum using a scalpel, the testicles were pulled from the surrounding tissue and the scalpel was used to cut the testicles free. The tail was then docked using standard tail clippers. Both the castration site and tail were sprayed with betadine to disinfect the wounds.

3.3.4 Sample Collection

Blood samples (1 mL) were collected and transferred into lithium heparin tubes via the jugular catheter at 0 (baseline), 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36, and 48 h after drug administration. Blood samples were centrifuged at 3,500 × g and the plasma collected for analysis of total drug concentrations. Interstitial fluid samples were collected via the preplaced collection probes at 0 (baseline), 2, 4, 6, 8, 12, 24, 36, and 48 h post-dose and weighed to determine the volume collected. At the end of the experiment, the ISF probe was removed and the tubing length measured. A lag time for ISF collection was calculated based on the length of the probe and the rate of fluid collection from each probe. The ultrafiltration probes are 42.5 cm in length (before

cutting to a custom length) and hold 70 µL of fluid, and the custom lengths for each animal were accounted for in the lag time calculation. Interstitial fluid was used to quantify the free (protein unbound/pharmacologically active portion) drug concentrations in the tissues. Both plasma and ISF were frozen at −80°C until analysis.

3.3.5 Analytical Methods

Different analytical methods were used for plasma and ISF due to the small volume of ISF samples. Methods for ISF were developed after the plasma analysis was already completed. For all analytical methods, validation standards were prepared over a linear range for each drug in each matrix (meloxicam, flunixin, and ketoprofen in plasma and ISF) and were used to construct calibration curves. All calibration curves were linear with a R^2 value of 0.99 or higher. Limit of quantification, inter-day accuracy, and inter-day precision are presented in Table 3.1 for each analytical method.

Table 3.1. Analytical standard concentration range, limit of quantification (LOQ), inter-day accuracy (%) and inter-day precision (%) for analytical methods.

Drug	Matrix	Concentration Range	LOQ	Accuracy (%)	Precision (%)
		µg/mL	µg/mL	Mean ±SD	Mean ±SD
Meloxicam	Plasma	0.01 – 10	0.01	99 ±6	8 ±3
	ISF	0.001 – 0.05	0.001	99 ±10	2 ±2
Flunixin	Plasma	0.0005 – 5	0.0005	103 ±7	8 ±5
	ISF	0.0005 – 0.2	0.0005	100 ±8	3 ±2
S(+)-Ketoprofen	Plasma	0.05 – 10	0.05	101 ±4	7 ±5
	ISF	0.001 – 0.5	0.001	101 ±11	7 ±6
R(-)-Ketoprofen	Plasma	0.05 – 10	0.05	100 ±3	7 ±5
	ISF	0.001 – 0.5	0.001	107 ±6	6 ±3

SD; standard deviation**.**

Meloxicam Plasma Analysis

Plasma concentrations of meloxicam were determined using high-performance liquid chromatography (HPLC; 1260 Infinity HPLC system with a multiwavelength detector, Agilent Technologies, Wilmington, DE, USA). The UV detector was set at a wavelength of 365 nm. The column was a 4.6 × 150 mm C18 column (Zorbax SB-C18; MAC-MODAnalytical, Inc., Chadds Ford, PA, USA) kept at a constant temperature of 40°C, and a flow rate of 1 mL/min. Mobile phase consisted of 60% 0.05M sodium acetate buffer (pH 3.75) and 40% acetonitrile (ACN). Meloxicam plasma samples, calibration samples, and blank (control) were prepared using solid phase extraction (1cc Waters Oasis Extraction Cartridges, Waters Corporation, Milford, MA, USA), conditioned with 1 mL methanol followed by 1 mL distilled water. A plasma sample (200 µL) was added to a conditioned cartridge, washed with 1 mL water: methanol (95:5 v/v), and then eluted with 1 mL 100% methanol. Samples were then evaporated at 40°C for 15–20 min. Each sample was then reconstituted with 200 µL of mobile phase and vortexed. Twenty-five microliters were then injected into the HPLC system.

Ketoprofen Plasma Analysis

Plasma concentrations of ketoprofen were analyzed using the same HPLC system as the meloxicam plasma samples. For ketoprofen, the UV detector was set at a wavelength of 255 nm. The column was a 4.6 × 150 mm, 5 µm chiral column (Agilent Ultron ES-OVM; Agilent Technologies), kept at 25°C. Mobile phase consisted of 89% 0.02 M potassium monobasic phosphate buffer and 11% ACN. Ketoprofen plasma samples, calibration samples, and blank (control) were prepared using solid phase extraction (3cc Waters Oasis Extraction Cartridges, Waters), conditioned with 1 mL methanol followed by 1 mL distilled water. A plasma sample (200 µL) was added to a conditioned cartridge, washed with 1 mL water: ammonium hydroxide (95:5

v/v), and then eluted with 1 mL methanol:formic acid (98:2). Samples were then evaporated at 30°C for 20–30 min. Each sample was then reconstituted with 200 µL of water and vortexed. Thirty microliters were then injected into the HPLC system. Standards spiked with S(–)-ketoprofen only, were also analyzed at the same time to determine the retention time, allowing separate identification of the S(+)- and R(-)-enantiomer.

Flunixin Plasma Analysis

Flunixin plasma concentrations were quantified by ultra-high-pressure liquid chromatography (UPLC) with mass spectrometric (MS/MS) detection (Waters Corp., Milford, MA, USA). The UPLC-MS/MS system consisted of a Xevo TQD tandem quadrupole mass spectrometer (Waters Corp.) For all flunixin sample matrices (plasma and ISF), the UPLC-MS/MS analysis consisted of a 2.1 × 100 mm, 1.8 um HSS T3 column (Waters Corp.) A gradient was used, and the initial mobile phase was 0.1% formic acid in water: 0.1% formic acid in acetonitrile (70:30 v/v) with a flow rate of 0.4 mL/min for the first 2.5 min. The mobile phase then switched to (10:90 v/v) from 2.5–3.5 min. For the last 1.5 min of the run, the mobile phase was (70:30 v/v). The MS/MS was run in ESI+ mode. The quantification trace used was 297 → 279. Column temperature was 35°C and sample temperature was ambient. Flunixin plasma samples were combined with 250 µL 0.5% citric acid in ACN, vortexed thoroughly and then centrifuged for 10 min at 3,000 × g. The supernatant was collected and evaporated at 55°C for 60 min under an 18-psi stream of air. Each sample was then reconstituted with 100 µL of water:ACN (50:50 v/v) and vortexed, filtered through a 0.2 µm filter and then injected.

Meloxicam ISF Analysis

Meloxicam ISF concentrations were quantified by UPLC-MS/MS (system information as mentioned previously). UPLC-MS/MS analysis consisted of a 2.1 × 50 mm, 1.7 µm Waters

Acquity BEH C18 column (Waters Corp.) A gradient was used, and the initial mobile phase was 0.1% formic acid in water: 0.1% formic acid in acetonitrile (65:35 v/v) with a flow rate of 0.4 mL/min for the first minute. The mobile phase then switched to (10:90 v/v) from 1.0 to 1.1 min. For the last 1.9 min of the run, the mobile phase was (65:35 v/v). The MS/MS was run in ESI+ mode. The quantification trace used was 352.043 → 115. Column temperature was 35°C and sample temperature was 10°C. Fifteen microliters of ISF were combined with 50 µL MeOH, filtered through a 0.2 µm syringe filter and then injected directly onto the chromatography system.

Flunixin ISF Analysis

Flunixin ISF samples were analyzed using the same UPLC-MS/MS system as previously described and using the same analytical method as for flunixin in plasma. The flunixin ISF sample preparation was the same as described for meloxicam ISF.

Ketoprofen ISF Analysis

Ketoprofen ISF samples were analyzed using the same UPLC system as previously described. The samples were prepared using solid phase extraction. OASIS HLB µElution Plates (Waters, Milford, MA, USA) were used. These were conditioned sequentially with 500 µL of methanol and 500 µL of ultrapure water. Fifty microliters (50 µL) of ISF were loaded into the plate. The loaded plates were washed with 50 µL of 90:10 water: methanol (v/v). Then, the retained ketoprofen was eluted with a total of 50 µL of 80:20 water: acetonitrile (v/v, eluted twice with 25 µL). Five microliters (5 µL) of the eluted solution was directly injected in the UPLC-MS/MS.

The UPLC-MS/MS analysis consisted of a 100 × 3.0 mm, 1.6 µm Chiralpak® IG-U column (Chiral Technologies, Inc., West Chester, PA, USA). A gradient was used, and the initial mobile phase was 0.1% formic acid in water: 0.1% formic acid in methanol (21:79 v/v) with a flow rate of 0.35 mL/min for the first 5.5 min. The mobile phase then switched to (5:95 v/v) from 5.5 to 7.0

min. For the last 2.0 min of the run, the mobile phase was (21:79 v/v). The MS/MS was run in ESI+ mode. The quantification trace used was 255.19 → 104.943, and the R(-)- and S(+)-ketoprofen enantiomers were separated by retention time. Column temperature was 25°C and sample temperature was 15°C.

3.3.6 Pharmacokinetic Analysis

Pharmacokinetic analysis of drug concentration vs. time profiles was performed with Phoenix WinNonLin software (version 8.0; Certara, Princeton, NJ, USA). A non-compartmental analysis was used to derive the mean residence time (MRT; h), slope of the terminal phase (λz; 1/h), and the half-life ($T_{1/2}$; h). The area under the plasma concentration–time curve from time zero to infinity (AUC0→ ∞; h × μg/mL) was calculated by the linear trapezoidal rule. The volume of distribution (per fraction absorbed) (Vd/F; L/kg) and clearance per fraction absorbed (Cl/F; L/h/kg) were also determined and values for maximum concentration (Cmax; μg/mL) and time to maximum concentration (Tmax; h) were taken directly from the data.

3.4 Results

3.4.1 Meloxicam

Mean plasma and ISF meloxicam concentrations over time following a single IM injection of 0.4 mg/kg are presented in Figure 3.1. Parameters describing the plasma pharmacokinetics of meloxicam following a single IM injection are presented in Table 3.2. Parameters describing the disposition of meloxicam in ISF are presented in Table 3.3. Meloxicam concentrations in plasma fell below the LOQ of 0.01 μg/mL after 36 h in all piglets. Meloxicam was still detected above the LOQ of 0.001 μg/mL in ISF at the end of the study (48 h). The plasma pharmacokinetics of meloxicam after IM administration were characterized by rapid absorption and a brief apparent

terminal half-life (4.46 ± 1.52 h), compared to ISF in which meloxicam persisted for a longer time (apparent half-life 11.26 ± 4.15 h).

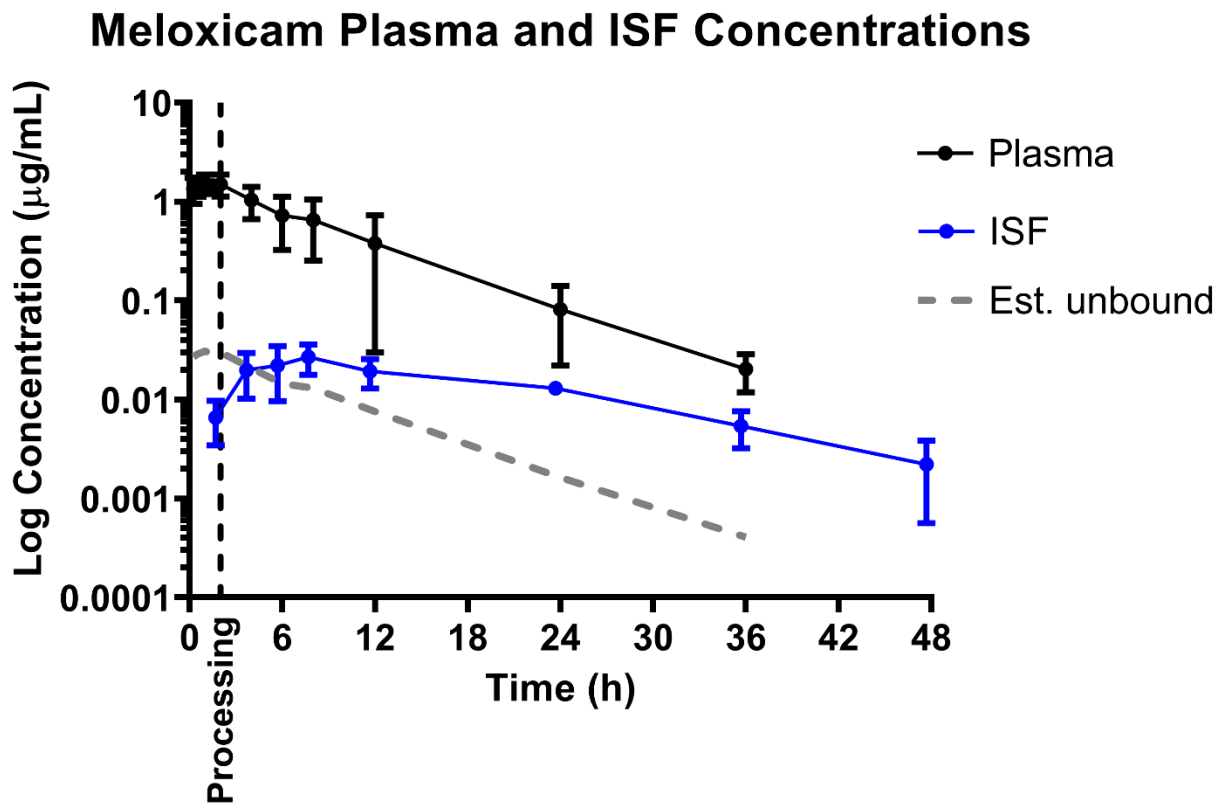

Figure 3.1. Total plasma concentrations and free/unbound ISF concentration over time following intramuscular injection of 0.4 mg/kg meloxicam in 6-day-old piglets*. ISF plotted with lag time (0.31h). Dose was administered at 0h and processing (castration and tail-docking) was performed at 2h, as indicated by the vertical dotted line. Data are represented as mean ± standard deviation. Est. unbound represents the percentage of the total plasma concentration that is estimated to be free or unbound from plasma proteins, assuming meloxicam is normally 98% protein bound (31).*

3.4.2 Flunixin

Mean plasma and ISF flunixin concentrations over time following a single IM injection of 2.2 mg/kg are presented in Figure 3.2. Parameters describing the plasma pharmacokinetics of flunixin following a single IM injection are presented in Table 3.2. Parameters describing the disposition of flunixin in ISF are presented in Table 3.3. Flunixin concentrations in both plasma and ISF were still detected above the LOQ of 0.0005 μg/mL at the end of the study (48 h) in all piglets. The plasma pharmacokinetics of flunixin after IM administration were characterized by rapid absorption, large volume of distribution/F (0.92 ± 0.21 L/kg) and an apparent terminal half-life (7.93 ±2.91 h) which was short compared to ISF in which flunixin persisted for a longer time (apparent half-life 16.34 ± 7.09 h).

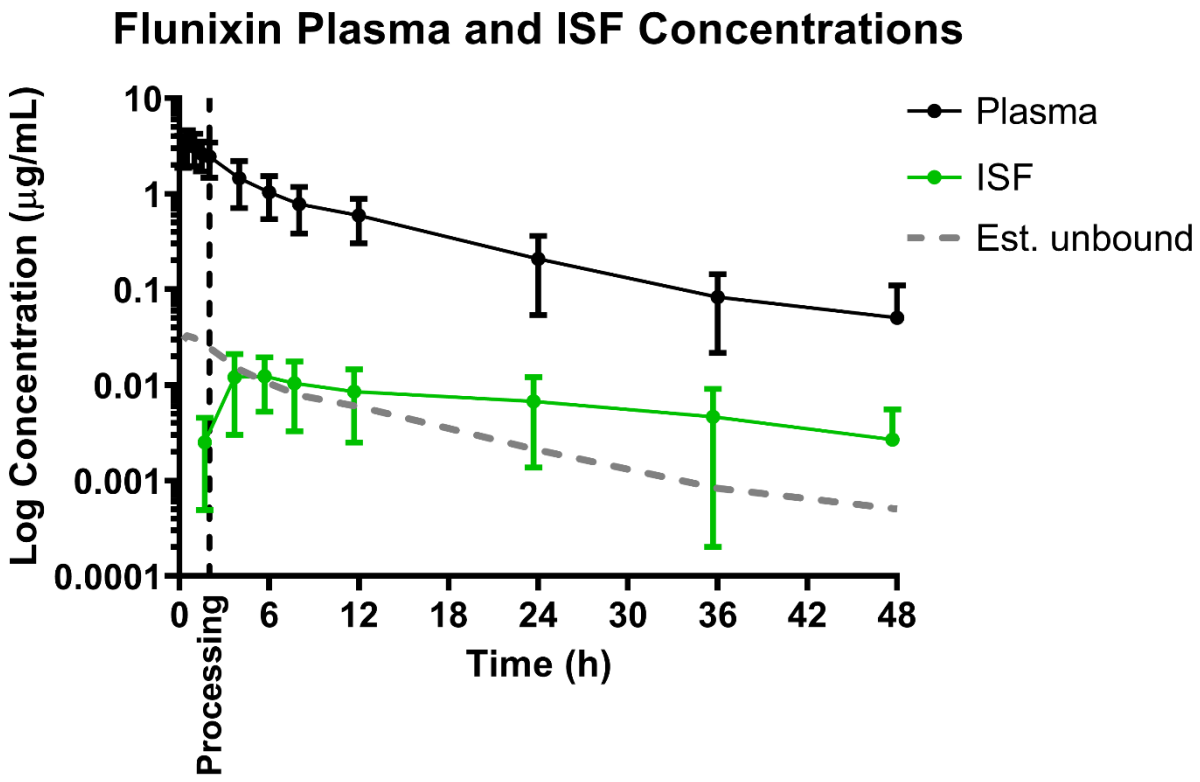

Figure 3.2. Total plasma concentrations and free/unbound ISF concentration over time following intramuscular injection of 2.2 mg/kg flunixin in 6-day-old piglets*. ISF plotted with lag time (0.31h). Dose was administered at 0h and processing (castration and tail-docking) was performed at 2h, as indicated by the vertical dotted line. Data are represented as mean ± standard deviation. Est. unbound represents the percentage of the total plasma concentration that is estimated to be free or unbound from plasma proteins, assuming flunixin is normally 99% protein bound (32).*

3.4.3 Ketoprofen

Mean plasma and ISF ketoprofen concentrations over time following a single IM injection of 3.0 mg/kg are presented in Figure 3.1. Parameters describing the plasma pharmacokinetics of ketoprofen following a single IM injection are presented in Table 3.2. Parameters describing the disposition of ketoprofen in ISF are presented in Table 3.3. Ketoprofen concentrations in plasma

fell below the LOQ of 0.05 μg/mL after 24 h in all piglets for both enantiomers. S(+)- and R(-)-ketoprofen were still detected above the LOQ of 0.001 μg/mL in ISF at the end of the study (48 h). The plasma pharmacokinetics of S(+)-ketoprofen after IM administration were characterized by rapid absorption and a short apparent terminal half-life (3.50 ± 0.80 h), as well as a relatively short apparent half-life in ISF (5.54 ± 0.99 h). Unfortunately, pharmacokinetic parameters could not be calculated for R(-)-ketoprofen. However, the plasma pharmacokinetics were characterized by rapid decrease in concentration in a short amount of time [R(-)-ketoprofen was last detected at 4 h after administration of the dose], compared to ISF in which the concentration of R(-)-ketoprofen persisted for much longer.

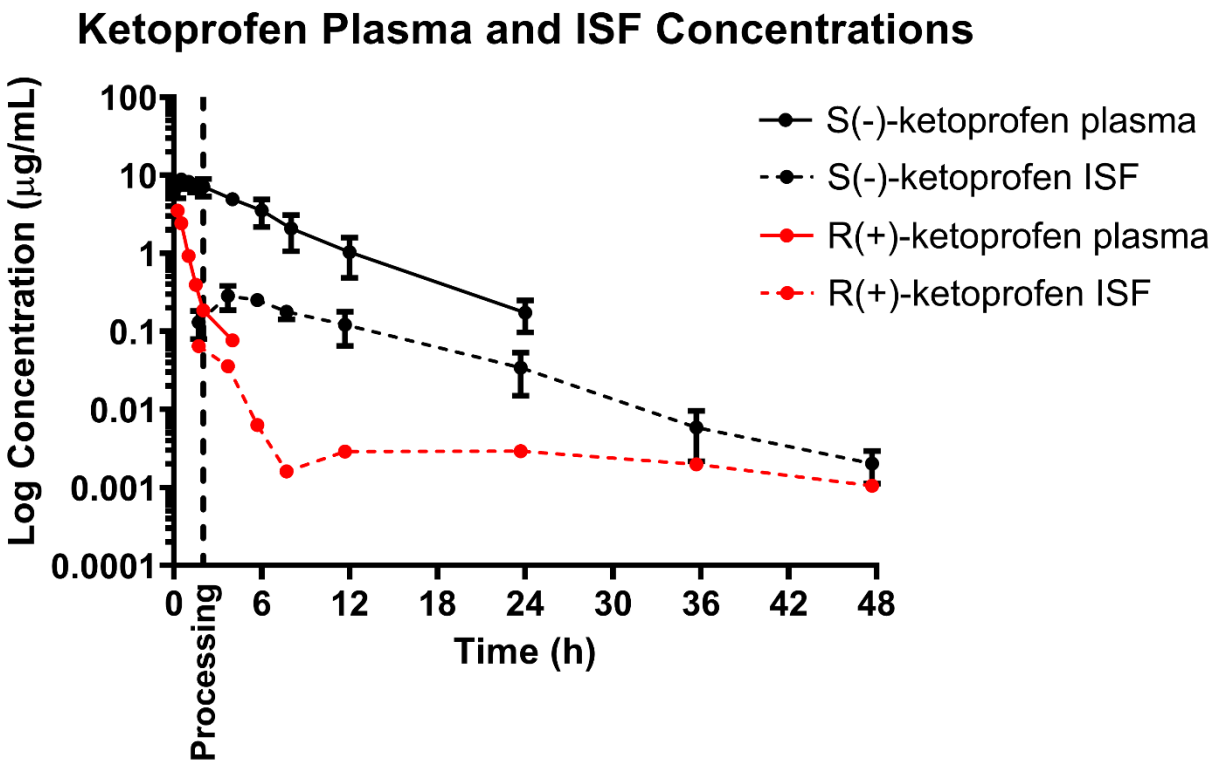

Figure 3.3. Total plasma concentration over time following intramuscular injection of 3 mg/kg ketoprofen in 6-day-old piglets. *Both the S(+)- and R(-)-enantiomers of ketoprofen are shown. Dose was administered at 0h and processing (castration and tail-docking) was performed at 2h, as indicated on the plot by a vertical dotted line. Data are represented as mean ± standard deviation. Protein binding has not been measured for the separate enantiomers so estimated unbound concentration in the plasma has not been shown in this figure.*

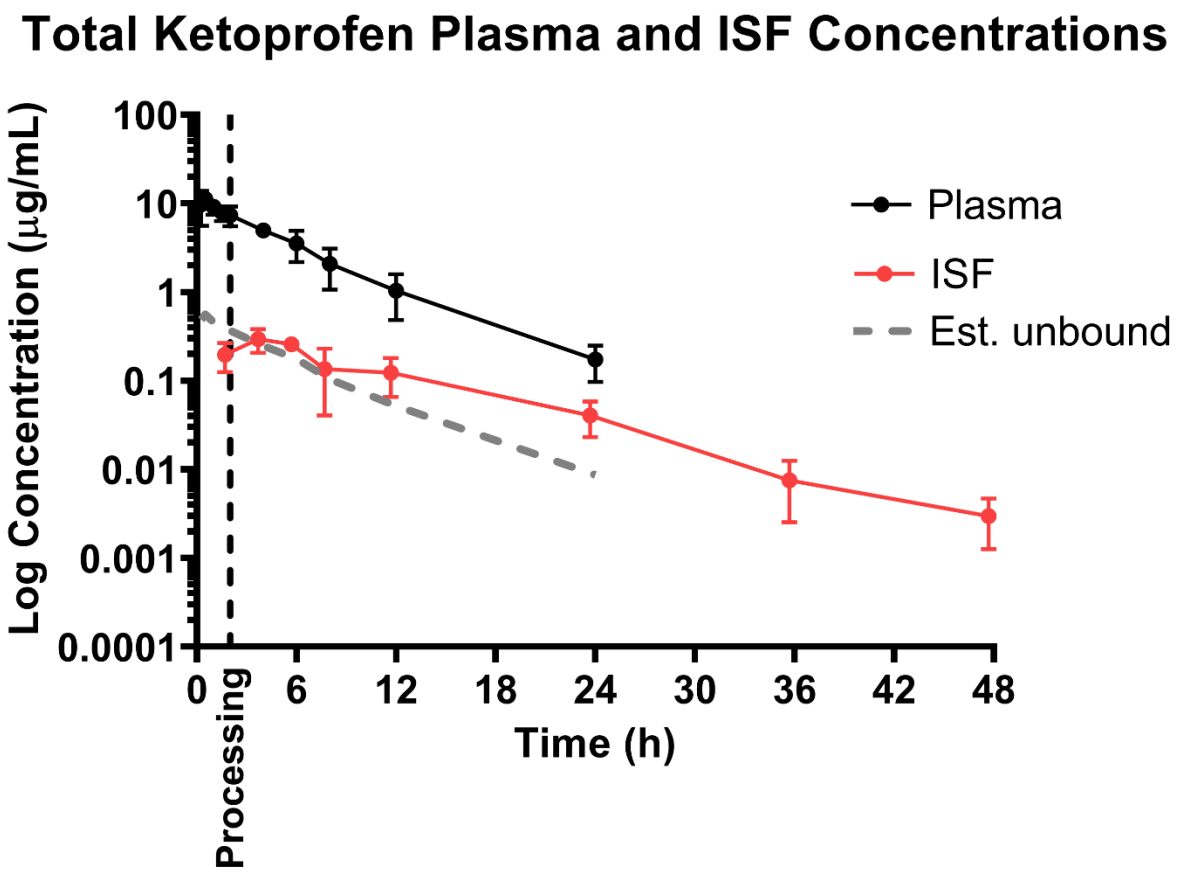

Figure 3.4. Total ketoprofen plasma concentration over time following intramuscular injection of 3 mg/kg ketoprofen in 6-day-old piglets [sum of both S(+)- and R(-)-ketoprofen]. *Dose was administered at 0h and processing (castration and tail-docking) was performed at 2h, as indicated on the plot by a vertical dotted line. Data are represented as mean ± standard deviation, and total ketoprofen was calculated by addition of the two ketoprofen enantiomers. Est. unbound represents the percentage of the total plasma concentration that is estimated to be free or unbound from plasma proteins, assuming ketoprofen is normally 95% protein bound (36).*

Table 3.2. Non-compartmental plasma pharmacokinetic parameters following intramuscular administration of NSAIDs (meloxicam 0.4 mg/kg, flunixin 2.2 mg/kg and ketoprofen 3 mg/kg) to 6-day-old piglets. Data are shown as mean (standard deviation).

Parameter	Units	Meloxicam	Flunixin	S(+)-Ketoprofen
Dose	mg/kg	0.40	2.20	3.00
MRT	h	5.80 (2.23)	9.77 (3.64)	5.11 (1.19)
$T_{1/2}$	h	4.46 (1.52)	7.93 (2.91)	3.50 (0.80)
λ_z	1/h	0.18 (0.09)	0.10 (0.04)	0.21 (0.05)
T_{max}	h	1.21 (0.68)	0.85 (0.70)	0.59 (0.27)
C_{max}	µg/mL	1.58 (0.34)	3.94 (0.86)	9.13 (1.75)
AUC_{last}	h.µg/mL	10.34 (3.97)	27.25 (9.06)	52.26 (14.61)
AUC_{inf}	h.µg/mL	10.75 (4.41)	28.06 (9.91)	53.74 (14.79)
AUC_{extrap}	%	3.75 (4.41)	2.35 (2.91)	2.82 (2.30)
Vd/F	L/kg	0.24 (0.04)	0.92 (0.21)	0.29 (0.04)
Cl/F	L/h/kg	0.04 (0.02)	0.09 (0.03)	0.06 (0.02)

Table 3.3. Non-compartmental ISF pharmacokinetic parameters following intramuscular administration of NSAIDs (meloxicam 0.4 mg/kg, flunixin 2.2 mg/kg or ketoprofen 3.0 mg/kg) to 6-day-old piglets. Data are shown as mean (standard deviation).

Parameter	Units	Meloxicam	Flunixin	S(+)-Ketoprofen
Dose	mg/kg	0.40	2.20	3.00
MRT	h	16.23 (5.00)	24.24 (9.98)	8.57 (2.05)
$T_{1/2}$	h	11.26 (4.15)	16.34 (7.09)	5.54 (0.99)
T_{max}	h	4.19 (1.00)	5.02 (1.63)	4.36 (1.03)
C_{max}	µg/mL	0.032 (0.004)	0.024 (0.009)	0.300 (0.079)
AUC_{last}	h.µg/mL	0.537 (0.240)	0.476 (0.211)	3.058 (0.669)
AUC_{inf}	h.µg/mL	0.613 (0.216)	0.577 (0.295)	3.081 (0.672)
AUC_{extrap}	%	15.86 (16.41)	14.18 (10.07)	0.73 (0.72)

3.5 Discussion

Following intramuscular administration of 0.4 mg/kg meloxicam, the peak plasma concentration was reached in 1.21 h, which was delayed compared to the previously reported 0.50 h in 8-day-old piglets given an intramuscular dose of 1 mg/kg (17). However, the apparent terminal half-life was comparable to the previous report [4.46 h in this study compared to 3.94 h; (17)]. Both of these values in piglets are shorter than 6.15 h, which is the terminal half-life reported in mature sows following an intravenous dose of 0.5 mg/kg (33). While these studies used different doses and routes of administration, this difference in half-life may suggest that drug elimination in piglets may be more rapid than that of mature pigs, which could be clinically important in the duration of analgesia provided post-processing. However, given that terminal half-life is a hybrid parameter that incorporates both volume of distribution and clearance, mechanisms for differences in plasma terminal half-life remain unknown at this time. Typically, the neonate has reduced clearance of many drugs compared with older individuals largely due to the greater body water content leading to a higher volume of distribution, a larger fraction of body mass that consists of highly perfused tissues, a lower plasma concentration of proteins that bind drugs and incomplete maturation of their hepatic-enzymes systems. Differences in sampling and analytical methodologies between studies could account for some of these differences, but without a direct comparison, it is unclear.

Following intramuscular administration of 2.2 mg/kg flunixin, the peak plasma concentration was reached in 0.85 h, which is comparable to the previously reported Tmax of 0.61 h following intramuscular administration to gilts (34). The apparent terminal half-life was also comparable [7.93 h in this study compared to 7.49 h (34)]. However, both of these were longer than previously reported in 10-day-old piglets following intravenous administration of 2.2 and 4.4

mg/kg flunixin [4.82 and 5.15 h, respectively (20)]. This is likely due to the different route of administration, as absorption continues following intramuscular administration while elimination is occurring, compared to intravenous that does not require absorption.

Following intramuscular administration of 3.0 mg/kg racemic ketoprofen, the peak plasma concentration of S(+)-ketoprofen was reached in 0.59 h, which was the most rapid of the NSAIDs investigated in this study, and was comparable to the previously reported Tmax of 0.68 h in 11 day old piglets given an intramuscular dose of 6.6 mg/kg (21). The apparent terminal half-life was almost identical to that previously reported [3.50 h in this study compared to 3.51 h (21)], and was similar to reports following intravenous doses of 6.6 mg/kg in piglets 6 and 21 days-of-age [3.4 and 3.3 h, respectively (22)]. The plasma concentration-time profile was very similar to that of 11-day-old piglets given 6.6 mg/kg IM, including R(-)-ketoprofen which rapidly decreased in concentration and was last detected by 4 h after administration of the dose in both studies.

Volume of distribution per fraction absorbed (Vd/F) in this study for meloxicam was comparable to that of other studies investigating piglets 8–23 days of age, given doses in the range of 0.4–1.0 mg/kg and given via intramuscular or intravenous routes of administration (17, 19, 20). S(–)-ketoprofen also had a comparable Vd/F (0.29 L/kg) compared to previous reports following intramuscular administration of 6.6 mg/kg in piglets 8–17 days-of-age [0.30 L/kg (21)]. There are no reports of volume of distribution/F following an intramuscular dose for flunixin, but the value reported in the present study (0.92 L/kg) is greater than that previously reported following IV doses in 10-day-old piglets [0.25 L/kg and 0.26 L/kg, (20)]. However, it is also much lower than that reported in juvenile pigs (18–27 kg body weight) following IV dose [1.83 L/kg, (32)]. In gilts, bioavailability of flunixin has been reported at 76% following an intramuscular dose of 2.2 mg/kg compared to an IV dose of the same amount (34), but there have been no bioavailability studies in

piglets. These differences could be due to differences in the route of administration (volume of distribution at steady state compared to volume of distribution not corrected for bioavailability), differences in early sampling time points or modeling methods, and/or differences in total body water composition as a result of age differences. Interestingly, flunixin had the longest apparent terminal half-life of the three NSAIDs studied, despite also displaying the highest clearance/F. This demonstrates the effect of volume of distribution on half-life, as flunixin also demonstrated the largest Vd/F in this study, which may indicate greater tissue penetration and presence at the site of action, although this cannot be confirmed without bioavailability data for these NSAIDs in this population of piglets.

Overall, plasma NSAID concentrations did not predict or reflect the tissue concentration data. For example, the time to maximum concentration and half-life were longer in the tissues as demonstrated by the ISF data. Although maximum concentrations were lower in the tissues, these data tentatively reflect only plasma unbound drug concentrations. These plasma unbound concentrations are more pharmacodynamically relevant, and are expected to be lower, because most NSAIDs are highly protein bound (generally > 95%) (23).

Based on the tissue (ISF) pharmacokinetic data, administration of each NSAID 2 h prior to castration and tail-docking is preferred over administering administer these drugs immediately prior to processing, as maximum tissue concentrations were achieved within 4–5 h of administration. NSAIDs are highly protein bound in the plasma, so tissue concentrations of drugs are more likely to be representative of the effective concentration at the site of action. As can be seen in Figures 3.1–3, tissue concentrations of both meloxicam, flunixin and S(–)-ketoprofen were detected at the last time point assessed (48 h). These results are different from those previously reported for meloxicam tissue exudate, which reported concentrations only to 12 h (19). Higher

concentrations were reported, which is likely reflective of the sampling methodology which was a tissue cage vs. an ultrafiltration probe. However, care should be taken when comparing pharmacokinetic parameters or concentrations between plasma and ISF in this study, due to the different limits of quantification for each assay. This is because new methods for ISF were developed due to the small sample volume, and these methods were developed after the plasma analysis was already completed.

The ability to study the distribution and anti-inflammatory effects of NSAIDs directly at sites of action (in this case, at the tissue level) can improve understanding of drug effects and allow the application of appropriate dosage regimens (23, 25, 35). Specifically, this knowledge is important when assessing NSAIDs, as plasma drug concentrations have not been correlated with therapeutic efficacy (24). Interstitial fluid can be analyzed for only the pharmacologically active, protein-unbound drug concentrations directly at the tissue level. In addition, unbound drug concentrations can be correlated with objective biomarkers of inflammation in the future, such as prostaglandin E2, thereby establishing therapeutic drug concentrations directly at the effect site. As seen in Figures 3.1, 3.2, and 3.4, the concentrations of estimated unbound plasma concentrations (dotted line) do not reflect those seen at the tissue level (ISF). Specifically, tissue depletion of NSAIDs tends to be slower. Therefore, measuring unbound concentrations found in plasma may not be appropriate to estimate the pharmacodynamic effects of NSAIDs, and unbound tissue concentrations may correlate better with therapeutic efficacy.

It is additionally important to consider the stereoselective pharmacokinetics of ketoprofen, rather than simply total ketoprofen concentration, as plasma concentration and pharmacodynamic effect of each enantiomer differs between species (37). Previous studies in pigs have reported that the S(–)-enantiomer is a more potent cyclooxygenase-inhibitor, and therefore displays greater anti-

inflammatory effects compared to the R(-)-enantiomer (22, 38). However, the R(-)-ketoprofen enantiomer may be a more potent analgesic according to a study assessing mechanical nociceptive threshold (21). In many species, S(+)-ketoprofen predominates over R(-)-ketoprofen in terms of plasma exposure following intramuscular administration of racemic ketoprofen (22, 37–39). The current study also found this to be true in piglets, and additionally found that this was also true for interstitial fluid exposure. Further investigation of the activity of the S(+)- and R(-)-enantiomers will attempt to elucidate the anti-inflammatory and analgesic effect in relation to both the plasma and tissue pharmacokinetics.

When making comparisons between pharmacokinetic parameters of each NSAID, care should be taken as these were given at different doses. However, this information is still important as the doses given were clinically relevant doses that are already being used in practice in the EU, USA or Canada. Secondly, a limitation of the study lies in the comparisons of volume of distribution and clearance are on the basis of "per fraction absorbed," as the drugs were administered intramuscularly and bioavailability in this age piglet is unknown.

This study was the first to describe both the plasma and tissue pharmacokinetics of each NSAID in the intended population of animals: 6-day old piglets undergoing surgical castration and tail docking. The plasma pharmacokinetic results are comparable to previous reports on pharmacokinetics of meloxicam, flunixin and ketoprofen in piglets of similar age or older, although across studies the routes of administration, doses, and methods of pharmacokinetic analysis differ slightly. This study is the first to report on the tissue pharmacokinetics of each of these drugs in piglets, using a novel, minimally invasive sampling technique of *in vivo* ultrafiltration, and demonstrated not only the feasibility of this technique in neonatal piglets for the first time, but the differences in tissue pharmacokinetics compared to plasma

pharmacokinetics for each NSAID. Future studies are currently aimed at establishing a relationship between the ISF concentration-time profiles with pain alleviation.

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CHAPTER 4

Plasma, Urine and Tissue Concentrations of Flunixin and Meloxicam in Pigs

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An erratum is being submitted to the journal regarding interstitial fluid lag times.

4.1 Abstract

Background

The objective of this study was to determine the renal clearance of flunixin and meloxicam in pigs and compare plasma and urine concentrations and tissue residues. Urine clearance is important for livestock show animals where urine is routinely tested for these drugs. Fourteen Yorkshire/Landrace cross pigs were housed in individual metabolism cages to facilitate urine collection. This is a unique feature of this study compared to other reports. Animals received either 2.2 mg/kg flunixin or 0.4 mg/kg meloxicam via intramuscular injection and samples analyzed by mass spectrometry. Pigs were euthanized when drugs were no longer detected in urine and liver and kidneys were collected to quantify residues.

Results

Drug levels in urine reached peak concentrations between 4 and 8 h post-dose for both flunixin and meloxicam. Flunixin urine concentrations were higher than maximum levels in plasma. Urine concentrations for flunixin and meloxicam were last detected above the limit of quantification at 120 h and 48 h, respectively. The renal clearance of flunixin and meloxicam was 4.72 ± 2.98 mL/h/kg and 0.16 ± 0.04 mL/h/kg, respectively. Mean apparent elimination half-life in plasma was 5.00 ± 1.89 h and 3.22 ± 1.52 h for flunixin and meloxicam, respectively. Six of seven pigs had detectable liver concentrations of flunixin (range 0.0001–0.0012 µg/g) following negative urine samples at 96 and 168 h, however all samples at 168 h were below the FDA tolerance level (0.03 µg/g). Meloxicam was detected in a single liver sample (0.0054 µg/g) at 72 h but was below the EU MRL (0.065 µg/g).

Conclusions

These data suggest that pigs given a single intramuscular dose of meloxicam at 0.4 mg/kg or flunixin at 2.2 mg/kg are likely to have detectable levels of the parent drug in urine up to 2 days and 5 days, respectively, after the first dose, but unlikely to have tissue residues above the US FDA tolerance or EU MRL following negative urine testing. This information will assist veterinarians in the therapeutic use of these drugs prior to livestock shows and also inform livestock show authorities involved in testing for these substances.

4.2 Introduction

Livestock show authorities drug test the urine in show animals in order to ensure fair competition. One of the groups of drugs of importance in this setting is nonsteroidal anti-inflammatory drugs (NSAIDs), which may be used to conceal an injury that would otherwise prevent a show animal from competing. Show animals are also sold soon after a livestock show competition, and there has always been the assumption that if the urine is clear of the drug, then the organs responsible for drug clearance will be free of drug residues. While, urine concentrations may correlate with plasma concentrations, using urine concentrations to assess potential tissue residues could lead to inaccurate predictions of target tissue concentrations and can result in violative drug residues [1,2,3,4].

There is only one US FDA-approved NSAID for use in pigs: flunixin meglumine for the control of pyrexia associated with swine respiratory disease. Another commonly prescribed NSAID, meloxicam, is not approved for use in the US in pigs, but is in the EU and Canada. The FDA has an established tolerance for flunixin in pigs, which is 30 part per billion (ppb) in liver (the target tissue) and 25 ppb in muscle [5]. No tolerance has been established for meloxicam in pigs, and therefore detection of any tissue residue would be considered a violation by USDA Food Safety and Inspection Service (FSIS).

There are numerous studies describing flunixin plasma and urine concentrations in cattle, goats, horses, camels and dogs [2, 3, 6, 7, 8, 9], as well as pigs [10, 11]. It should also be noted that relationship between plasma and urine has been the background for doping control in horses [8, 12]. Of the two studies that examined flunixin in urine of pigs, only one of those attempted to establish a correlation with tissue concentrations. However, that study used a single spot urine sample taken at necropsy to predict the residue depletion profile in edible tissues. Those results

showed that earlier urine samples (24 h) were highly variable in concentration and are affected by many factors, including the urinary output, voiding intervals, last voiding time, postvoid residual urine volume [10]. The author recommended that future studies should consider using metabolism cages to collect cumulative urine samples to improve the prediction of tissue concentrations. This was a primary goal of our current study. The second study [11] calculated 6.8% of the parent drug was excreted in urine following administration of 2.2 mg/kg flunixin intramuscularly once daily for 3 days in 3 month old pigs. However this study did not assess tissue concentrations of flunixin in pigs. There are far fewer data available regarding meloxicam concentration-time profiles in urine in large animal species, with only two studies in horses and goats [2, 13], and no published studies have assessed the relationship between meloxicam concentrations in plasma, tissues and urine in pigs.

The objective of this study was to compare plasma and interstitial fluid; ISF concentrations to urine concentrations of both meloxicam and flunixin, with a focus on estimating the renal clearance of these parent drugs in pigs which has not been reported before. Show animals are often sold for slaughter soon after a livestock show, therefore, another aim of this project was not to substitute tissue residue testing with urine testing, but to be able to inform owners of show animals that even though the urine may be cleared of the drug during the show, there may or may not be violative tissue residues in their show animals.

4.3 Methods

4.3.1 Animals and housing

North Carolina State University Institutional Animal Care and Use Committee approved this study. All animals were acquired from the North Carolina State University Swine Education Unit and transferred to the North Carolina State University College of Veterinary Medicine, where

they were housed individually in metabolism cages (72^{o}F), with a 12:12 light:dark cycle, fed LabDiet 5084 (LabDiet, St. Louis, MO, USA) twice a day and had access to freshwater *ad libitum*. A total of fourteen healthy, castrated, male Yorkshire/Landrace cross pigs (weighing 23.1–35.4 kg) were enrolled to receive either flunixin or meloxicam (n = 7 per treatment group). There is no hypothesis testing involved in this pharmacokinetics study, and therefore a power analysis is not required for estimating sample size as previously described [**30**]. This study excluded any animals with hernias, diarrhea, lameness or any other clinical signs of disease and inclusion criteria were no prior treatment with flunixin or meloxicam. Pigs appeared healthy on physical examination by lack of any clinical signs. During catheter placement and interstitial fluid probe placement, temperature, heart rate and respiratory rate were monitored, and no abnormalities were noted. Pigs were randomly assigned to metabolism cages at any given trial by individuals not involved in the study. There were three trials consisting of 4 pigs/trial with 2 pigs being treated with flunixin and 2 pigs being treated with meloxicam, and one trial with one pig treated with flunixin and other pig with meloxicam. This allowed us to account for the effect of litter. Investigators were not blinded to sample collection or sample analysis at any stage of the study.

4.3.2 Catheter and Interstitial Probe Placement

Prior to the start of the study, pigs were moved to individual metabolism cages and allowed 4 days of acclimation. After the adjustment period pigs were sedated using an intramuscular injection of a combination of Telazol® (50 mg/mL tiletamine HCl and 50 mg/mL zolazepam HCl), ketamine (100 mg/mL) and xylazine (100 mg/mL) at a concentration of 0.6 mL/kg body weight. Using sterile technique, an 18 Ga x 15 cm catheter (SA1815; Mila International, Inc., Florence, KY, USA) was inserted into the right jugular vein and sutured to the skin using 2 − 0 monofilament suture and an extension attached.

At the time of catheter placement, an ultrafiltration probe (Canine UF Probe, BASi systems, W. LaFayette, IN, USA) was placed subcutaneously along the epaxial muscles using a previously described technique [**31**]. The interstitial probe allowed for continuous collection of interstitial fluid (ISF). Pigs were able to recover for 36–48 hours following the placement of instrumentation. During this recovery period, patency of the catheter was maintained by removing the heparin lock (100 mg/mL), flushing the catheter with saline and replacing the heparin lock every 12 hours.

4.3.3 Drug Administration and Sample Collection

Pigs were administered a single intramuscular dose of either 0.4 mg/kg meloxicam (Meloxicam solution for injection 5 mg/mL, Putney, Inc., Portland, ME, USA), the labeled dose for pigs in Europe, or 2.2 mg/kg flunixin meglumine (Banamine-S®, Merck Animal Health, Summit, NJ, USA), the labeled dose for pigs in the US. The injection location was the neck of the pigs in accordance to the label instructions. Blood samples (3 mL) were collected via the jugular catheter and transferred to lithium heparinized tubes at 0 (baseline), 0.08, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 18, 24, 36, 48, 60, 72, 84, 96, 108, 132, 156 and 180 h post-administration of flunixin or meloxicam. Blood samples were centrifuged at 3500 x g and the plasma collected for analysis of total drug concentrations.

Interstitial fluid samples were collected via the preplaced collection probes at 0 (baseline), 2, 4, 6, 8, 10, 12, 18, 24, 36, 48, 60, 72, 84, 96, 108, 132, 156 and 180 h post-dose and weighed to determine the volume collected. At the end of the experiment, the ISF probe was removed and the tubing length measured. A lag time for the ISF collection was calculated to account for the time taken for the sample to travel along the ISF probe tubing. Interstitial fluid was used to quantify the free (protein unbound/pharmacologically active portion) drug concentrations in the tissues.

In order to determine drug concentrations in urine, animals were housed individually in metabolism cages to allow for collection of urine samples. Urine samples were collected at 0 (pretreatment), 4, 8, 12, 24, 48, 72, 96, 120, 144 and 168 h after drug administration. Urine was collected via a tray under the metabolism cages with a spout at the front of the cage and a stainless-steel bucket underneath to catch the urine and limit fecal contamination. Further details of the sample collection and calculation are described in our previous study in goats by Bublitz et al. [**2**]. All plasma, ISF and urine samples were frozen at -80 ºC prior to analysis.

4.3.4 Tissue collection

After two consecutive negative (drug-free) urine samples (36–72 h for meloxicam, and 96–168 h for flunixin), each pig was euthanized. In order to minimize stress associated with euthanasia, the pigs were first sedated via intramuscular injection of 50:50 ketamine (100 mg/mL) and xylazine (100 mg/mL), equivalent to a final dose of 2.2 mg/kg ketamine and 2.2 mg/kg xylazine. After sedation, Euthasol® was administered through the jugular vein catheter at a dose equivalent to 85.9 mg/kg pentobarbital sodium and 11 mg/kg phenytoin sodium. Several biopsy punches were taken from each lobe of the liver and the entire left and right kidneys were taken from each pig in order to analyze tissues when the drug was no longer detectable in urine. These tissue samples were frozen at -20ºC until analysis.

4.3.5 Drug Analysis

Plasma and Urine Sample Preparation

Flunixin and meloxicam plasma and urine samples were prepared using solid-phase extraction prior to UPLC-MS/MS analysis. Samples (300 µL) were pretreated with 300 µL of 4% phosphoric acid and vortexed for 10 seconds. Then, 500 µL of this pretreated sample was loaded onto an Oasis 1 mL 30 mg PRiME HLB cartridge (Waters Corp.), washed with 1 mL of methanol,

and eluted from the cartridge with 500 µL of 90:10 (vol/vol) acetonitrile:methanol. The eluate was filtered through a 0.2 µm PTFE Whatman Mini-UniPrep Syringeless Filter vial (GE Healthcare UK Limited., Buckinghamshire, UK) and then injected onto the UPLC-MS/MS system.

Tissue Sample Preparation

For all kidney and liver samples, a 0.1 g sub-sample was weighed into a 2-mL bead mill tube containing 2.8-mm ceramic beads (Fisher Scientific, Hampton, NH, USA). Then, 1 mL of acetonitrile was added to the tube and the contents homogenized 3 times for 15 seconds during each cycle at a speed of 5 m/s for kidney, and 4 m/s for liver, with a 10 second rest between cycles (BeadMill24, Fisher Scientific). Following homogenization, the tubes were centrifuged at 10,000 x g for 5 minutes. Then, 800 µL of supernatant was transferred to a 16 × 100 mm borosilicate glass tube containing 800 µL of acetonitrile and 400 µL of water. This mixture was vortexed gently for 10 seconds and then eluted through a 3 mL Captiva EMR-Lipid cartridge (Agilent Technologies, Inc., Santa Clara, CA, USA). The eluate was then evaporated to dryness at 55 °C for 25 minutes. The sample was reconstituted in 300 µL of 1:1 acetonitrile:water, vortexed for 30 seconds, and the contents transferred a 0.2 µm PTFE Whatman Mini-UniPrep Syringeless Filter vial and then injected onto the UPLC-MS/MS system.

UPLC-MS/MS Conditions

All samples were quantified by ultra-high-pressure liquid chromatography (UPLC) with mass spectrometric (MS/MS) detection (Waters Corp., Milford, MA, USA). The UPLC-MS/MS system consisted of a Xevo TQD tandem quadrupole mass spectrometer (Waters Corp.)

For flunixin samples, separation was achieved with a 2.1 mm x 100 mm, 1.7 µm Waters Acquity BEH Phenyl column (Waters Corp.). A gradient was used, and the initial mobile phase was 0.1% formic acid in water: 0.1% formic acid in acetonitrile (70:30 v/v) with a flow rate of

0.4 mL/min for the first 2.5 minutes. The mobile phase then switched to (10:90 v/v) from 2.5 min − 3.5 min. For the last 1.5 min of the run, the mobile phase was (70:30 v/v). The MS/MS was run in ESI + mode. The quantification trace used was 297 → 279. Column temperature was 35 °C and sample temperature was ambient.

For meloxicam samples, separation was achieved with a 2.1 mm x 50 mm, 1.7 um Waters Acquity BEH C18 column (Waters Corp.) A gradient was used, and the initial mobile phase was 0.1% formic acid in water: 0.1% formic acid in acetonitrile (65:35 v/v) with a flow rate of 0.4 mL/min for the first minute. The mobile phase then switched to (10:90 v/v) from 1.0 min to 1.1 min. For the last 1.9 min of the run, the mobile phase was (65:35 v/v). The MS/MS was run in ESI + mode. The quantification trace used was 352.043 → 115. Column temperature was 35 °C and sample temperature was 10 °C.

Validation standards were prepared over a linear range for each matrix (plasma, urine, kidney and liver) and were used to construct calibration curves. For the inter-day accuracy and precision, standards were repeated over 3 days. The concentrations analyzed varied by drug and by matrix and are shown in Table 4.1 below.

All calibration curves were linear with a R^2 value of 0.99 or higher. Limit of quantification, inter-day accuracy and inter-day precision are presented in Table 4.2 for each analytical method.

Table 4.1. Concentrations and replicates used for the assay validation.

Drug	Matrix	# of concentrations	Concentrations (µg/mL)
Flunixin	Plasma	6	0.0005, 0.001, 0.01, 0.1, 0.5, 1
	Urine	7	0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5
	ISF	6	0.0005, 0.001, 0.002, 0.005, 0.01, 0.05
	Liver	6	0.0001, 0.0005, 0.001, 0.01, 0.05, 0.1
	Kidney	5	0.0005, 0.001, 0.01, 0.05, 0.1
Meloxicam	Plasma	7	0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1
	Urine	6	0.005, 0.01, 0.05, 0.1, 0.5, 1
	ISF	6	0.0005, 0.002, 0.005, 0.01, 0.025, 0.05
	Liver	5	0.005, 0.01, 0.1, 0.5, 1
	Kidney	6	0.005, 0.01, 0.05, 0.1, 0.5, 1

Table 4.2. Limit of quantification (LOQ; µg/mL for fluids or µg/g for tissues), inter-day accuracy (%) and inter-day precision (%) for analytical methods.

Drug	Tissue	LOQ	Accuracy (%)		Precision (%)	
		µg/mL or µg/g	Mean	±SD	Mean	±SD
Flunixin	Plasma	0.0005	107	(6)	5	(3)
	ISF	0.0005	99	(7)	6	(5)
	Urine	0.0005	103	(5)	6	(3)
	Liver	0.0001	100	(5)	5	(2)
	Kidney	0.0001	100	(7)	8	(5)
Meloxicam	Plasma	0.001	100	(5)	6	(5)
	ISF	0.001	97	(6)	6	(2)
	Urine	0.001	104	(5)	4	(2)
	Liver	0.005	100	(5)	7	(2)
	Kidney	0.005	100	(4)	8	(2)

SD; standard deviation

4.3.6 Pharmacokinetic analysis

A noncompartmental analysis of drug plasma concentration vs. time profiles was performed with Phoenix WinNonLin software (version 8.0; Certara, Princeton, NJ, USA). The area under the plasma concentration–time curve from time zero to infinity (AUC0→∞; h*µg/mL)

was calculated by linear log trapezoid method. The AUC0→∞ was used to calculate clearance per fraction absorbed (Cl/F; L/h/kg) and half-life (T1/2; h). The volume of distribution (per fraction absorbed) (Vd/F; L/kg) was also calculated. Peak concentration (Cmax; µg/mL) and time at which maximum concentration occurs (Tmax; h) in plasma and urine was taken directly from the data from each pig.

Individual renal clearance values were estimated for each pig using the following equation: Renal Clearance (mL/h) = [(Ae/AUC)] [32], where Ae is the cumulative amount of drug excreted unchanged in the urine and AUC is the area under the plasma concentration-time curve to infinity. These values were then corrected for body weight for comparison with the total body clearance and reported as mL/h/kg in the Table 4.4.

4.4 Results

All pigs (n = 7 for each treatment) completed the study with no adverse effects. One pig from the pilot study was excluded from the flunixin urine results due to carryover in the collection tray. One pig was excluded from the flunixin plasma and ISF results due to loss of the catheter and ISF probe.

4.4.1 Flunixin

Plasma and ISF

Mean plasma and ISF flunixin concentrations over time following a single IM injection of 2.2 mg/kg are presented in Figure 4.1. Parameters describing the pharmacokinetics of flunixin following a single IM injection are presented in Table 4.3. Flunixin concentrations in plasma were last detected above the LOQ of 0.0005 µg/mL at 60 h. The plasma pharmacokinetics of flunixin after IM administration were characterized by rapid absorption, an apparent large volume of distribution/F and an apparent elimination half-life which was relatively short. In interstitial fluid,

the average free maximum concentration of flunixin (Cmax) was 0.0039 µg/mL at 5.03 hours (Tmax) after flunixin administration.

Urine

The highest concentration of flunixin in urine (Cmax; 1.55 ± 0.96 µg/mL) was detected in the first sample collected from each pig (Tmax; 5.33 ± 3.27 h, as not all pigs urinated for the first 4 h sample). After 120 h, urine concentrations for all pigs fell below the LOQ of 0.0005 µg/mL. Renal clearance for flunixin was 5.29 ± 2.98 mL/h/kg (Table 4.4).

Liver and Kidney

Flunixin was detected in liver samples from six out of seven pigs at necropsy following two consecutive negative urine samples (96–168 h; concentration range 0.0001–0.0012 µg/g). However, flunixin concentrations in all liver samples were far below the FDA tolerance of 0.03 µg/g [5] and EMA MRL of 0.2 µg/g [14]. Only one kidney sample tested positive for flunixin (right kidney 0.0002 µg/g). Although there is no FDA tolerance level for kidney, this was far below the FSIS confirmatory limit of detection of 0.0125 µg/g [15] and EMA MRL of 0.03 µg/g [14].

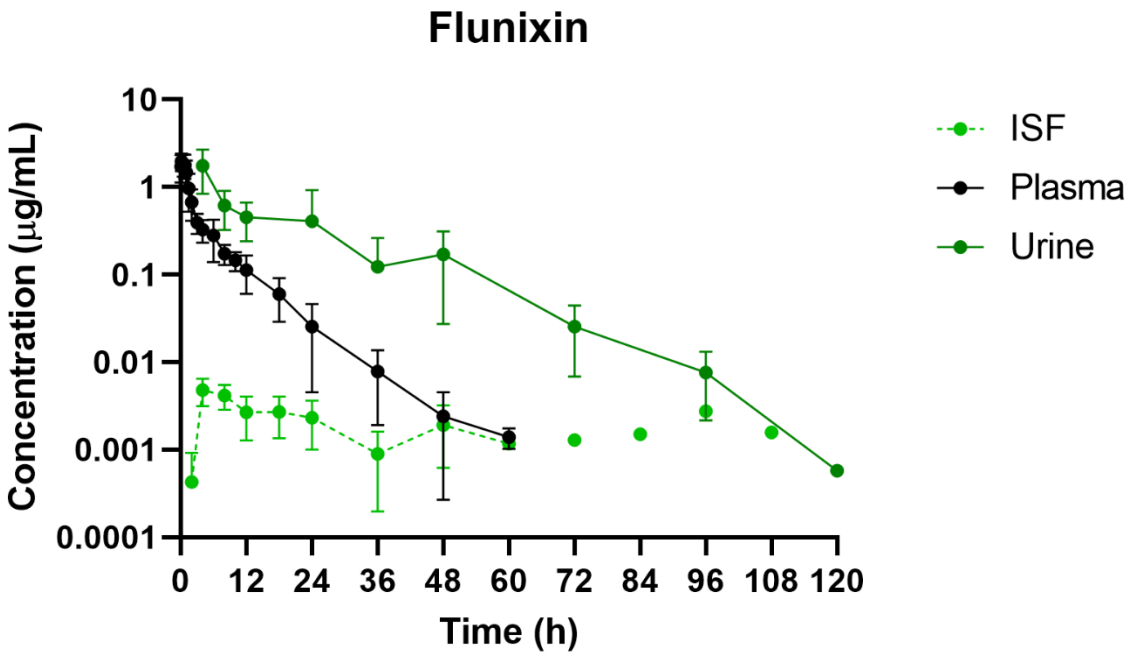

Figure 4.1. Plasma, urine and interstitial fluid flunixin concentration-time profiles following intramuscular administration of 2.2 mg/kg flunixin to pigs*. Data are represented as mean ± standard deviation. Interstitial fluid concentrations shown at 72–108 h are for a single pig only.*

4.4.2 Meloxicam

Plasma and ISF

Mean plasma and ISF meloxicam concentrations over time following a single IM injection of 0.4 mg/kg are presented in Figure 4.2. Parameters describing the pharmacokinetics of meloxicam following a single IM injection are presented in Table 4.3. Meloxicam concentrations in plasma were last detected above the LOQ of 0.001 µg/mL at 36 h. The plasma pharmacokinetics of meloxicam after IM administration were characterized by rapid absorption and a relatively short apparent elimination half-life. In interstitial fluid, the average maximum concentration of meloxicam (Cmax) was 0.0078 µg/mL at 7.64 hours (Tmax) after meloxicam administration.

Urine

The highest concentration of meloxicam in urine (Cmax; 0.05 ± 0.01 µg/mL) was detected in the first sample collected from each pig (Tmax; 4.57 ± 1.51 h, as not all pigs urinated for the first 4 h sample). After 48 h, urine concentrations for all pigs fell below the LOQ of 0.001 µg/mL. Renal clearance for meloxicam was 0.17 ± 0.04 mL/h/kg (Table 4.4).

Liver and Kidney

Meloxicam was detected in a single liver sample at necropsy following two consecutive negative urine samples (36–72 h; caudal lobe 0.0054 µg/g, although meloxicam was not detected in samples taken from other lobes of this pig's liver). While there is no FDA tolerance for meloxicam in pigs, this was below the EMA MRL of 0.065 µg/g [16]. Meloxicam was not detected in any kidney sample.

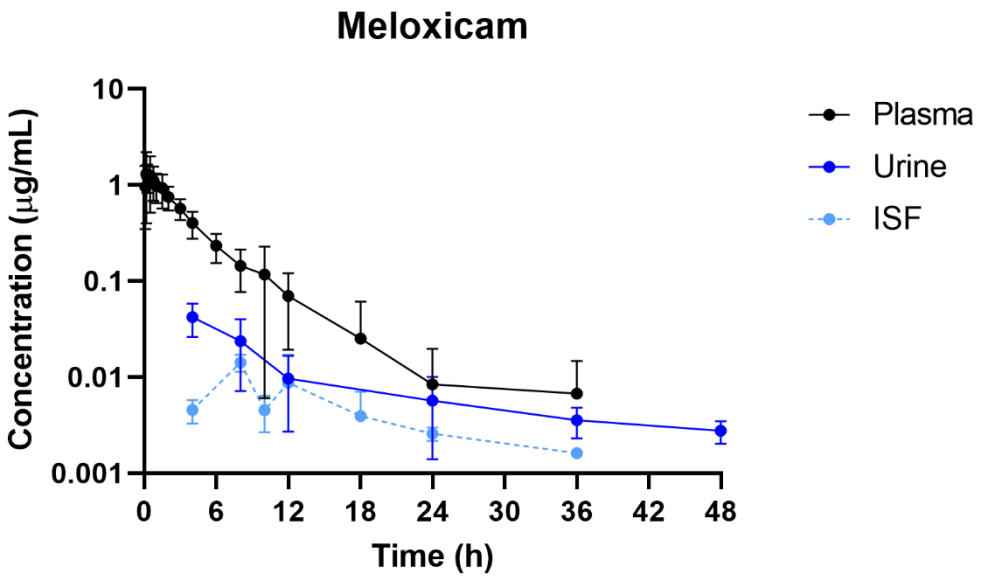

Figure 4.2. Plasma, urine and interstitial fluid meloxicam concentration-time profiles following intramuscular administration of 0.4 mg/kg meloxicam to pigs*. Data are represented as mean ± standard deviation.*

Table 4.3. Plasma pharmacokinetic parameters following intramuscular administration of either flunixin meglumine 2.2 mg/kg (n=6) or meloxicam 0.4 mg/kg (n=7). *Data are shown as geometric mean and range.*

		Flunixin		Meloxicam	
Parameter	**Units**	**Mean**	**Range**	**Mean**	**Range**
Dose	mg/kg	2.20		0.40	
MRT	h	5.63	(3.44 - 8.58)	4.15	(3.23 - 6.37)
$T_{1/2}$	h	5.41	(3.01 - 9.34)	3.34	(2.35 - 6.37)
λ_z	h	0.13	(0.07 - 0.23)	0.21	(0.11 - 0.29)
T_{max}	h	0.22	(0.08 - 0.75)	0.37	(0.17 - 1.50)
C_{max}	µg/mL	2.18	(1.82 - 2.87)	1.20	(0.75 - 2.85)
AUC_{last}	h*µg/mL	5.96	(3.95 - 7.83)	4.63	(3.01 - 8.55)
AUC_{inf}	h*µg/mL	5.97	(3.97 - 7.85)	4.65	(3.02 - 8.67)
AUC_{extrap}	%	0.23	(0.10 - 0.38)	0.31	(0.10 - 1.31)
Vd/F	L/kg	2.88	(1.22 - 7.47)	0.41	(0.23 - 0.58)
Cl/F	L/h/kg	0.37	(0.28 - 0.55)	0.09	(0.05 - 0.13)

MRT; mean residence time, $T_{1/2}$; apparent elimination half-life, λz; slope of the terminal phase, Tmax; time to maximal concentration, Cmax; maximum concentration, AUC; area under the concentration-time curve, Vd/F; volume of distribution per fraction absorbed, Cl/F; total body clearance per fraction absorbed.

Table 4.4. Renal clearance values, % contribution of renal clearance to total systemic clearance (Cl/F) and % total dose excreted in urine following intramuscular administration of 2.2 mg/kg flunixin (n=5) or 0.4 mg/kg meloxicam (n=7).

Parameter	**Units**	**Flunixin**		**Meloxicam**	
Renal Clearance	mL/h/kg	4.72	(2.98)	0.16	(0.04)
Renal clearance component of Cl/F	%	1.31	(0.42)	0.19	(0.06)
Percent total dose excreted in urine as parent drug	%	1.39	(0.42)	0.20	(0.06)

4.5 Discussion

4.5.1 Plasma pharmacokinetics

Following intramuscular administration of 2.2 mg/kg flunixin, the peak plasma concentration was reached in 0.22 h, which was slightly less than the previously reported Tmax of 0.61 h and 0.85 h following intramuscular administration to gilts [17] and 6-day-old piglets [18], respectively. The apparent elimination half-life (5.41 h) was similar to previously reported $t_{1/2}$ in 10-day-old piglets following intravenous administration of 2.2 mg/kg and 4.4 mg/kg flunixin (4.82 h and 5.15 h, respectively [19]), but less than previously reported in gilts and 6-day-old piglets administered 2.2 mg/kg intramuscularly (7.49 h and 7.93 h) [17, 18]. Elimination half-life is a hybrid variable and can be altered by flip-flop kinetics and changes in drug distribution and clearance. Variation between studies (including multiple injection sites in the gilt study) could be responsible for these differences in half-lives.

Only one previous study reported the volume of distribution/F (Vd/F) following an intramuscular dose of flunixin, and the reported value was much less than the Vd/F in the present study (0.92 L/kg for 6-day-old piglets [18] compared to 2.88 L/kg in this study). The Vd/F is affected by the bioavailability (F) and the degree of plasma and tissue protein binding, as well as the drug's lipophilicity [20]. Lipid-soluble drugs such as flunixin have high apparent volumes of distribution, and flunixin has been shown to be highly bound to plasma proteins in pigs (99% [21]). The body fat-to-water ratio increases with age, resulting in increased sequestration of flunixin in adipose tissue [20], which may explain the variation in Vd/F between 6-day-old piglets and the juvenile pigs in the present study, both administered 2.2 mg/kg flunixin intramuscularly.

Following intramuscular administration of 0.4 mg/kg meloxicam, the peak plasma concentration was reached around 0.37 h), which was comparable to previously reported in 8-day-old piglets given a dose of 1 mg/kg (0.50 h [22]), but less than that reported in 5-day-old piglets

given 0.4 mg/kg and 2-week-old piglets given 0.6 mg/kg (1.21 h and 1.1 h, respectively [18, 23]. However, the apparent elimination half-life was comparable to most previous reports (3.34 h in this study compared to 2.6 h, 3.94 h and 4.46 h; [18, 22, 23]), except for the elimination half-life reported in sows following an intravenous dose of 0.5 mg/kg (6.15 h; [24]). Despite differences in routes of administration, this difference may suggest that drug elimination may be slower in mature pigs.

Volume of distribution/F in this study for meloxicam (0.41 L/kg) was comparable to that of other studies investigating piglets 5–23 days of age, as well as mature sows, given doses in the range of 0.4-1.0 mg/kg and given via intramuscular or intravenous routes of administration [18, 19, 22, 24, 25].

4.5.2 Urine pharmacokinetics and renal clearance

As a general rule, NSAIDs are primarily eliminated by hepatic biotransformation, with renal excretion of the parent compound contributing to a small amount of total excretion (< 5% [26]). In this study, the percent of the total dose that was excreted as unchanged parent drug in the urine was low for both flunixin and meloxicam (1.31% and 0.19%, respectively). The total body clearance for each of these NSAIDs was comparable to previous studies [19, 21, 22, 23, 25], and represents elimination from the whole body of the parent drug and not its metabolites, including hepatic and renal elimination. The metabolites are predominantly glucuronide or oxidative metabolites of these drugs which were not targeted in our urine analysis, but have been reported in the urine of several species [12, 27]. There is no evidence of phase 2 metabolism of meloxicam in pigs [28], but evidence of several phase one metabolites with 5-hydroxymethyl metabolites being the predominant metabolite. Biotransformation of meloxicam governs meloxicam's clearance in most species and explains why little of the parent drug is found in the urine and this

is not unusual for the oxicams class of drugs. The relative contribution of renal clearance to the overall systemic clearance was low, suggesting that the main route of elimination is hepatic metabolism, although this study is limited in that the metabolites of either NSAID were not measured. Renal clearance of parent flunixin or meloxicam in pigs has not been previously reported, which is a unique feature of this study.

The relationship between plasma and urine flunixin concentrations across all time points indicates that urine flunixin concentrations were higher than those measured in plasma at any given time point, similar to previously reported in both cattle and goats [2, 6], however this was the opposite for meloxicam, with plasma concentrations being similar to or higher than that of the urine, again, similar to previously reported in goats [2].

Our data also allowed us to propose a plausible mechanism of renal clearance of these parent drugs. For example, by assuming glomerular filtration rate (GFR) is 259 mL/h/kg [29] and assuming fraction unbound in plasma, fu = 0.01 for flunixin and for meloxicam, as they are known to be highly bound to plasma proteins, then the clearance by filtration for both drugs can be estimated to be 2.59 mL/h/kg. This value is less than our measured renal clearance of 4.72 mL/h/kg for flunixin, but is greater than our measure renal clearance of 0.16 ml/h/kg for meloxicam. Based on these calculations, one can infer that filtration and active secretion contributed to renal clearance of flunixin but filtration and reabsorption for renal clearance of meloxicam. The latter is consistent with very little meloxicam (< 0.2%) of parent drug appearing in the urine of most species. It should be reiterated here that this pertains to the clearance of the parent dugs and not the metabolites.

4.5.3 Tissue residues

The FDA established tolerance levels are based on edible tissues, or the slowest depleting organs (target tissues) which often refers to the liver or kidneys. For livestock shows, it is not

possible to directly test these target tissues, and plasma samples are not convenient, and urine is tested instead. Detection of drug in urine may be a violation of livestock show rules.

Interstitial fluid (ISF) was collected to create a concentration-time profile in an attempt to reflect the tissue concentrations across multiple time points for each pig. However, these concentrations did not correlate well with plasma or urine concentrations, particularly for flunixin in which the ISF concentrations were prolonged but at a low level. However, the ISF concentrations for both meloxicam and flunixin fell below the LOQ before the urine concentrations.

Flunixin concentrations detected in the liver following negative urine samples were far below the FDA tolerance and present no food safety concerns or violations of livestock show rules. Meloxicam was detected in a single liver sample at necropsy. While there is no FDA tolerance for meloxicam in pigs, this was below the EMA MRL for meloxicam in liver. However, the presence of meloxicam at any level in pig liver in the US would be a violation according to US FSIS.

While the liver is regarded as the main target tissue when examining residues of NSAIDs, this study also measured drug concentrations in the kidneys. Only one kidney sample tested positive for flunixin. Although there is no FDA tolerance level for kidney, the concentration detected was far below the FSIS confirmatory limit of detection. However, limits of detection change as analytical methods improve. Any detectable amount of flunixin in the kidneys would technically be violative. Meloxicam was not detected in any kidney sample, which is consistent with previous work and the renal clearance reported here in the present study.

4.5.4 Limitations of this study

This study had a number of limitations that should be taken into consideration when reviewing data and applying to livestock show scenarios. As was highlighted earlier, this study did

not have a companion intravenous study from which the bioavailability, F, could have been determined, and therefore the clearance and volume of distribution values are approximations and need to be expressed as Cl/F and Vd/F. Nonspecific binding to the ISF probes was not determined. While there is a possibility for drug binding to the ISF probes, these probes are inert materials (polyacrylonitrile) and they were placed 36–48 hours prior to the start of the trial to allow adequate time for equilibration with body fluids. Assessment of drug binding to the probes has been investigated previously for various drugs including NSAIDs such as carprofen and flunixin in other animal species but have not been reported in the literature. In these studies, there was no accumulation when assessed *in vitro*, but we cannot assume this is the same for *in vivo* as there are critical differences in the experimental conditions (for example, the flow rate in vitro is much higher than *in vivo*). This study was focused on estimating the renal clearance of the parent drug and not metabolites, as the primary reason for this study was to determine when parent drug concentrations in urine were below detectable levels so that urine in show pigs are clear of these two drugs. Should livestock show officials target the urinary metabolites for testing, this information may not be applicable. Related to this limitation is the fact that plasma concentration is the driving force controlling all other concentrations including urine concentration, and uncertainty with urine concentrations occurs for many other reasons such as the extent of urine dilution, urinary pH (not reported here), analytical issues, and plasma and urine concentration can be out of phase.

4.6 Conclusions

The primary purpose of this study was to determine the urinary excretion of two frequently used NSAIDs, flunixin and meloxicam, in pigs and relate these findings to its plasma pharmacokinetics and potential tissue residues. Prior to this study there were data gaps pertaining

to the renal clearance of this drug and how long it will take for the parent form of these drugs to clear from urine. While pharmacokinetics parameters from our study were comparable to earlier studies in pigs for these two drugs and a small percentage of the parent drug is cleared in the urine as demonstrated in other species, our study is the first to report the renal clearance of these two NSAIDs in pigs. The data also suggest that the renal clearance mechanism for flunixin was predominantly by active secretion while the predominant clearance mechanism associated with meloxicam was likely by tubular reabsorption. Flunixin urine concentrations were always higher than plasma and ISF concentrations up to 5 days post administration. This was the complete opposite for meloxicam albeit urine levels at 2 days were higher than plasma and ISF levels. When urine levels were negative at 7 days for flunixin and 3 days for meloxicam, the pigs were slaughtered and liver and kidney concentrations were below either the FDA tolerance or the detection level of current US FSIS. However, this study was not able to determine the converse, i.e., whether detectable concentrations of meloxicam or flunixin in the urine correlated with drug concentrations above the tolerance or above detectable levels in the liver. The present study demonstrated that following a single intramuscular dose of 2.2 mg/kg flunixin, the drug should be undetectable in the urine if the label meat withdrawal time (12 days) is followed appropriately. There is no approved label in the US for meloxicam and therefore no established withdrawal time for meloxicam in pigs; however, this study suggests that pigs given a single intramuscular dose 0.4 mg/kg may test positive in urine for up to 2 days post-dose. On the other hand, following negative urine samples, meloxicam may still be detected in the liver (albeit below current FSIS testing limits). As there is no label for meloxicam in pigs, any level detected in the tissues would be considered violative in the US. This study provides useful information that can help livestock show authorities and veterinarians determine an appropriate elimination period for show animals

whose urine may be tested prior to competition, and it may help provide data on which to base penalties for detection of flunixin or meloxicam in urine in show pigs.

4.7 Funding

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CHAPTER 5

Comparative Effects of Nonsteroidal Anti-Inflammatory Drugs at Castration and Tail-Docking in Neonatal Piglets

Nixon, E., Carlson, A.R., Routh, P.A., Hernandez, L., Almond, G. W., Baynes, R. E., & Messenger, K. M. (2021) Comparative effects of nonsteroidal anti-inflammatory drugs at castration and tail-docking in neonatal piglets. *PLOS ONE.* (*In press).*

5.1 Abstract

This study assessed the efficacy of meloxicam, flunixin, and ketoprofen in piglets undergoing routine castration and tail-docking. Six-day-old male piglets (8/group) received one of five randomized treatments: intramuscular saline (SAL PROC), meloxicam (MEL; 0.4 mg/kg), flunixin (FLU; 2.2 mg/kg), ketoprofen (KETO; 3.0 mg/kg) or sham (SAL SHAM; saline injection, no processing). Two hours post-dose, piglets were castrated and tail-docked. Plasma cortisol, interstitial fluid (ISF) prostaglandin E2 (PGE2) and activity levels via Actical® monitoring were used to estimate pain. SAL SHAM and FLU exhibited lower cortisol concentrations than SAL PROC at the time of processing ($p=0.003$ and $p=0.049$, respectively), and all NSAIDs exhibited lower PGE2 than SAL PROC at 3.69 hours (MEL $p=0.050$; FLU $p=0.043$ and KETO $p=0.031$). While not statistically significant, PGE2 was higher in SAL PROC piglets vs. other treatment groups at most time points. There was also a high degree of variability between piglets, especially for SAL PROC. Activity levels were significantly decreased at multiple time points in SAL PROC and MEL piglets following processing. However, FLU and KETO piglets had increased activity levels closer to that of the SAL SHAM group, suggesting that these NSAIDs are more effective than MEL in providing analgesia. These results demonstrate that management strategies including administration of intramuscular flunixin or ketoprofen to reduce pain associated with processing will likely improve piglet health and welfare in the United States.

5.2 Introduction

Piglets in the United States routinely undergo painful procedures, collectively known as processing, which can include castration, tail-docking, teeth clipping, ear notching/tagging, and injections. Castration (i.e., removal of the testicles or destruction of testicular formation) is commonly performed to prevent unwanted breeding, reduce aggression, and reduce boar taint (improving meat quality) [1] and tail docking (i.e., removal of a portion of the tail) is intended to reduce tail biting severity and total events [2] . To improve piglet welfare standards, Europe and Canada have implemented legislation requiring that piglets receive anesthetic or analgesic drugs in conjunction with processing procedures [3,4]. Additionally, legislation is in place to prevent routine tail-docking in the EU unless there is evidence that tail biting has occurred [5].

In 2018, the reported number of pigs produced in the United States was approximately 133.5 million [6]. Assuming half of those pigs are male, an estimated 66.8 million piglets will likely undergo castration and tail docking each year. Castration and tail docking are often performed simultaneously; both cause tissue damage, and the acute consequences of these invasive procedures in terms of pain and stress are well described [7,8,17–19,9–16]. While not currently required in the United States, the provision of analgesia for piglets is a critical welfare issue, and many large retailers are requiring pain mitigation throughout their supply chains [20]. However, for legislation to be implemented to require pain management, drugs with proven efficacy and FDA approval are needed.

Data supporting the use of NSAIDs to manage pain is conflicting between studies [19,21–25], which may be due to the doses or methodology used to assess efficacy. There is also only one other study that assessed the pharmacodynamics of multiple NSAIDs in direct comparison [26] .

In that study, both ketoprofen and meloxicam were determined to be ineffective for castration-associated pain in piglets (Viscardi and Turner, 2018).

The objectives of the present study were to describe the pharmacodynamics of three different NSAIDs (meloxicam, flunixin, and ketoprofen) in neonatal piglets. This article only focuses on the comparative analgesic efficacy aspect of the study; the pharmacokinetics portion of the study is reported elsewhere [27]. Given that pain is a complex and multidimensional phenomenon, and there is conflicting data in the open literature on the efficacy of various NSAIDs at piglet processing, this study used a multimodal approach to assess the severity of pain and inflammation in piglets at castration and tail docking. This study achieved a direct comparison of three NSAIDs (meloxicam, ketoprofen, and flunixin), using objective measures of prostaglandin E2, cortisol, and activity levels, while also comparing to untreated controls (both processed and sham processed).

5.3 Materials and methods

5.3.1 Animals

This study was approved by the North Carolina State University Institutional Animal Care and Use Committee (protocol #17-088-A). A total of 46 Yorkshire/Landrace cross male piglets (weighing 3.80-3.21 kg and from 14 different litters) were individually housed in cages arranged so they were able to see each other. Lighting consisted of 12 hours of light and 12 hours of dark and ambient room temperature was maintained at 26-30 degrees Celsius. Heat lamps were hung above the piglets on one end of the individual cages. Piglets were fed non-medicated swine milk replacer (Milk Specialties Global, Eden Prairie, MN, USA) and offered fresh water every 4 hours from 7 am to 12 am. Piglets were weighed daily on a calibrated scale (i.e., 48 and 24 hours pre-dose and 0, 24, and 48 hours post-dose). The piglets were humanely euthanized at the end of the

study. First, the piglets were sedated via intramuscular injection of 50:50 ketamine (100 mg/mL) and xylazine (100 mg/mL) equivalent to a final dose of 2.2 mg/kg ketamine and 2.2 mg/kg xylazine. After sedation, Euthasol® was administered through the jugular vein catheter at a dose equivalent to 85.9 mg/kg pentobarbital sodium and 11 mg/kg phenytoin sodium. Death was confirmed by thoracic auscultation (lack of both heart and respiratory sounds), as well as lack of a corneal reflex.

5.3.2 Catheter and Interstitial Fluid Probe Placement

At 4 days-of-age (±1 day), a catheter (22 Ga, 10 cm small animal long-term venous catheterization kit, MILA International, Inc., Florence, KY, USA) was placed aseptically into the jugular vein under sevoflurane anesthesia. At the time of catheter placement, an ultrafiltration probe (RUF-3-12 Reinforced In Vivo Ultrafiltration Sampling Probes, BASi systems, W. LaFayette, IN, USA) was placed subcutaneously along the epaxial muscles. Details for each procedure were previously reported [27] . Finally, activity monitors (Actical, Philips Respironics, Bend, OR, USA) were also secured to the back of the piglets' neck with Ioban™ (3M, St. Paul, MN, USA). Piglets were then allowed to recover for 36–48 hours before the start of the study. During the recovery period, catheter patency was maintained by removing the heparin lock, flushing the catheter with saline, and replacing the heparin lock every 12 hours.

5.3.3 Drug Administration and Processing

In the present study, "processing" was defined as castration and tail-docking only. At 6 days-of-age (±1 day) piglets were randomized using a random number generator and allocated to one of five treatment groups; Saline with sham processing (SAL SHAM; n=9), saline with processing (SAL PROC; n=8), meloxicam and processing (MEL; n=8), flunixin and processing (FLU; n=7) and ketoprofen and processing (KETO; n=8). Forty out of 46 piglets completed the

study. Six piglets were removed from the data analysis due to illnesses unrelated to the study (severe diarrhea, malaise, abdominal cyst), and their data were not included in the results. Two piglets used in a pilot study were not included due to differences in housing and handling.

All treatments were administered intramuscularly as follows; One hundred microliters of saline (both SAL SHAM and SAL PROC), 0.4 mg/kg meloxicam (Meloxicam solution for injection 5 mg/mL, Putney, Inc., Portland, ME, USA), 2.2 mg/kg flunixin (Banamine-S®, Merck Animal Health, Summit, NJ, USA) or 3.0 mg/kg ketoprofen (Ketofen®, Zoetis, Inc., Kalamazoo, MI, USA). These doses were chosen based on existing labels for piglets at castration (meloxicam and ketoprofen) in Europe and an existing label for other indications in pigs (flunixin) in the United States.

Two hours after drug administration, one researcher restrained the piglets to expose the anogenital region and a second person (with extensive experience in piglet processing procedures) performed the castration and tail-docking. Incisions were made with a scalpel on each side of the scrotum, the testicles were pulled from the surrounding tissue and the scalpel was used to cut the testicles free. Side-cutter pliers were used to dock the tail per industry standard in the United States. Then, the castration site and tail were sprayed with Betadine® surgical scrub (Emerson Healthcare LLC., Wayne, PA, USA) to disinfect the wounds. SAL SHAM piglets were handled for approximately the same length of time as other pigs, and handled similarly, but not castrated or tail docked.

5.3.4 Sample Collection

Blood samples (1 mL) were collected via the jugular catheter and transferred into lithium heparin tubes (BD Vacutainer TM , Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at the following time points: 0 (baseline), 0.25, 0.5, 1, 1.5, 2, 4, 6, 8, 12, 24, 36 and 48 hours post-

dose. Plasma was collected by centrifugation of the blood at 3500 x g and used for the analysis of plasma cortisol concentrations. Interstitial fluid samples were collected at 0 (baseline), 2, 4, 6, 8, 12, 24, 36, and 48 hours post-dose and used to quantify prostaglandin E2, a biomarker of inflammation. A lag time for ISF collection was calculated based on the length of the probe and the rate of fluid collection from each probe. The ultrafiltration probes are 42.5 cm in length and hold 70 μL of fluid, and the probes were cut to custom lengths for each animal which was accounted for in the lag time calculation. Both plasma and ISF were frozen at -80 ºC until analysis.

5.3.5 Plasma Cortisol Analysis

Plasma cortisol samples were analyzed using a commercial radioimmunoassay (RIA) kit (ImmuChem™ Cortisol Coated Tube RIA Kit, MP Biomedicals, LLC., CA, USA). The samples were assayed in triplicate and analyzed on a Packard Cobra gamma counter. Calibration curves were within the range of 1.0 – 25.0 μg/dL and all R 2 values were >0.9970. The inter-day assay variability was 2.95 ±1.15% and the intra-day assay variability was 7.88 ±8.36%.

5.3.6 Interstitial Fluid Prostaglandin E2 Analysis

The concentration of interstitial fluid prostaglandin E2 (PGE2) was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Co., Ann Arbor, MI, USA). R 2 for all calibration curves were >0.96 and within the range of 7.81– 1000 pg/mL. The inter-day assay variability was 10.8% and the intra-day assay variability was 3.1%. All samples were analyzed in duplicate.

5.3.7 Activity Monitoring

Actical monitors were placed on the back of the neck at the time of catheter and interstitial fluid probe placement. Actical monitors are 17 g omnidirectional accelerometers which collect

data each minute on spontaneous activity. The activity monitors were active from the morning of catheter and probe placement, but only the data from 24 hours prior to drug administration, until 48 hours after drug administration were analyzed. The 24 hours immediately before dose were used as the baseline. The Actical monitors provide an output of activity counts when the data are downloaded.

5.3.8 Statistical Analysis

All statistical analyses were performed in GraphPad Prism (version 8.4.3, GraphPad Software, Inc., San Diego, CA, USA). All data were tested for normality by the Shapiro-Wilks test. PGE2 data were not normally distributed but found to be log-normally distributed, and therefore were log-transformed before statistical testing. All data except activity were analyzed using a mixed model procedure, including treatment, time, and treatment x time interaction. Time was a repeated measure with piglet as the experimental unit. A post hoc Tukey's test was conducted for significant outcomes.

5.3.9 Activity Count Statistical Analysis

For statistical analysis, activity was split into three 24-hour periods; baseline, 0-24 h post-dose, and 24-48 h post-dose. Similarly to a previous study analyzing activity data [28] , total activity counts for 6-hour periods were calculated and described as quarters (Q1, 08:00–13:59; Q2, 14:00–19:59; Q3, 20:00–01:59; and Q4, 02:00–07:59). The values for each quarter post-dose were compared to the baseline values within each treatment group. Data were analyzed using a mixed model procedure, with multiple comparisons and Tukey's test for significant outcomes. Time was a repeated measure with piglet as the experimental unit. The treatment groups were also compared to the control (SAL SHAM) at each time point. This was also analyzed using a mixed model procedure but this time with Dunnett's post hoc test.

In addition to the above, a functional linear modeling (FLM) approach was also used. This type of analysis is designed for actigraphy time-series data, allowing statistical characterization of activity patterns, while avoiding summary statistics that risk masking differences between groups and changes in patterns over time [29,30]. This analysis was performed using R software [31] and the package “actigraphy” [32], which applies a non-parametric permutation F test. Significance was calculated by counting the proportion of permutation F values that are larger than the F statistics for the observed pairing. In this case, a point-wise test (bspline method with 500 permutations) was used to generate a curve representing the proportion of all permutation F values at each point in the time series [29,30,33].

5.4 Results

5.4.1 Plasma Cortisol

There was an effect of time ($p=0.001$) but not treatment ($p=0.315$) or the interaction between time and treatment ($p=0.209$) on plasma cortisol concentrations. However, for the pairwise comparisons, both the SAL SHAM and FLU groups exhibited significantly lower plasma cortisol levels at 2 h than the SAL PROC group ($p=0.003$ and $p=0.049$, respectively). FLU piglets also had significantly lower cortisol than KETO piglets at 24 h ($p=0.022$). The plasma cortisol results for each group are presented in Figure 5.1.

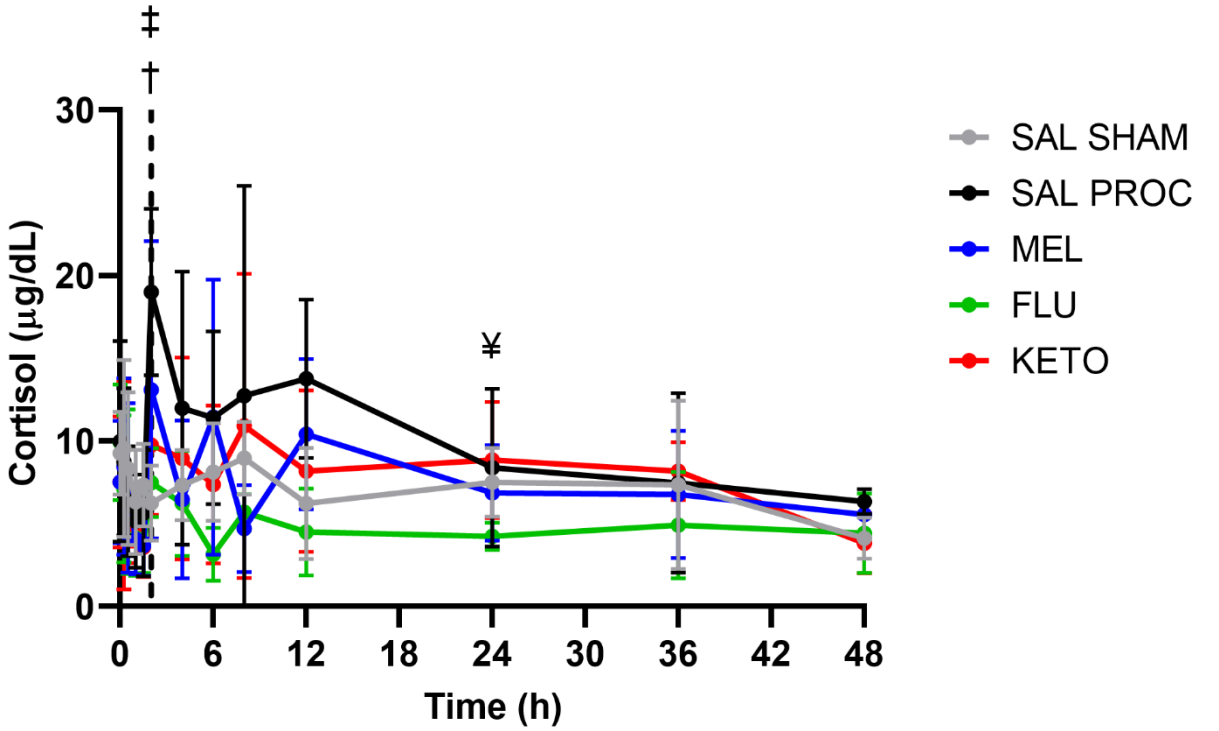

Figure 5.1. Total plasma cortisol concentration over time following intramuscular administration of either 0.1 mL saline, 0.4 mg/kg meloxicam, 2.2 mg/kg flunixin or 3.0 mg/kg ketoprofen in 6-day-old piglets. *All piglets were processed (castrated and tail-docked) at 2 h as shown by the vertical dotted line, except for the SAL SHAM group. Data are represented as mean ± standard deviation. † indicates significant difference between SAL PROC and SAL SHAM; ‡ indicates significant difference between SAL PROC and FLU; ¥ indicates significant difference between FLU and KETO.*

5.4.2 ISF Prostaglandin E2

The calculated lag time for the interstitial fluid collection was 0.31 hours. That is, it takes 0.31 hours for the sample to travel along the tubing, and this is accounted for in the results. The effect of treatment was significant ($p=0.048$), but the effect of time or the interaction between treatment and time was not significant ($p>0.05$). All NSAID treatments exhibited statistically

lower PGE2 at 3.69 hours post-dose than the SAL PROC group (MEL p=0.050; FLU p=0.043 and KETO p=0.031), and the MEL and FLU groups also had lower PGE2 than the SAL SHAM group at 2.313.69 (MEL p=0.050 and FLU p=0.043), 5.69 (MEL p=0.020 and FLU p=0.008) and 7.69 hours (MEL p=0.012 and FLU p=0.020). KETO also had lower PGE2 than the SAL SHAM group at 7.69 hours (p=0.043). While not statistically significant, SAL PROC PGE2 concentrations were higher than that of other treatment groups at most time points. There was also high variability in PGE2 concentrations between piglets, especially for the SAL PROC group. The ISF PGE2 results for each group are presented in Figure 5.2.

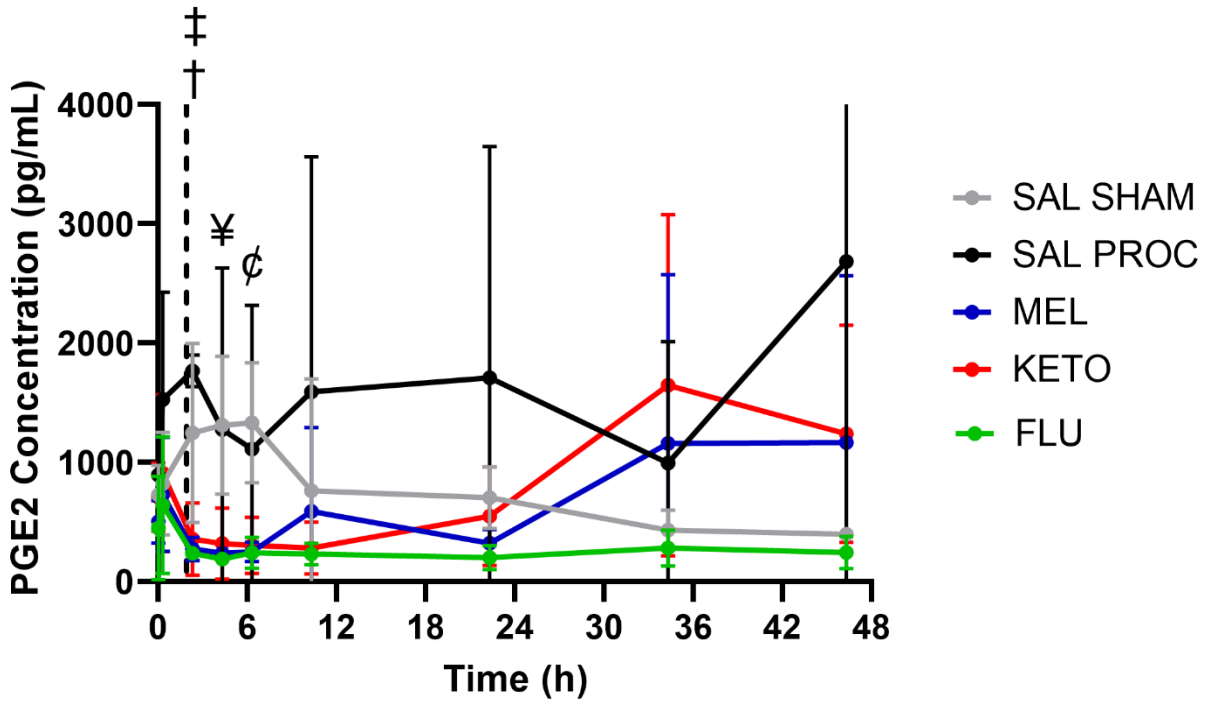

Figure 5.2. Total ISF PGE2 concentration over time (accounting for ISF probe lag time) following intramuscular administration of either 0.1 mL saline, 0.4 mg/kg meloxicam, 2.2 mg/kg flunixin or 3.0 mg/kg ketoprofen in 6-day-old piglets*. All piglets were processed (castrated and tail-docked) at 2 h as shown by the vertical dotted line, except for the SAL SHAM group. Data are represented as mean ± standard deviation. † indicates significant difference between SAL PROC and all NSAID groups; ‡ indicates significant difference between SAL SHAM and MEL/FLU; ¥ indicates significant difference between SAL SHAM and MEL/FLU; ¢ indicates significant difference between SAL SHAM and all NSAID groups.*

5.4.3 Activity

For activity levels, the mixed model showed that treatment and time were statistically significant ($p=0.035$ and $p<0.001$, respectively; Figure 5.3a and 5.3b). SAL PROC and MEL piglets at 0-24h Q3 and Q4 had significantly lower activity compared to baseline at Q3 (SAL

PROC, p=0.032; MEL, p=0.048) and Q4 (SAL PROC, p=0.023; MEL, p=0.023). However, FLU and KETO piglets were significantly more active when compared to SAL PROC at 24-48 h Q3 (p=0.012 and p=0.024, respectively). FLU piglets were also significantly more active than SAL PROC piglets at 24-48 h Q1 (p=0.027). Finally, the FLU group was significantly more active at 24-48 h compared to 0-24 h at Q3 (p=0.014).

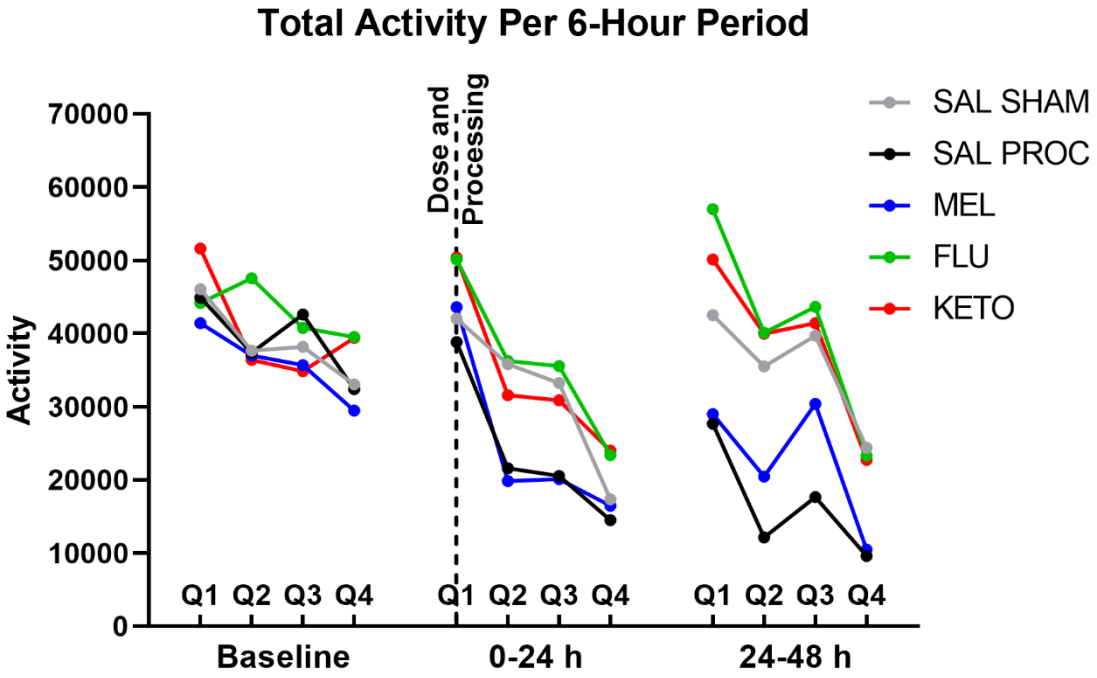

Figure 5.3a. Shows the total activity levels for each quarter (Q, 6-hour period) Q1, 8:00-13:59; Q2, 14:00-19:59; Q3, 20:00-01:59; Q4, 02:00-07:59.

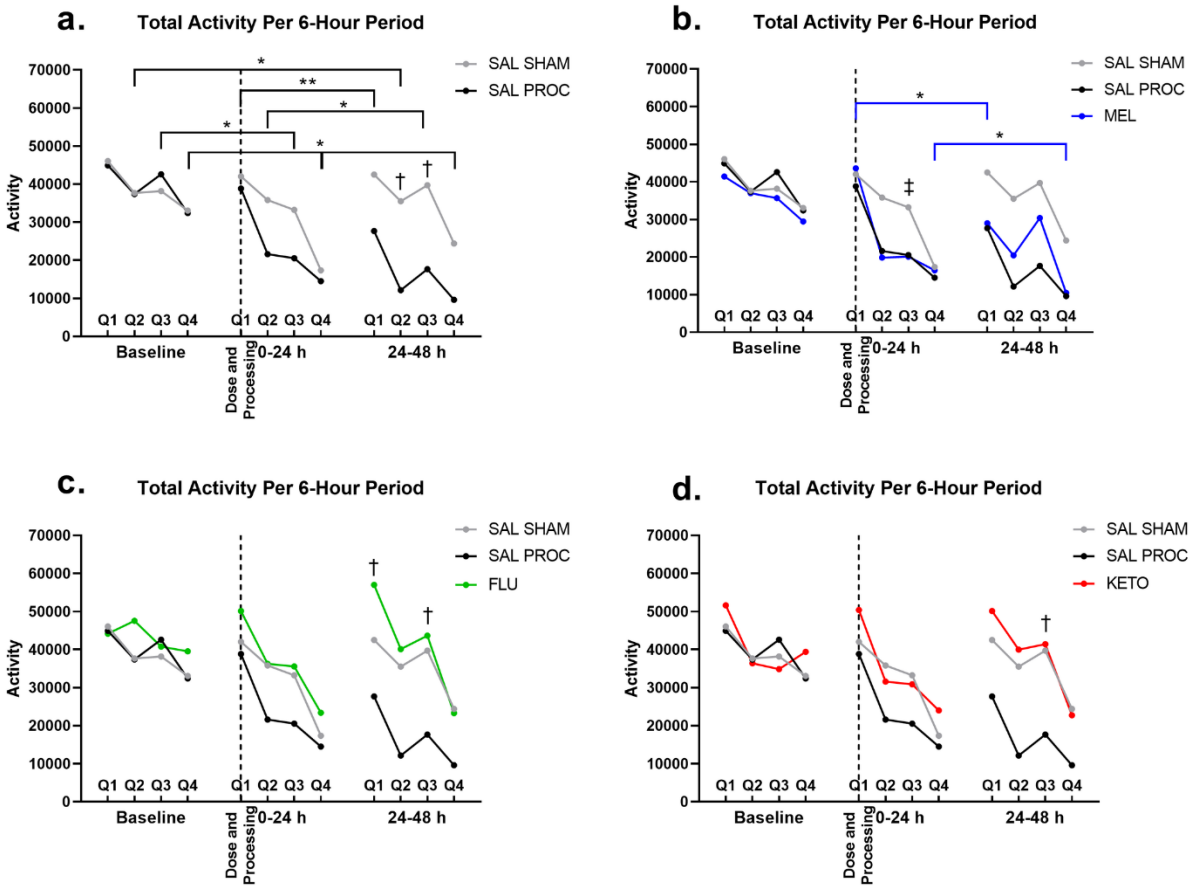

Figure 5.3b. The same data as Figure 5.3a but divided by treatment. Total activity levels for each quarter (Q, 6-hour period) Q1, 8:00-13:59; Q2, 14:00-19:59; Q3, 20:00-01:59; Q4, 02:00-07:59. ** indicates significant difference (p<0.05) between the indicated time periods for SAL PROC (graph a) and MEL (graph b). ‡ indicates significant difference between MEL and SAL SHAM (p<0.05). † indicates significant difference between SAL PROC and SAL SHAM (graph a) or NSAID-treated group (graphs b-d).*

The FLM demonstrated changes in 24-hour activity patterns after processing, particularly for the SAL PROC and MEL treatment groups between approximately 18-42 hours after dosing (16-40 hours post-processing; Figure 5.4). FLU and KETO appeared to be effective in maintaining

sleep-wake patterns closer to that of baseline, similar to the SAL SHAM piglets, and overall activity was reduced for the SAL PROC and MEL piglets.

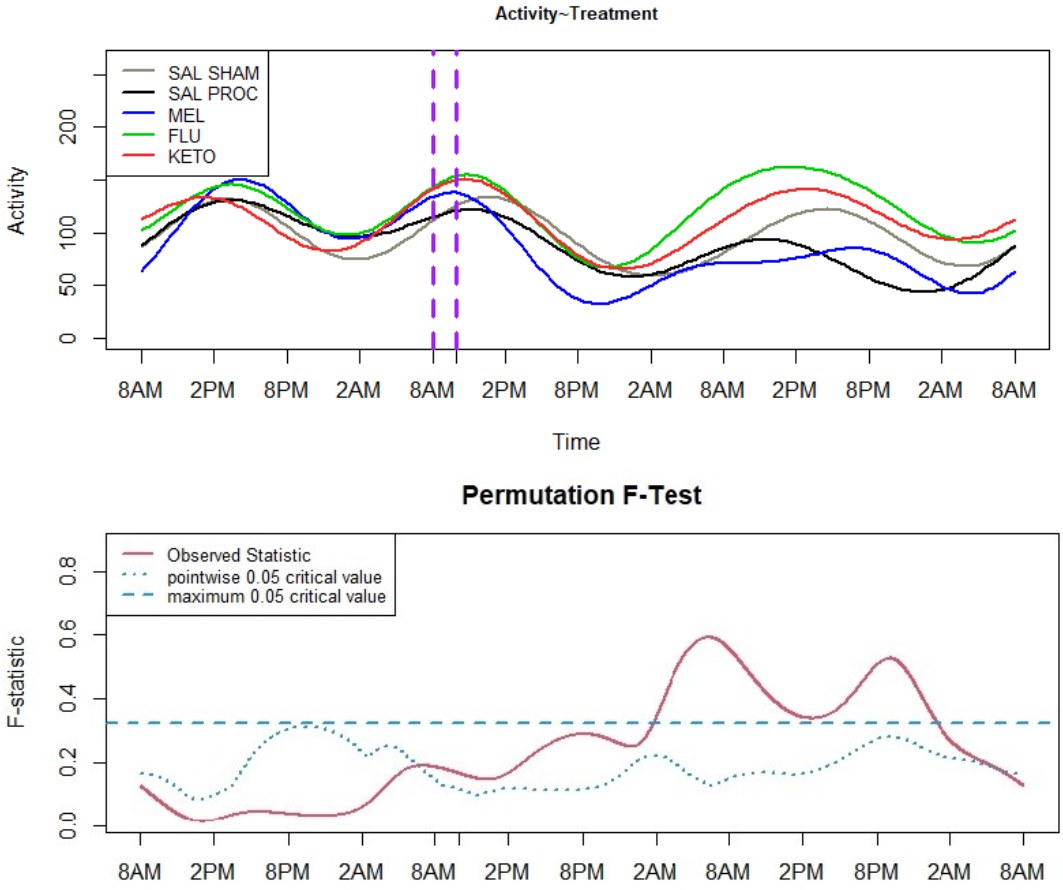

Figure 5.4. Functional linear modeling (FLM) of activity data following intramuscular administration of either 0.1 mL saline, 0.4 mg/kg meloxicam, 2.2 mg/kg flunixin or 3.0 mg/kg ketoprofen in 6-day-old piglets. *All injections were given at the time represented by the first vertical dashed line, and piglets were processed (castrated and tail-docked) 2 hours later, as shown by the second vertical dashed line, except for the SAL SHAM group. The bottom panel shows the point-wise critical value (dotted line), the proportion of all permutation F values at each time point at the significance level of 0.05. When the observed F-statistic (solid red line) are above the dotted line, the groups are considered significantly different.*

5.4.4 Bodyweight

Daily change in body weight was not significantly different between treatment groups (Figure 5.5). All piglets gained weight over the course of the study regardless of treatment.

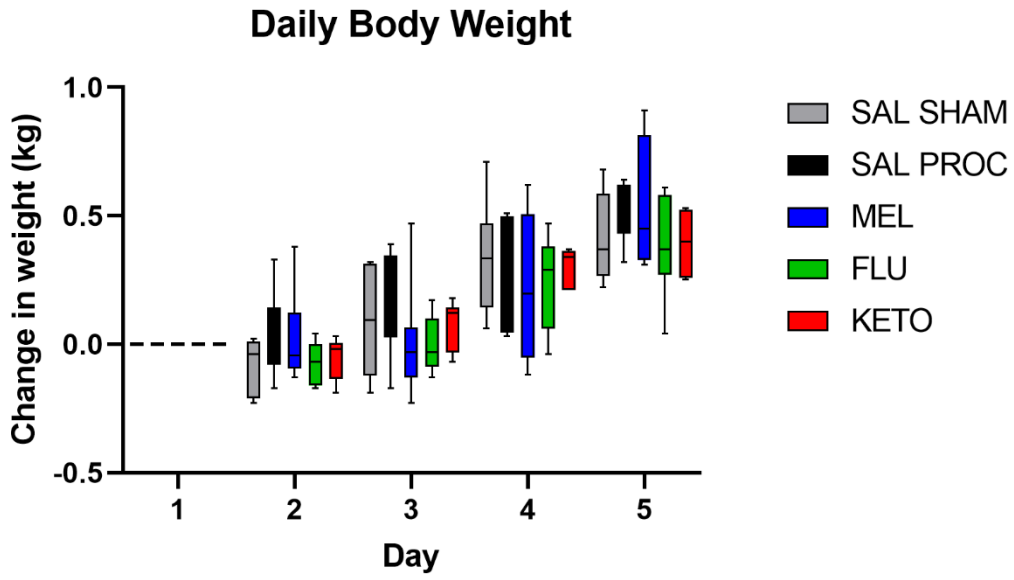

Figure 5.5. Daily change in bodyweight from baseline over the course of the study, where day 0 is when catheters and ISF probes were placed, and day 3 is the day of processing.

5.5 Discussion

NSAIDs are commonly administered analgesics for treating pain at piglet processing, however, there are conflicting data supporting the use of NSAIDs across studies [19,21–25]. The present study is unique in that it provides a direct comparison between three different NSAIDs simultaneously, as only one other study has directly compared the pharmacodynamics of two NSAIDs at piglet processing [26]. The results of this study demonstrated that flunixin may be the most effective NSAID in alleviating processing-associated pain in piglets, followed by ketoprofen. Similarly to previous reports [34], meloxicam showed minimal efficacy at the EU/Canada label dose. The sham-processed piglets showed negligible effects of handling or intramuscular injection

(saline) on the measured outcomes. There was a small increase in PGE2 following the injection, which returned to baseline in under 12 hours. The activity was also slightly reduced overnight after saline administration and sham processing, but again, quickly returned to baseline by 24 hours.

While the utility of measuring cortisol as a proxy for pain is controversial, cortisol was included in this study as it has been commonly assessed in previous piglet pain studies. As expected, SAL PROC piglets had significantly higher plasma cortisol concentrations immediately after processing compared to SAL SHAM piglets. Overall, flunixin achieved the greatest reduction in plasma cortisol throughout the duration of the study, although this was only statistically significant at 24 hours after drug administration (22 hours after processing). All NSAIDs also decreased PGE2 compared to SAL PROC soon after processing, but flunixin maintained this inhibition beyond 24 hours post-dose. This longer duration of action is consistent with the fact that flunixin has a longer half-life than meloxicam or ketoprofen in pigs [27].

Accelerometry has been used in other species to measure activity levels in relation to painful conditions [30,35,36] , and in sows to detect lameness [37–39] . However, to the best of the author's knowledge, the present study is the first to use accelerometry in assessing piglet pain. Activity levels were significantly decreased in SAL PROC and MEL piglets following processing, while FLU and KETO treated pigs had increased activity levels, closer to that of the SAL SHAM group suggesting that these NSAIDs are more effective than meloxicam in the provision of analgesia. In addition, following FLM, the 24-hour activity patterns appear to be altered following processing, with significant differences between approximately 18-42 hours after dosing (16-40 hours post-processing). The results suggest that these changes may be mitigated by treating with flunixin or ketoprofen.

Finally, there were no significant differences in weight gain in the first 48 hours after dosing. While not significantly different, flunixin and ketoprofen-treated piglets appeared to gain slightly less weight than meloxicam-treated piglets. This could indicate a link between increased activity and decreased weight gain. Weight gain may not be a helpful measure for determining pain as painful piglets are more likely to have reduced activity, therefore reduced calorie expenditure and possibly increased weight gain. There is conflicting evidence to support whether analgesia/anesthesia can improve weight gain following castration, and very few efficacy studies measured the long-term weight gain of piglets. Of those that did, different analgesics/anesthetics were administered, so it is difficult to draw any meaningful conclusions [40–42] . Future studies would need to monitor weight gain and activity for a longer time to determine any longer-term effects, as the meloxicam-treated piglets in this study had not returned to baseline activity levels by 48 hours (the end of the study).

5.6 Limitations

This study has some limitations worth consideration. Piglets were removed from their litters/sows and individually housed. While this is not reflective of farm management, it was necessary to maintain patency of IV catheters/ISF probes and to prevent damage to the accelerometer. Removal from the sow, individual housing and use of milk replacer are all possible sources of additional stress, and this must be considered before attempting to extrapolate the results of this study to on-farm conditions. All piglets were treated, handled, and housed identically; thus, it is unlikely that this limitation affected the primary outcomes (i.e., the comparison of meloxicam, flunixin and ketoprofen within this study), however, on-farm efficacy studies would need to be performed to validate these results. Additionally, NSAIDs were administered 2 hours before processing. In a production setting, to administer a drug and then return 2 hours later may not be

practical. This timing was chosen purposefully to ensure NSAID concentrations would reach maximum levels in the tissues- one of the main sites of anti-inflammatory and analgesic actions of these drugs- by the time of processing [27]. Finally, it is difficult to differentiate between stress, pain, and inflammation, and none of the measures used in this study directly measure 'pain', which can also be a subjective experience. However, in combination, these objective measures can provide some insight into the analgesic effects of these NSAIDs.

5.7 Conclusion

In summary, flunixin (2.2 mg/kg) or ketoprofen (3.0 mg/kg) administered intramuscularly 2 hours before processing, were shown to be the most efficacious treatments in this study within the limitation of the three objective parameters that were assessed for analgesic effects. Additionally, meloxicam appears to be inferior as an NSAID analgesic to prevent pain associated with piglet processing (at a dose of 0.4 mg/kg), despite its current use in the EU and Canada. Similar findings have been reported by other investigators [26]. This study was the first to investigate changes in activity associated with processing using a wearable accelerometer in piglets. These results demonstrate that management strategies including administration of intramuscular flunixin or ketoprofen to reduce pain associated with processing will likely improve piglet health and welfare in the United States. Future investigation will involve PK/PD modeling to correlate plasma and tissue (interstitial fluid) concentrations with efficacy, based on the promising parameters in this paper, with the goal of establishing a standard in drug concentrations and physiological/behavioral outcomes to which other analgesics can be compared.

5.8 Funding

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CHAPTER 6

Pharmacokinetic/Pharmacodynamic Modeling of Ketoprofen and Flunixin at Piglet Castration and Tail-Docking

6.1 Abstract

This study performed population-pharmacokinetic/pharmacodynamic (pop-PK/PD) modeling of ketoprofen and flunixin in piglets undergoing routine castration and tail-docking, utilizing previously published data. Six-day-old male piglets (8/group) received either ketoprofen (3.0 mg/kg) or flunixin (2.2 mg/kg) intramuscularly. Two hours post-dose, piglets were castrated and tail docked. Inhibitory indirect response models were developed utilizing plasma cortisol or interstitial fluid prostaglandin E2 (PGE2) concentration data. IC50 for ketoprofen utilizing PGE2 as a biomarker was 0.08 μg/mL, and ED50 for was 5.83 mg/kg. The ED50 calculated using cortisol was 4.36 mg/kg, however, the IC50 was high, at 2.56 μg/ml. There was also a large degree of inter-individual variability (124.08%) associated with the cortisol IC50 following ketoprofen administration. IC50 for flunixin utilizing cortisol as a biomarker was 0.06 μg/mL, and ED50 was 0.51 mg/kg. The results show that the currently marketed doses of ketoprofen (3.0 mg/kg) and flunixin (2.2 mg/kg) correspond to drug responses of 33.97% (ketoprofen-PGE2), 40.75% (ketoprofen-cortisol), and 81.05% (flunixin-cortisol) of the maximal possible responses. Given this information, flunixin may be the best NSAID to use in mitigating castration and tail-docking pain at the current label dose.

6.2 Introduction

In the United States, piglets are subjected to routine yet painful husbandry procedures such as castration and tail-docking without analgesia. In the EU and Canada, certain nonsteroidal anti-inflammatory drugs (NSAIDs) are approved to reduce pain associated with castration; however, there are no FDA-approved medications indicated to treat pain in pigs in the US. Several studies have assessed the effectiveness of NSAIDs to reduce pain associated with castration; however, there are conflicting results regarding their analgesic efficacy, and pharmacodynamic effects are highly variable (1–7). In addition, there is limited knowledge regarding the optimal dose required to provide effective analgesic and anti-inflammatory effects.

The primary mechanism of action for NSAIDs is inhibition of the cyclooxygenase (COX) enzyme. The COX-2 isoform is inducible and upregulated by tissue damage and inflammatory stimuli, increasing production of prostaglandins, and prostaglandin E2 (PGE2) increases following castration and tail docking in piglets (8,9). Prostaglandins contribute to pain signaling by activating and sensitizing nociceptors (10), leading to an increase in the magnitude of response to noxious stimulation. Inhibition of the COX-2 isoform is the most likely mechanism for NSAID-mediated analgesia (11); therefore, reduction of PGE2 should decrease nociception following castration and tail docking (1). In response to stress, corticotrophin-releasing hormone (CRH) is released by the hypothalamus, stimulating the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary which acts on the adrenal gland to produce cortisol. Prostaglandins also directly stimulate ACTH and cortisol release (12), and piglet castration research commonly uses cortisol as an indirect measure of pain (1,3,8,13–16) . A panel of experts performed a systematic review of available data related to pain mitigation during piglet husbandry procedures. Most of the panel

decisions relied primarily on cortisol as an outcome, leading to a weak recommendation for the use of NSAIDs (17).

Several PK/PD models are published that describe the effect of NSAIDs on pain and inflammation in other veterinary species, and IC50 values (specifically describing PGE2 suppression) have been generated for NSAIDs in other species as well (Table 6.1). However, there are relatively few PK/PD models for NSAIDs in pigs (18,19). Both previously published PK/PD models for NSAIDs in pigs were developed via an induced inflammation model rather than routine castration, and these models also did not establish IC50 values for suppression of PGE2. In addition, studies have suggested that plasma drug concentrations do not always reflect tissue drug concentrations, particularly for NSAIDs, which may become "trapped" at sites of inflammation (20–22). The previously published portion of this study shows that NSAIDs given before processing procedures may reduce pain and inflammation associated with castration and tail docking (8), and interstitial fluid drug concentrations were also previously reported (23). Interstitial fluid collected via *in vivo* ultrafiltration allows the measurement of only the pharmacologically active, protein-unbound drug concentrations, critical to assess drug concentrations directly at the tissue level and may better correlate with the anti-inflammatory effect than plasma concentrations. The authors are not currently aware of any PK/PD models that assess the impact of NSAIDs on cortisol concentrations in any species, and there are no reported IC50 values specifically describing the effect on cortisol.

The objective of this study was to study the anti-inflammatory and analgesic effects of flunixin and ketoprofen at piglet castration and tail-docking and to establish critical pharmacodynamic parameters for these effects via pop-PK/PD modeling.

Table 6.1. Overview of meloxicam, flunixin, and ketoprofen (racemic and S-(+)-ketoprofen) pharmacodynamic parameters collected from existing literature for various species, where PGE2 inhibition was the outcome of interest.

Species	IC_{20}	IC_{50}	IC_{80}	Imax	Source
	µg/mL	µg/mL	µg/mL	%	
Meloxicam					
Dog	-	0.160*	-	-	(24)
Horse	-	0.040**	4.129**	-	(25)
Human	-	0.246*	-	-	(26)
Flunixin					
Alpaca	-	-	0.230	-	(27)
Calf	-	-	0.026 (pain)	-	(28)
	-	-	0.039 (no pain)	-	(28)
	0.002*	0.009*	0.049*	123.00	(29)
Horse	-	0.033*	-	-	(30)
	-	0.063**	0.895**	-	(25)
	-	0.019	-	109.04	(31)
	-	0.053*	-	-	(24)
Racemic ketoprofen					
Calf	-	0.086	-	-	(32)
Cat	-	0.046	-	-	(33)
Dog	-	0.06*	-	-	(24)
Goat	-	0.028	-	112.00	(34)
Horse	-	0.057	-	100.88	(31)
Sheep	-	0.012	-	92.00	(35)
S-(+)-ketoprofen					
Calf	-	0.042	-	99.00	(36)
Goat	-	0.003	-	100.00	(34)
Horse	-	0.033	-	-	(37)
Sheep	-	0.007	-	94.00	(35)

*converted units from µM
**converted units from (-log M)

6.3 Materials and Methods

6.3.1 The data source for model development

The data used in developing these models was previously published (8,23). Briefly, 6-day-old male piglets (8/group) received one of five randomized treatments: intramuscular saline, meloxicam (0.4 mg/kg), flunixin (2.2 mg/kg), ketoprofen (3.0 mg/kg) or sham (saline injection, no processing). Two hours post-dose, piglets were castrated and tail docked. The previous report examined various efficacy measures; however, these models only utilize plasma cortisol or interstitial fluid PGE2 concentration data. Meloxicam was excluded from the PK/PD modeling for two reasons; 1) the PK/PD models for meloxicam did not pass the validation process due to poor model fits, 2) meloxicam was the least effective of the three NSAIDs overall, and so it is more useful to focus on flunixin and ketoprofen. The flunixin-PGE2 model was also excluded from the PK/PD modeling due to an inability to pass the validation process.

6.3.2 Population-Pharmacokinetic/Pharmacodynamic Analysis

Sequential analysis of the pharmacokinetic (PK) and pharmacodynamic (PD) data was performed using a population modeling approach with Phoenix® NLME (Version 8.3, Certara, St. Louis, MO), using first-order conditional estimation with the extended least squares algorithm. The PK model was built first to describe the time course of NSAID concentrations in plasma. Subsequently, PD modeling analysis of the anti-inflammatory effect of NSAIDs was performed. The model success was judged by the fits (observed vs. predicted data), visual predictive checks, the coefficient of variation (CV%) of parameter estimates, and the values of the Akaike Information Criterion and the Bayesian Information Criterion. The fitted parameters were assumed to be log-normally distributed. The stability and performance of the final pop-PK/PD models were assessed by a bootstrap method performed in Phoenix NLME. Bootstrap

resampling was repeated 100 times, and the values of the parameters were compared with those obtained from the original data set.

The Pharmacokinetic Models

Several alternative pop-PK models (e.g., single compartment, different residual error models, parameterization by clearance) were tested and discarded due to inferior performance before the selection of the final pop-PK base model. Secondary parameter estimates were obtained using standard compartmental equations (38).

Ketoprofen

For ketoprofen, the S-(+)-enantiomer is a much more potent COX inhibitor (39), and S-(+)-ketoprofen plasma concentration predominates over R-(-)-ketoprofen following intramuscular administration of racemic ketoprofen in piglets (23,40). Therefore, the pop-PK/PD modeling in this study utilized only the S-(+)-ketoprofen concentration data. The final model used for the pop-PK analysis of S-(+)-ketoprofen (see Figures 6.1 and 6.2) assumed first-order kinetics of absorption following intramuscular administration, one-compartmental disposition, and micro constant parameterization. Inter-individual variability (variance of a parameter among different subjects, or random effects) was expressed using an exponential error model according to the equation:

$$Pi = \theta P * \exp(\eta iP) \qquad \textit{Equation 1}$$

Where Pi is the parameter of interest for the individual i, θP is the population estimate for the parameter of interest, and ηiP is the η for the individual and parameter of interest. The η values were assumed to be independent and have a normal distribution with a mean of zero and a variance of ω^2. A multiplicative model was used to describe the residual random variability (ε) of the data

for the plasma concentrations, where ε is the residual intrasubject variability with a mean of zero and a variance of σ2, according to the equation:

$$CObs = Cpred * (1 + \varepsilon) \qquad \textit{Equation 2}$$

Where Cobs is the observed plasma drug concentration for the individual and Cpred is the model predicted plasma drug concentration.

Flunixin

The final model used for the pop-PK analysis of flunixin (see Figure 6.3) assumed first-order kinetics of flunixin absorption following intramuscular administration, two-compartmental disposition, and micro constant parameterization. Similar to the S-(+)-ketoprofen pop-PK model, inter-individual variability was expressed using an exponential error model according to equation 1. A multiplicative model was used to describe the residual random variability (ε) of the data for the plasma concentrations according to equation 2.

The Pharmacodynamic Models

Raw ISF PGE2 and plasma cortisol concentrations for each of the NSAID-treated groups were expressed as a percent difference from the control group (piglets that were castrated and tail-docked without analgesia) before modeling. The pop-PK/PD relationships were described using indirect PD response models, in which the effect (E) represents the percent difference in PGE2 or cortisol concentrations compared to the control group. Several alternative PD models (e.g., indirect response models with stimulation of Kout, with or without the addition of a shape factor, with or without an effect compartment, and combination of these factors) were tested and discarded due to inferior performance before selection of the final PD models.

Ketoprofen-PGE2 Model

For the S-(+)-ketoprofen-PGE2 model, it was assumed that the pharmacological effect (reduction of PGE2) is related to the ISF S-(+)-ketoprofen concentration (see Figure 6.1), and an indirect response PD model with inhibition of Kin was applied to link the S-(+)-ketoprofen concentration and anti-inflammatory effect:

$$\frac{dCe}{dt} = Ke0in * C - Ke0out * Ce \qquad \textit{Equation 3}$$

$$\frac{dE}{dt} = Kin * \left(1 - \frac{Imax * Ce^{\gamma}}{Ce^{\gamma} + IC50^{\gamma}}\right) - Kout * E \qquad \textit{Equation 4}$$

Where dCe/dt is the rate of change in S-(+)-ketoprofen ISF concentration, Ke0in and Ke0out are rate constants describing drug movement between the central and effect (ISF) compartments, dE/dt is the rate of change in PGE2 concentration, Ce is the S-(+)-ketoprofen concentration in ISF, γ is a curve-fitting parameter, Imax is the maximal anti-inflammatory effect, IC50 is the concentration that leads to 50% of the maximal anti-inflammatory effect, Kin is a zero-order constant for basal PGE2 production, and Kout is a first-order rate constant for the removal of PGE2 from ISF. A multiplicative model was used to describe the residual random variability (ε) of the data for both ISF S-(+)-ketoprofen and PGE2 concentrations, where ε is the residual intrasubject variability with a mean of zero and a variance of σ2, according to the equations:

$$CeObs = Cepred * (1 + \varepsilon) \qquad \textit{Equation 5}$$

$$EObs = Epred * (1 + \varepsilon) \qquad \textit{Equation 6}$$

Where CeObs is the observed ISF concentration for the individual and Cepred is the model predicted ISF concentration, EObs is the observed PGE2 concentration for the individual, and Epred is the predicted PGE2 concentration. Inter-individual variability (variance of a parameter

among different subjects or random effects) was expressed using an exponential error model according to equation 1.

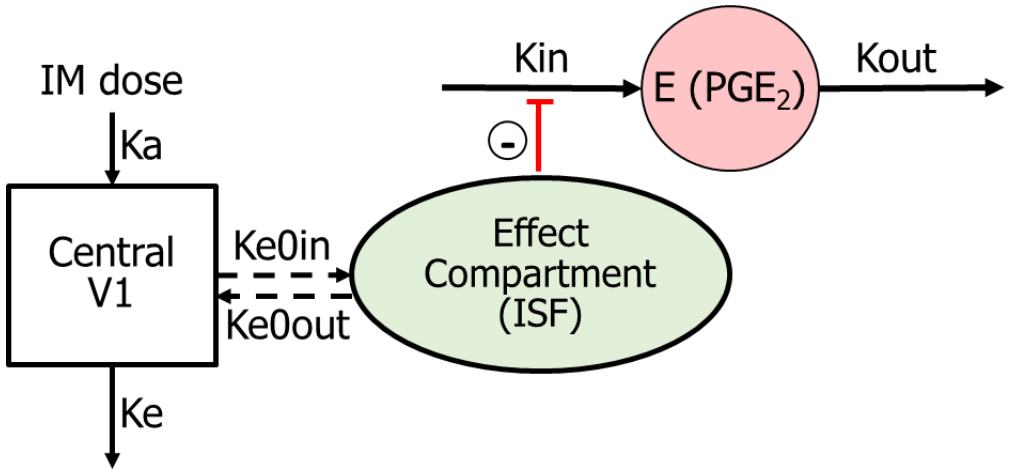


Figure 6.1. Schematic representation of the selected pharmacokinetic-pharmacodynamic model for ketoprofen-PGE2. *Following intramuscular administration, ketoprofen is absorbed into the central compartment with a first-order absorption rate constant Ka, with drug movement between the central and effect (ISF) compartments (rate constants Ke0in and Ke0out) and is eliminated with elimination constant, Ke. In the absence of the drug, elevated PGE2 concentration results from the balance of the PGE2 production (with zero-order rate constant Kin) and PGE2 removal (with first-order rate constant Kout). Ketoprofen that reaches the ISF induces an indirect anti-inflammatory effect by inhibiting PGE2 production (reduction of Kin).*

Ketoprofen-Cortisol Model

It was assumed that the pharmacological effect (reduction of cortisol) is indirectly related to the S-(+)-ketoprofen concentration in plasma (see Figure 6.2) via a hypothetical effect compartment, and a sigmoidal indirect response PD model with inhibition of Kin was applied to link the NSAID concentration and cortisol as in equation 4, where dE/dt is the rate of change in

cortisol concentration, Ce is the NSAID concentration in the effect compartment, γ is a curve-fitting parameter, Imax is the maximal inhibition of cortisol production, IC50 is the concentration that leads to 50% of the maximal inhibition of cortisol production, Kin is a zero-order constant for basal cortisol production, and Kout is a first-order rate constant for the removal of cortisol from plasma. A multiplicative model was used to describe the residual random variability (ε) of the data for cortisol concentrations, according to equation 6, where EObs is the observed cortisol concentration for the individual and Epred is the model predicted cortisol concentration. Inter-individual variability (variance of a parameter among different subjects or random effects) was expressed using an exponential error model according to equation 1.

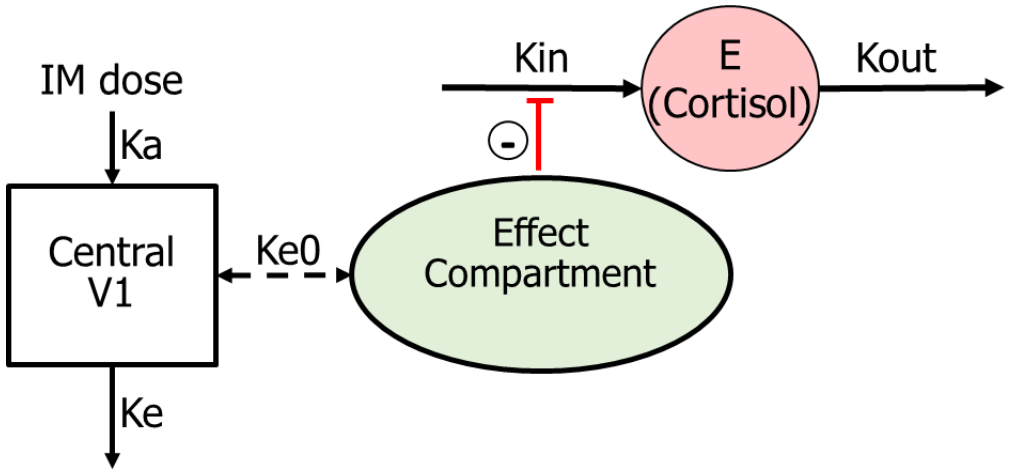


Figure 6.2. Schematic representation of the selected pharmacokinetic-pharmacodynamic model for ketoprofen-cortisol. *Following intramuscular administration, ketoprofen is absorbed into the central compartment with a first-order absorption rate constant Ka, with drug movement between the central and effect compartments (rate constant Ke0) and is eliminated with elimination constant, Ke. In the absence of the drug, elevated cortisol concentration results from the balance of cortisol production (with zero-order rate constant Kin) and cortisol removal (with first-order rate constant Kout). Ketoprofen induces an indirect reduction of cortisol (reduction of Kin).*

Flunixin-Cortisol Model

It was assumed that the pharmacological effect (reduction of cortisol) is indirectly related to the flunixin concentration in the plasma (see Figure 6.3), and an indirect response PD model with inhibition of Kin was applied to link the flunixin concentration and cortisol:

$$\frac{dE}{dt} = Kin * \left(1 - \frac{Imax * C}{C + IC50}\right) - Kout * E \qquad \textit{Equation 7}$$

Where dE/dt is the rate of change in cortisol concentration, C is the flunixin concentration in plasma, Imax is the maximal inhibition of cortisol production, IC50 is the concentration that leads to 50% of the maximal inhibition of cortisol production, Kin is a zero-order constant for basal cortisol production, and Kout is a first-order rate constant for the removal of cortisol from plasma. A Poisson model was used to describe the residual random variability (ε) of the data for cortisol concentrations:

$$EObs = Epred + (Epred^{0.5} * \varepsilon) \qquad \textit{Equation 8}$$

Where EObs is the observed percent difference in cortisol concentration for the individual and Epred is the model predicted percent difference cortisol concentration. Inter-individual variability (variance of a parameter among different subjects or random effects) was expressed using an exponential error model according to equation 1.

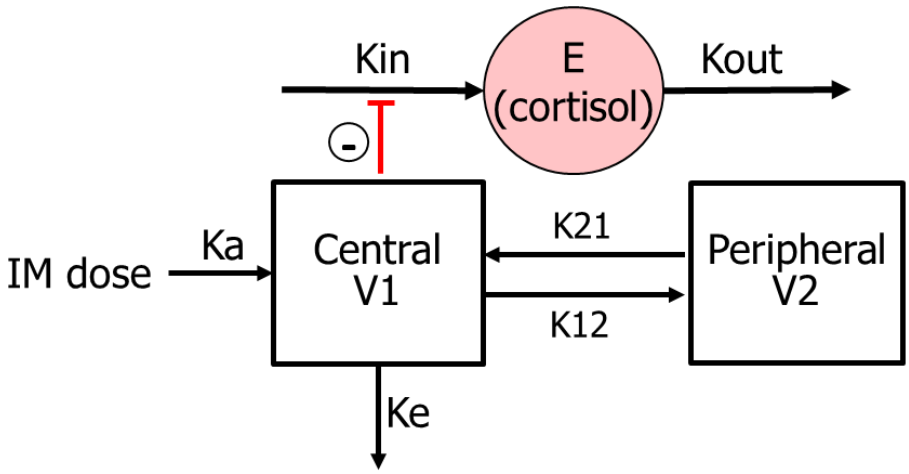


Figure 6.3. Schematic representation of the selected pharmacokinetic-pharmacodynamic model for flunixin-cortisol. *Following intramuscular administration, flunixin is absorbed into the central compartment with a first-order absorption rate constant Ka, with drug movement between the central and peripheral compartments (rate constants K12 and K21) eliminated with elimination constant, Ke. In the absence of the drug, elevated cortisol concentration results from the balance of cortisol production (with zero-order rate constant Kin) and cortisol removal (with first-order rate constant Kout). Flunixin induces an indirect reduction of cortisol (reduction of Kin).*

Median Effective Dose Calculation

Using the finalized pop-PK/PD models, the predicted time course of PGE2 and cortisol was simulated after single intramuscular doses of each NSAID at 15 doses ranging from 0 to 100 mg/kg. The area under the time-response curve (AUR) was then calculated at each simulated dose, and the net effect was determined by subtracting the AUR corresponding to an absence of NSAID (i.e., a simulated dose of 0 mg/kg). The average drug response was then expressed as a percentage of the maximum possible average drug response, i.e., the response obtainable for a very high dose of NSAID (e.g., 100 mg/kg). The maximum possible drug response percentage

was plotted against the simulated dose and used to interpolate the median effective dose (ED50; GraphPad Prism version 9.1.2, GraphPad Software, San Diego, California, USA).

6.4 Results

6.4.1 Ketoprofen-PGE2

The individual plasma concentration-time curves for S-(+)-ketoprofen are shown in Figure 6.4, and the individual interstitial fluid concentration-time curves are shown in Figure 6.5. The plasma and ISF PK parameters are presented in Tables 6.2 and 6.3. The absorption half-life was short, and peak S-(+)-ketoprofen plasma concentration was reached rapidly. The elimination half-life was 3.45 hours. Penetration to the ISF was only 6.64% of the plasma concentration. Figure 6.6 shows the individual percent difference in PGE2 concentrations over time compared to piglets that were castrated but not treated with an NSAID. The population fits for plasma S-(+)-ketoprofen, ISF S-(+)-ketoprofen, and ISF PGE2 are shown in Figure 6.7-9. The PD parameters are presented in Table 6.4. Pop-PK/PD modeling of PGE2 gave an IC50 of 0.08 μg/mL for S-(+)-ketoprofen and an ED50 of 5.83 mg/kg. Simulation of the dose-effect relationship showed that a dose of 3.0 mg/kg corresponds to a drug response of 33.97% of the maximal possible response (Figure 6.10). The visual predictive checks and the simulations of the effect following 15 different doses (0-100 mg/kg) are provided in the supplementary data (Appendix A Figure 6.S1-4).

Table 6.2. Population pharmacokinetic estimates for S-(+)-ketoprofen in piglet plasma, following administration to piglets intramuscularly at a dose of 3.0 mg/kg before castration and tail-docking.

Parameter	**Estimate**	**Units**	**CV%**	**2.5% CI**	**97.5% CI**	**IIV%**	**Bootstrap Median Estimates**	**Bootstrap CV%**
Tmax	0.61	h	10.12	0.49	0.74	-	-	-
Cmax	8.78	µg/mL	5.16	7.87	9.68	-	-	-
Ka	5.63	1/h	13.65	4.09	7.16	-	5.55	15.81
Ka $t_{1/2}$	0.12	h	13.65	0.09	0.16	-	-	-
Ke	0.20	1/h	8.43	0.17	0.23	23.31	0.20	8.65
Ke t1/2	3.45	h	7.64	2.87	4.03	-	-	-
MRT	4.98	h	8.43	4.14	5.82	-	-	-
$AUC_{0\text{-}48h}$	49.46	h.µg/mL	9.60	32.22	68.85	-	-	-
Vd/F	302.11	mL/kg	5.25	270.43	333.79	12.92	301.88	5.30
Cl/F	60.66	mL/h/kg	9.60	49.05	72.32	-	-	-

Tmax, time of maximal concentration; Cmax, maximal concentration; Ka, absorption rate constant; Ka t1/2, absorption half-life; Ke, elimination rate constant; Ke t1/2, elimination half-life; MRT, mean residence time; $AUC_{0\text{-}48h}$, area under the concentration vs. time curve from 0-48 hours; Vd/F, apparent volume of distribution (per fraction absorbed); Cl/F, apparent total body clearance (per fraction absorbed); IIV, inter-individual variability.

- not applicable

Table 6.3. Population pharmacokinetic estimates for S-(+)-ketoprofen in piglet interstitial fluid, following administration to piglets intramuscularly at a dose of 3.0 mg/kg before castration and tail-docking.

Parameter	**Estimate**	**Units**	**CV%**	**2.5% CI**	**97.5% CI**	**IIV%**	**Bootstrap Median Estimates**	**Bootstrap CV%**
Ke0in	0.01	1/h	8.36	0.010	0.013	8.63	0.01	13.30
Ke0in t1/2	59.91	h	8.36	49.95	69.88	-	-	-
Ke0out	0.17	1/h	10.57	0.14	0.21	9.22	0.17	16.02
Ke0out t1/2	3.98	h	10.57	3.14	4.82	-	-	-
AUC	3.58	h.µg/mL	20.95	2.07	4.08	-	-	-
Penetration factor	6.64	%	24.16	5.40	9.85	-	-	-

Tmax, time of maximal concentration; Cmax, maximal concentration; AUC, area under the concentration vs. time curve from 0-48 hours; Penetration factor, the ratio of AUC values of ISF and plasma; IIV, inter-individual variability. - not applicable.

Table 6.4. Population pharmacodynamic estimates describing the inhibitory effect of S-(+)-ketoprofen on interstitial fluid PGE2 production, following administration to piglets intramuscularly at a dose of 3.0 mg/kg before castration and tail-docking.

Parameter	**Estimate**	**Units**	**CV%**	**2.5% CI**	**97.5% CI**	**IIV%**	**Bootstrap Median Estimates**	**Bootstrap CV%**
Kin	207.20	%/h	36.26	57.70	356.69	-	195.90	60.41
Kout	2.34	1/h	24.09	1.22	3.46	-	2.19	38.62
Imax	97.33	%	1.51	94.41	100.25	-	96.80	11.93
IC50	0.08	ug/mL	39.37	0.02	0.15	6.39	0.07	53.19
Gamma	2.07		23.55	1.10	3.05	-	2.51	113.77
ED50	5.83	mg/kg	-	-	-	-	-	-

Kin, zero-order constant for basal PGE2 production; Kout, first-order rate constant for the removal of PGE2 from ISF; Imax, maximal anti-inflammatory effect; IC50, the concentration that leads to 50% of the maximal anti-inflammatory effect; gamma, exponent expressing sigmoidicity of the concentration-effect relationship; ED50, median effective dose; IIV, inter-individual variability. - not applicable.

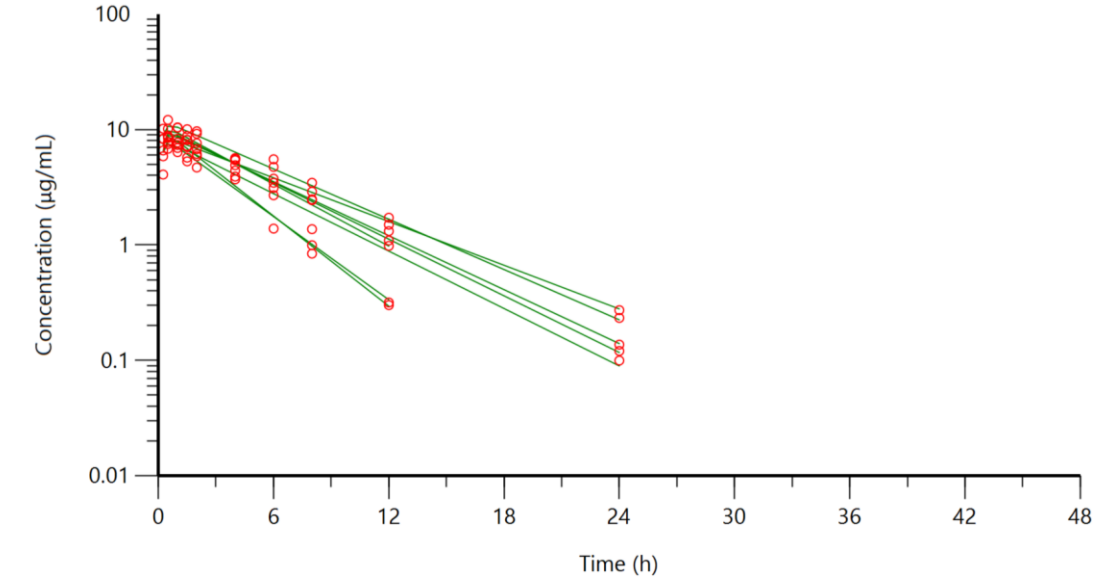


Figure 6.4. Individual fits of plasma concentrations for S-(+)-ketoprofen in piglets following an intramuscular dose of 3.0 mg/kg. *The observed individual plasma concentrations are represented by the open circles, and the model fits are shown by the solid lines.*

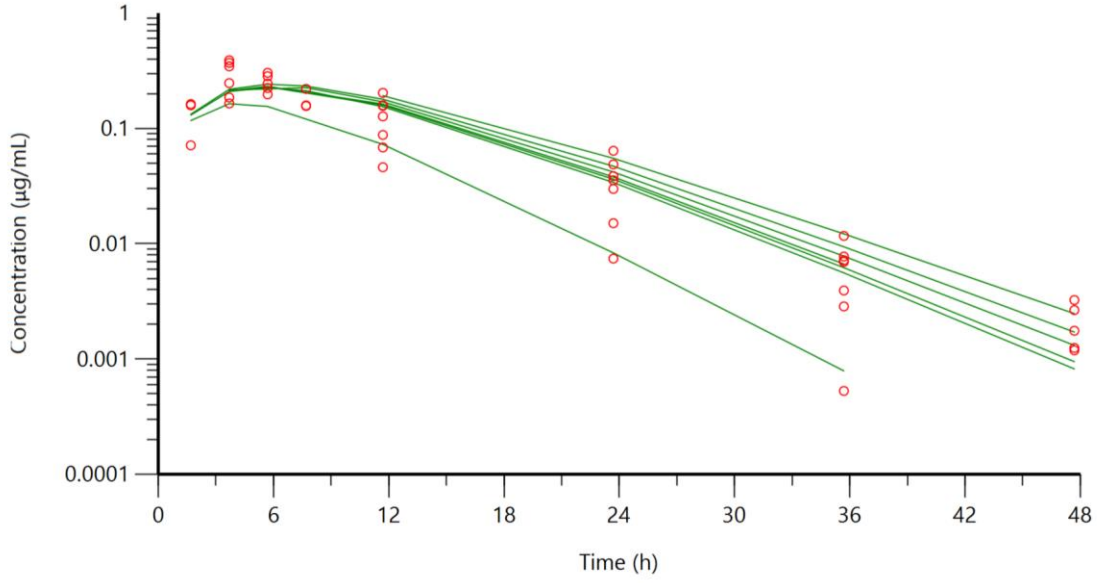


Figure 6.5. Individual fits of ISF concentrations for S-(+)-ketoprofen in piglets following an intramuscular dose of 3.0 mg/kg. *The observed individual ISF concentrations are represented by the open circles, and the model fits are shown by the solid lines.*

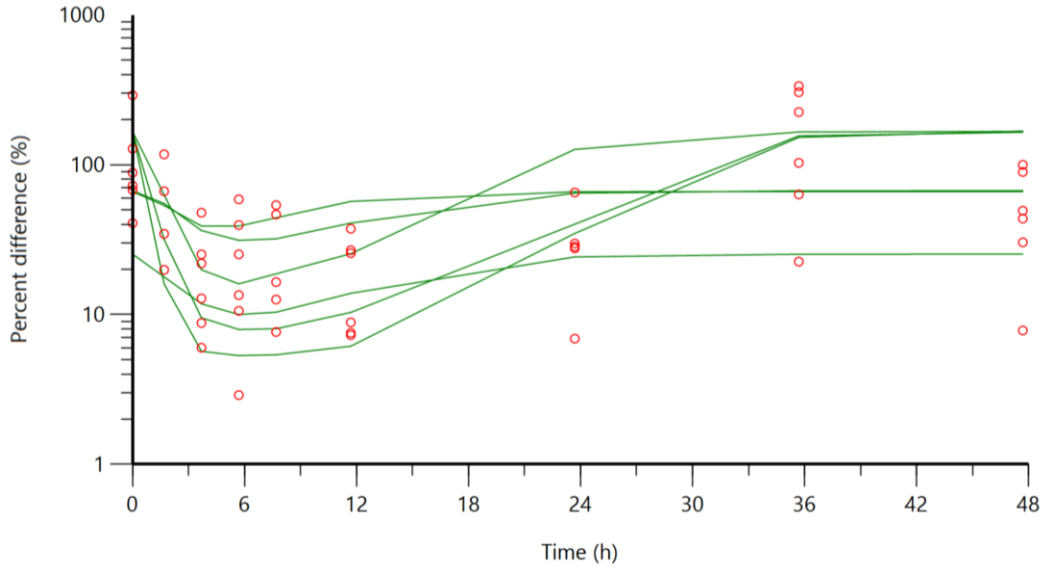


Figure 6.6. Individual fits of the percent difference in PGE2 concentrations in piglet ISF, compared to control piglets not given an analgesic, following an intramuscular dose of 3.0 mg/kg and castration and tail docking*. The observed values are represented by the open circles, and the model fits are shown by the solid lines.*

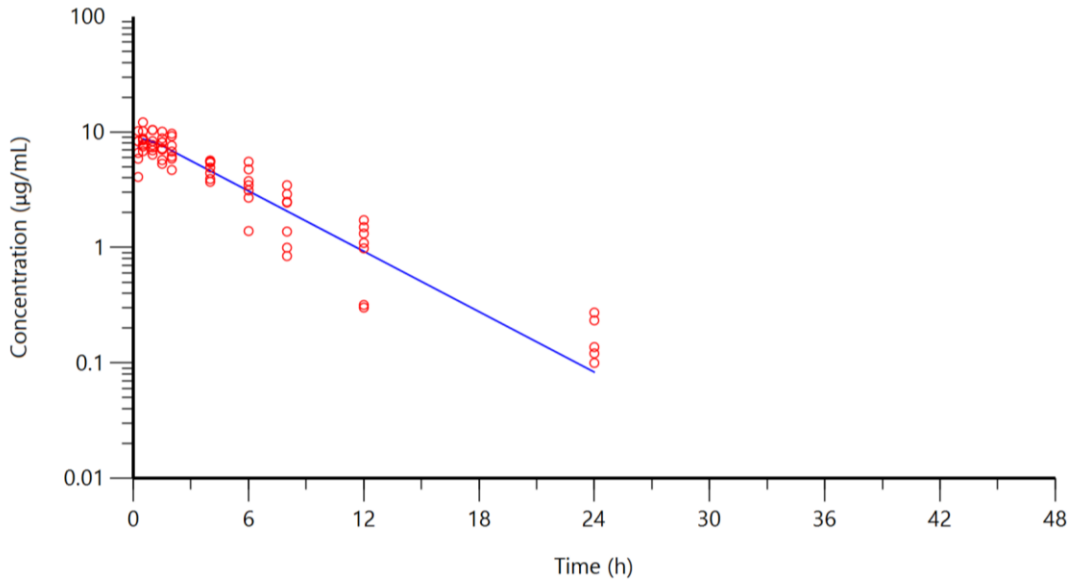


Figure 6.7. Population fit of plasma concentrations for S-(+)-ketoprofen in piglets following an intramuscular dose of 3.0 mg/kg. *The observed individual ISF concentrations are represented by the open circles, and the model fits are shown by the solid lines.*

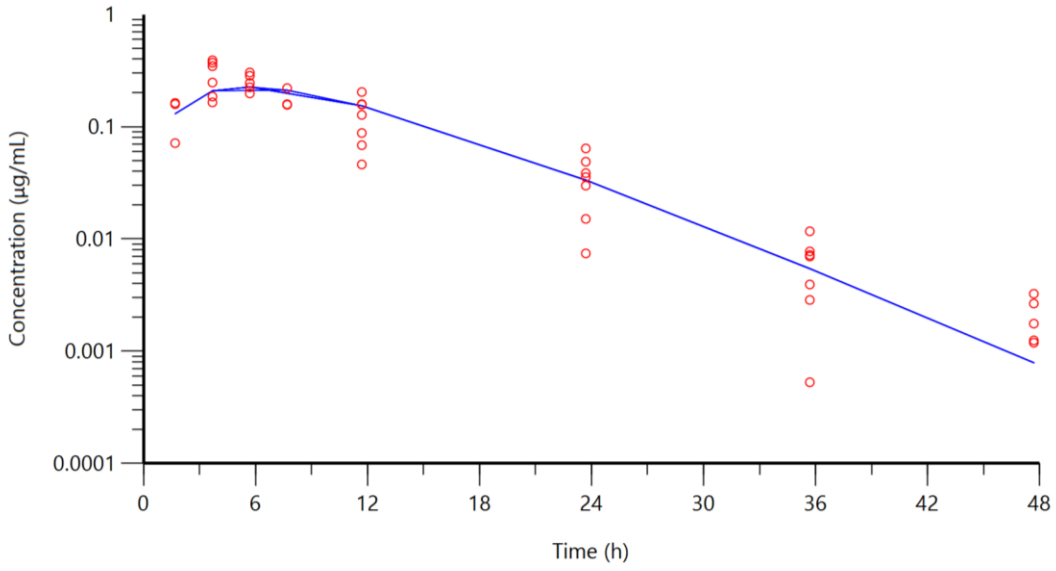


Figure 6.8. Population fit of ISF concentrations for S-(+)-ketoprofen in piglets following an intramuscular dose of 3.0 mg/kg. *The observed individual ISF concentrations are represented by the open circles, and the model fits are shown by the solid lines.*

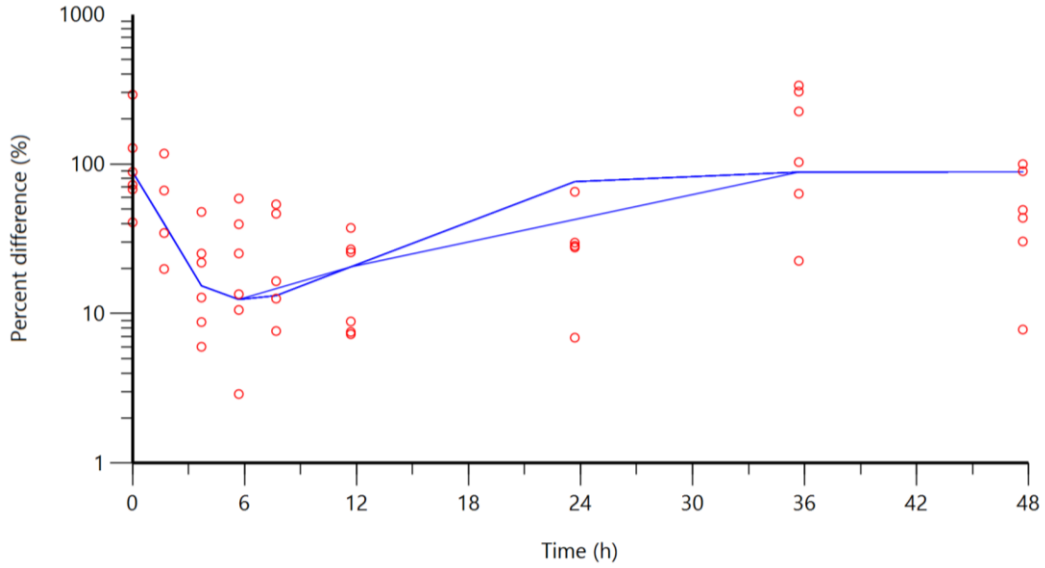


Figure 6.9. Population fit of the percent difference in PGE2 concentrations in piglet ISF, compared to control piglets not given an analgesic, following an intramuscular dose of 3.0 mg/kg and castration and tail docking. *The observed values are represented by the open circles, and the model fits are shown by the solid lines.*

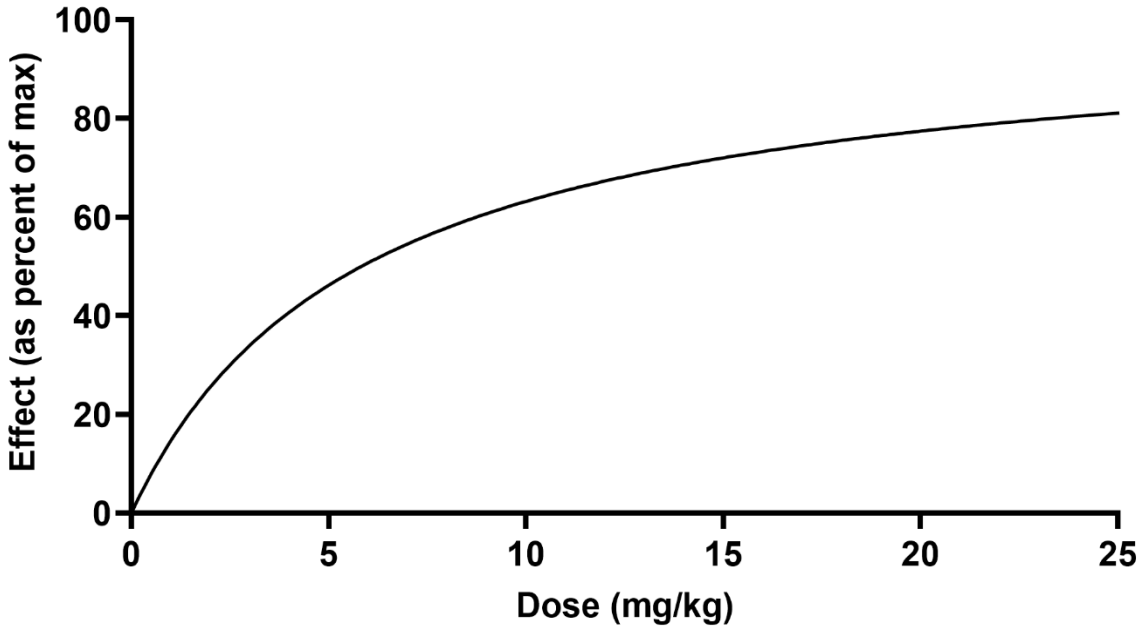


Figure 6.10. The relationship between ketoprofen administered intramuscularly and the inhibition of PGE2 production in piglets undergoing castration and tail-docking.

6.4.2 Ketoprofen-Cortisol

The results for the plasma pop-PK model are shown in the section above. Figure 6.11 shows the individual percent difference in cortisol concentrations over time compared to piglets that were castrated but not treated with an NSAID. The population fits for cortisol are shown in Figure 6.12. The PD parameters are presented in Table 6.5. Pop-PK/PD modeling of cortisol gave an IC50 of 2.56 μg/mL for S-(+)-ketoprofen and an ED50 of 4.36 mg/kg. Simulation of the dose-effect relationship showed that a dose of 3.0 mg/kg corresponds to a drug response of 40.75% of the maximal possible response (Figure 6.13). The visual predictive checks and the simulations of the effect following 15 different doses (0-100 mg/kg) are provided in the supplementary data (S5-6).

Table 6.5. Population pharmacodynamic estimates describing the inhibitory effect of S-(+)-ketoprofen on plasma cortisol production, following administration to piglets intramuscularly at a dose of 3.0 mg/kg prior to castration and tail-docking.

Parameter	**Estimate**	**Units**	**CV%**	**2.5% CI**	**97.5% CI**	**IIV%**	**Bootstrap Median Estimates**	**Bootstrap CV%**
Ke0	15.35		48.56	0.60	30.10	-	14.72	320.89
Kin	65.46	%/h	28.11	29.04	101.87	30.28	86.38	221.19
Kout	0.72	1/h	25.41	0.36	1.08	-	0.87	218.52
Imax	73.00	%	12.67	0.55	0.91	-	66.00	22.82
IC50	2.56	ug/mL	35.68	0.75	4.37	124.08	2.44	70.22
Gamma	1.90		29.86	0.78	3.03	78.87	2.95	222.20
ED50	4.36	mg/kg	-	-	-	-	-	-

Kin, zero-order constant for basal cortisol production; Kout, first-order rate constant for the removal of cortisol; Imax, maximal anti-inflammatory effect; IC50, the concentration that leads to 50% of the maximal anti-inflammatory effect; gamma, exponent expressing sigmoidicity of the concentration-effect relationship; ED50, median effective dose; IIV, inter-individual variability. - not applicable.

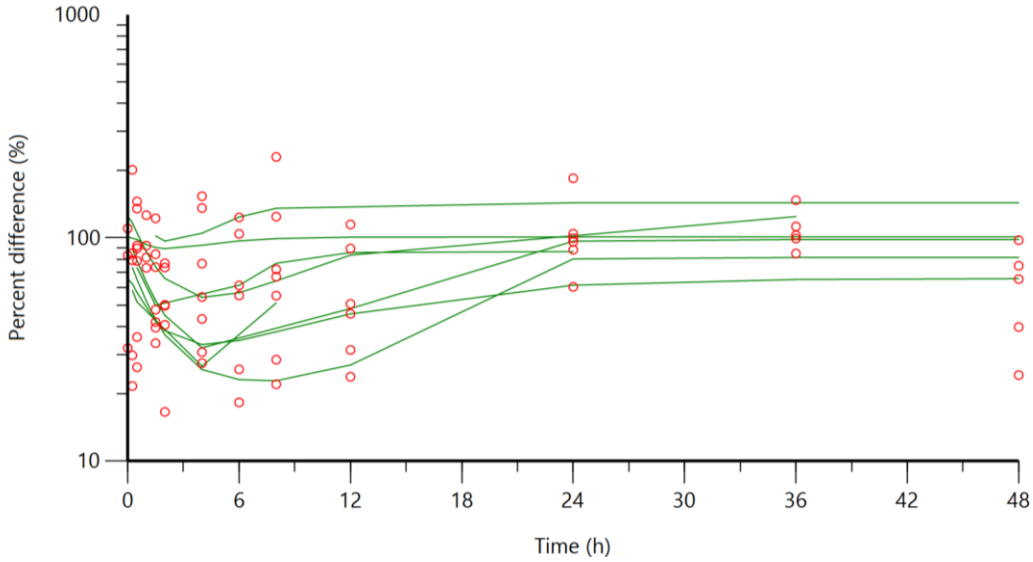


Figure 6.11. Individual fits of the percent difference in cortisol concentrations in piglet plasma, compared to control piglets not given an analgesic, following an intramuscular dose of 3.0 mg/kg and castration and tail docking*. The observed values are represented by the open circles, and the model fits are shown by the solid lines.*

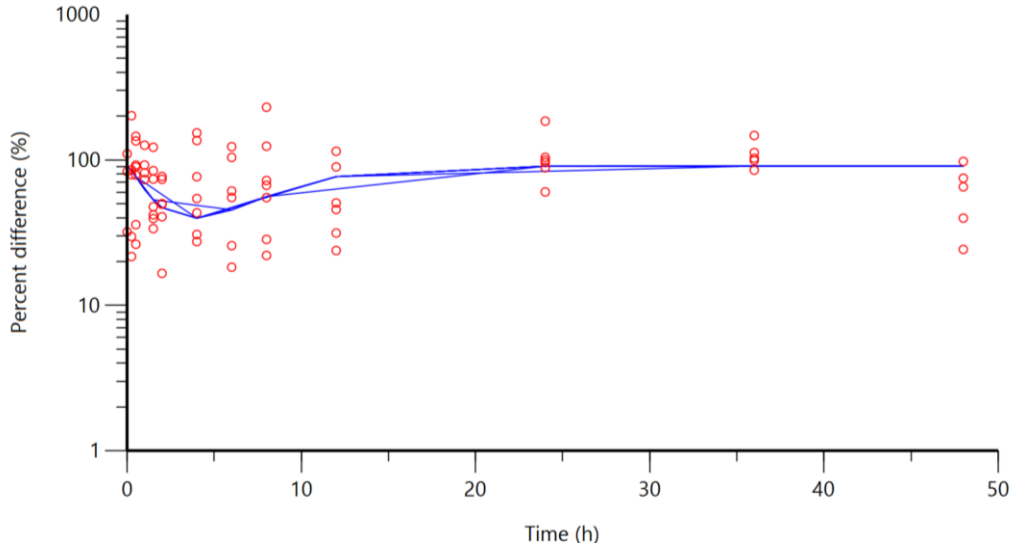
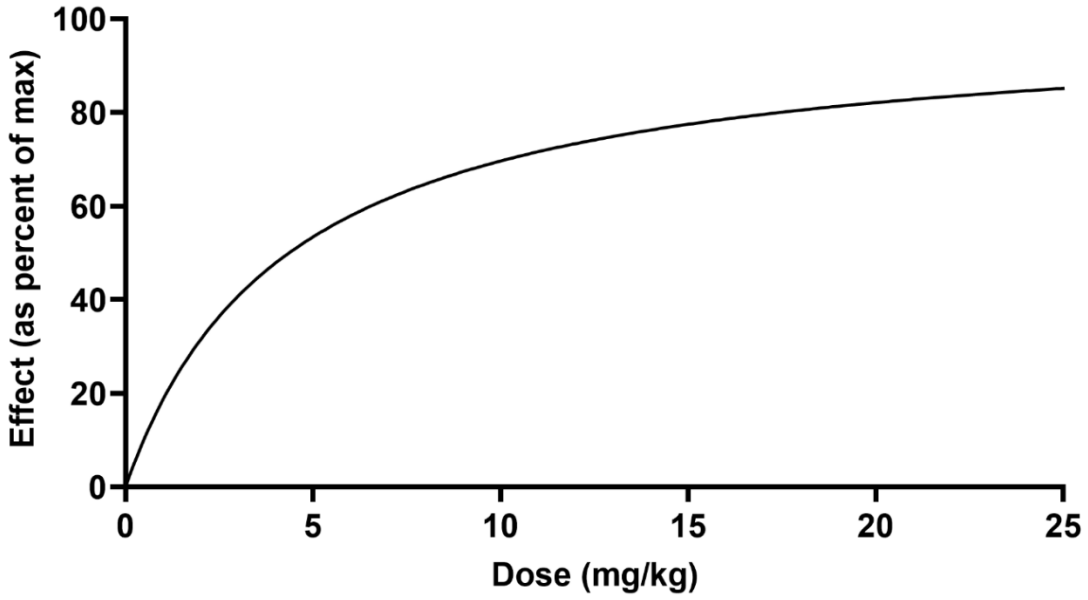


Figure 6.12. Population fit of the percent difference in cortisol concentrations in piglet plasma, compared to control piglets not given an analgesic, following an intramuscular dose of 3.0 mg/kg and castration and tail docking*. The observed values are represented by the open circles, and the model fits are shown by the solid lines.*



6.13. The relationship between ketoprofen administered intramuscularly and the inhibition of cortisol production in piglets undergoing castration and tail-docking.

6.4.3 Flunixin-Cortisol

The individual plasma concentration-time curves for flunixin are shown in Figure 6.14, and the plasma pop-PK parameters are presented in Table 6.6. The absorption half-life was short, and peak flunixin plasma concentration was reached rapidly. The elimination half-life was 4.42 hours. Figure 6.15 shows the individual percent difference in cortisol concentrations over time compared to piglets that were castrated but not treated with an NSAID. The population fits for plasma flunixin and plasma cortisol are shown in Figure 6.16-17. The PD parameters are presented in Table 6.7. Pop-PK/PD modeling of cortisol gave an IC50 of 0.06 μg/mL and an ED50 of 0.51 mg/kg for flunixin. Simulation of the dose-effect relationship showed that a dose of 2.2 mg/kg corresponds to a drug response of 81.05% of the maximal possible response (Figure 6.18). The visual predictive checks and the simulations of the effect following 15 different doses (0-100 mg/kg) are provided in the supplementary data (S7-9).

Table 6.6. Population pharmacokinetic estimates for flunixin in piglet plasma, following administration to piglets intramuscularly at a dose of 2.2 mg/kg before castration and tail-docking.

Parameter	**Estimate**	**Units**	**CV%**	**2.5% CI**	**97.5% CI**	**IIV%**	**Bootstrap Median Estimates**	**Bootstrap CV%**
Tmax	0.51	h	14.88	0.36	0.66	-	-	-
Cmax	3.03	µg/mL	16.01	2.06	4.00	-	-	-
Ka	6.68	1/h	22.29	3.71	9.65	-	6.66	23.66
Ka $t_{1/2}$	0.10	h	22.29	0.06	0.15	-	-	-
Ke	0.16	1/h	13.60	0.11	0.20	31.44	0.16	28.62
Ke t1/2	4.42	h	13.60	3.22	5.62	-	-	-
K12	0.11	1/h	40.66	0.02	0.20	71.98	0.11	78.69
K21	0.16	1/h	41.66	0.03	0.29	94.78	0.18	50.50
MRT	6.38	h	13.60	4.65	8.11	-	-	-
AUC	22.06	h.µg/mL	19.86	13.32	30.80	-	-	-
Vd/F	636.17	mL/kg	16.63	425.09	847.24	41.07	618.87	21.46
Cl/F	99.73	mL/h/kg	19.86	60.21	139.26	-	-	-

Tmax, time of maximal concentration; Cmax, maximal concentration; Ka, absorption rate constant; Ka t1/2, absorption half-life; Ke, elimination rate constant; Ke t1/2, elimination half-life; MRT, mean residence time; AUC, area under the concentration vs. time curve; Vd/F, apparent volume of distribution (per fraction absorbed); Cl/F, apparent total body clearance (per fraction absorbed); IIV, inter-individual variability.

- not applicable

Table 6.7. Population pharmacodynamic estimates describing the inhibitory effect of flunixin on plasma cortisol production, following administration to piglets intramuscularly at a dose of 2.2 mg/kg before castration and tail-docking.

Parameter	**Estimate**	**Units**	**CV%**	**2.5% CI**	**97.5% CI**	**IIV%**	**Bootstrap Median Estimates**	**Bootstrap CV%**
Kin	44.93	%/(h)	33.89	14.35	75.51	38.58	57.68	53.84
Kout	0.46	1/h	26.78	0.21	0.71	-	0.56	46.55
Imax	72.02	%	11.20	0.56	0.88	13.26	72.35	12.32
IC50	0.06	μg/mL	47.70	0.00	0.12	10.62	0.07	94.10
ED50	0.51	-	-	-	-	-	-	-

Kin, zero-order constant for basal cortisol production; Kout, first-order rate constant for the removal of cortisol; Imax, maximal anti-inflammatory effect; IC50, the concentration that leads to 50% of the maximal anti-inflammatory effect; ED50, median effective dose; IIV, inter-individual variability.

- not applicable.

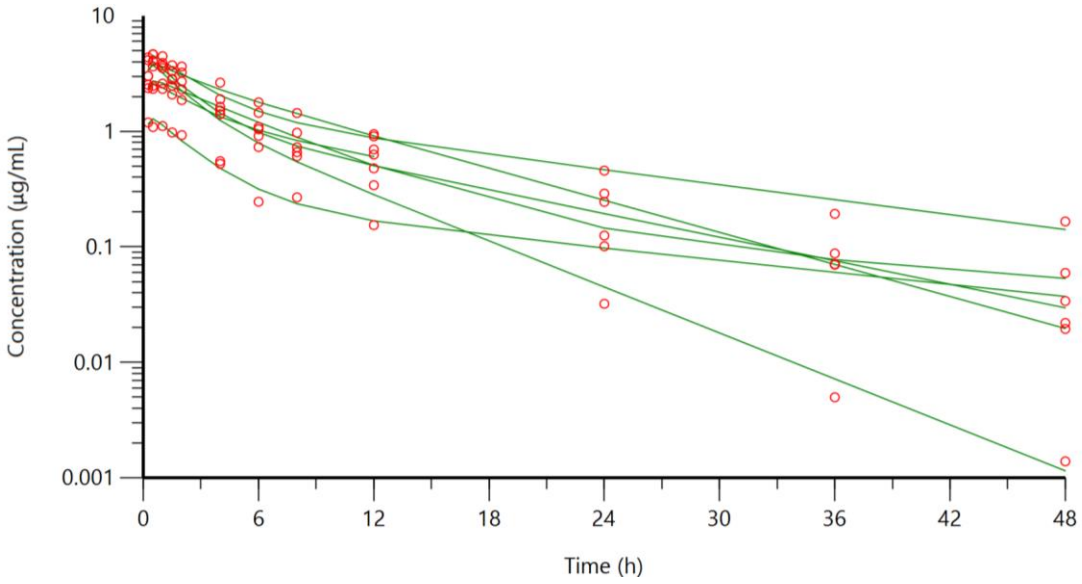


Figure 6.14. Individual fits of plasma concentrations for flunixin in piglets following an intramuscular dose of 2.2 mg/kg. *The observed individual plasma concentrations are represented by the open circles, and the model fits are shown by the solid lines.*

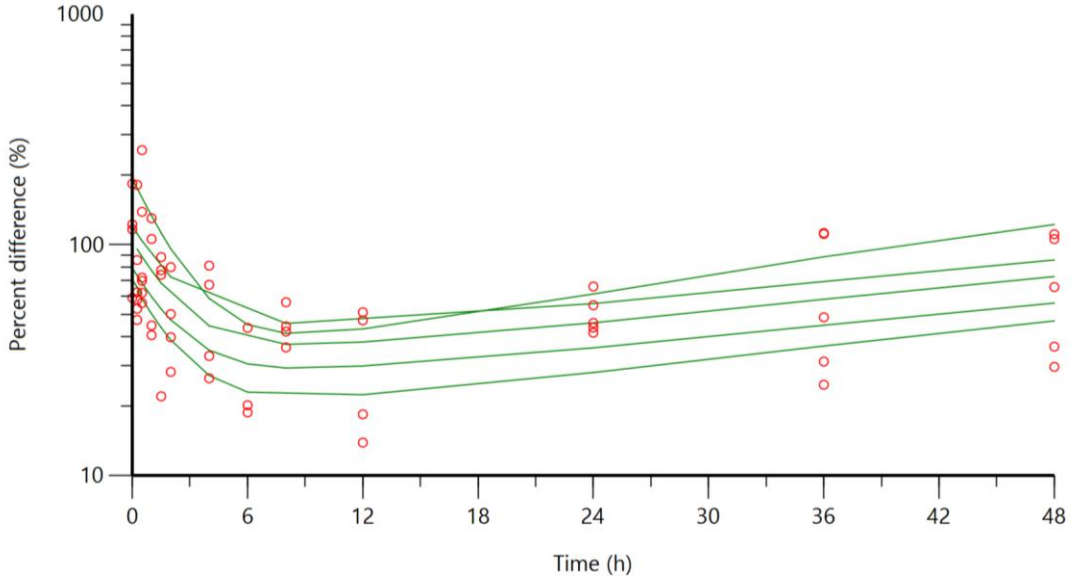


Figure 6.15. Individual fits of the percent difference in cortisol concentrations in piglet plasma, compared to control piglets not given an analgesic, following an intramuscular dose of 3.0 mg/kg and castration and tail docking. *The observed values are represented by the open circles, and the model fits are shown by the solid lines.*

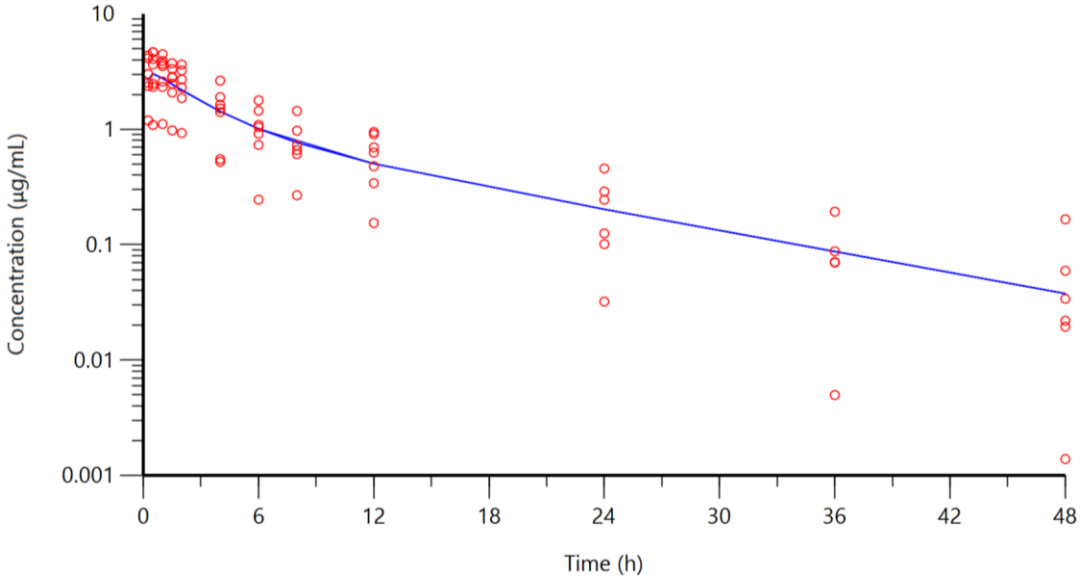


Figure 6.16. Population fit of plasma concentrations for flunixin in piglets following an intramuscular dose of 2.2 mg/kg. *The observed individual ISF concentrations are represented by the open circles, and the model fits are shown by the solid lines.*

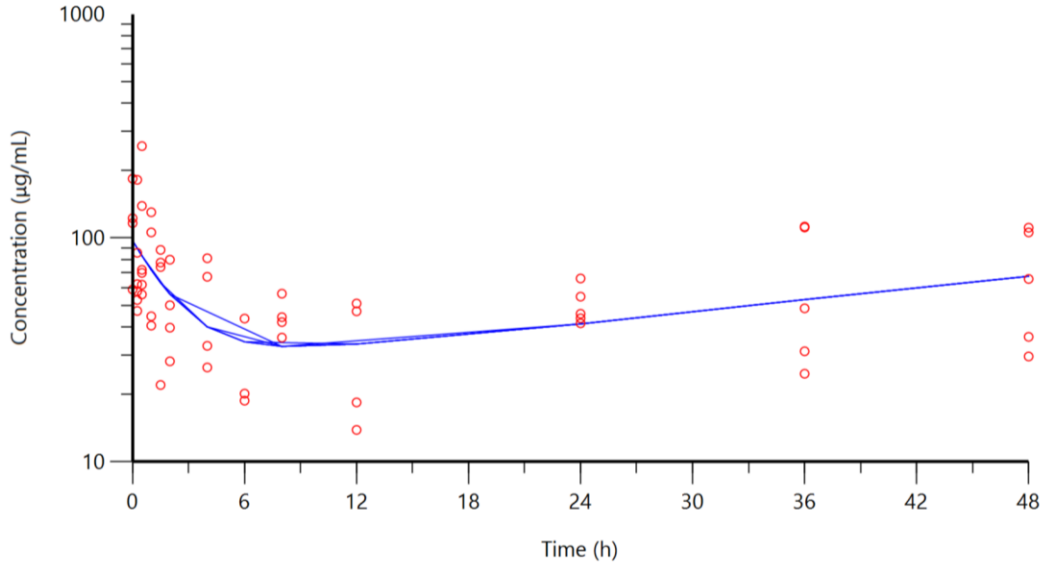


Figure 6.17. Population fit of the percent difference in cortisol concentrations in piglet plasma, compared to control piglets not given an analgesic, following an intramuscular dose of 3.0 mg/kg and castration and tail docking. *The observed values are represented by the open circles, and the model fits are shown by the solid lines.*

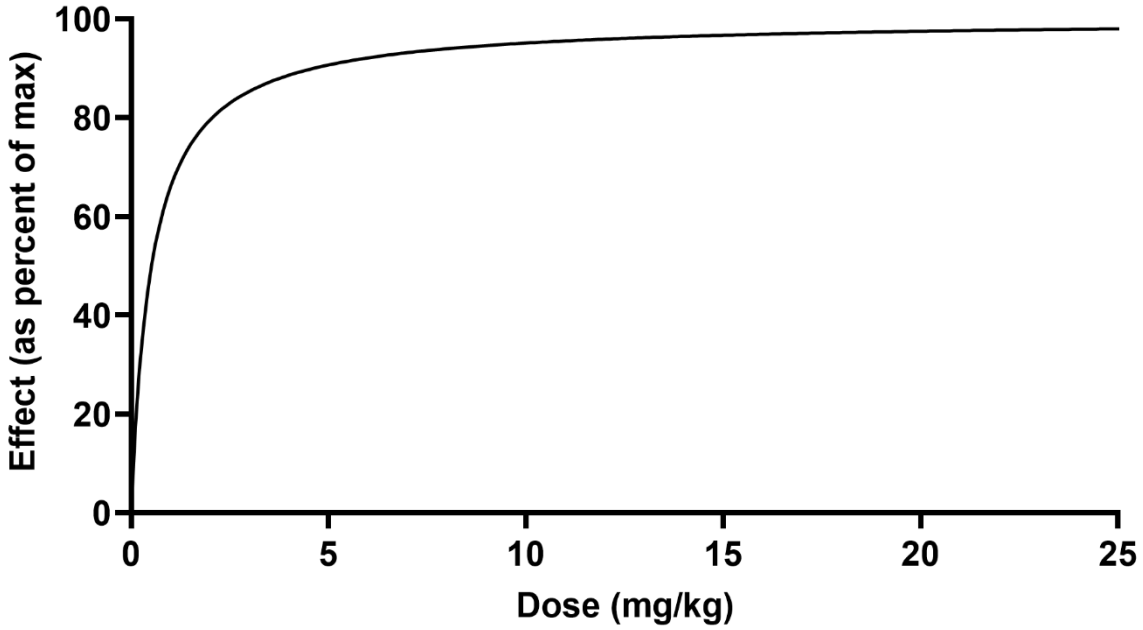


Figure 6.18. The relationship between flunixin administered intramuscularly and the inhibition of cortisol production in piglets undergoing castration and tail-docking.

6.5 Discussion

This study is the first to report an IC50 value associated with PGE2 reduction for any NSAID in pigs and the first to report an IC50 value associated with reduction of cortisol for NSAIDs in any species. This study is also the first to assess the PK/PD relationships directly in piglets that are surgically castrated and tail-docked, rather than using an induced inflammation model.

The S-(+)-enantiomers of 2-arylpropionic acid derivatives, or "profens," are much more potent inhibitors of COX than the R-(-)-enantiomers (39), and the S-(+)-enantiomers have therefore been considered the pharmacologically active enantiomers (41). In addition, S-(+)-ketoprofen predominates over R-(-)-ketoprofen in terms of plasma exposure following

intramuscular administration of racemic ketoprofen in piglets (23,40). Therefore, the pop-PK/PD modeling in this study was performed solely using S-(+)-enantiomer concentrations of ketoprofen.

The pharmacokinetic parameters Tmax, Cmax, and AUC of S-(+)-ketoprofen were similar to the previously reported noncompartmental analysis using this dataset (23). The elimination half-life of 3.45 hours was also similar to the previously reported terminal half-life of 3.50 hours. These values are also comparable to values reported for similar age piglets by other studies (19,40).The IC50 of 0.08 μg/mL for S-(+)-ketoprofen using PGE2 as a biomarker was higher than that reported in other large animal species; calf 0.042 μg/mL, goat 0.003 μg/mL, horse 0.033 μg/mL and sheep 0.007 μg/mL (34–37), however, it fell within the range reported for racemic ketoprofen in large animal species; calf 0.086 μg/mL, goat 0.028 μg/mL, horse 0.057 μg/mL and sheep 0.012 μg/mL (31,32,34,35). PK/PD modeling of ketoprofen in piglets has been performed previously. However, that study utilized a kaolin-induced inflammation model and mechanical nociceptive threshold (MNT) testing, an outcome with a different mechanism of action (19). Based on MNTs, the estimated ED50 from that study was 2.5 mg/kg, which is lower than the estimates in the present study (5.83 mg/kg when using PGE2 as a biomarker, and 4.36 mg/kg when using cortisol as a biomarker). However, IC50 calculated when using cortisol as a biomarker was high, at 2.56 μg/ml. As prostaglandins stimulate ACTH and cortisol release (12), the effect of NSAIDs on cortisol is more indirect, as it is further down the pathway in the mechanism of action. Therefore, cortisol may be less sensitive to small changes in NSAID concentration. There was also a significant degree of inter-individual variability (124.08%) associated with the IC50 for cortisol following ketoprofen administration. There is also marked individual variability in response to anti-inflammatories in other species (42,43).

The pharmacokinetic parameters for flunixin were slightly different from the previously reported noncompartmental analysis (NCA) using this dataset (23). A 2-compartment model enables the distribution and elimination phases to be accounted for separately, which likely caused these slight differences. In addition, the Tmax and Cmax are taken directly from the raw data in an NCA, whereas a compartmental analysis calculates these from the predicted model fits. Based on the compartmental model, the Tmax was reached more rapidly (0.51 hours vs. 0.85 hours), but still similar to the Tmax of 0.61 hours from another study in mature swine (44). The AUC was slightly lower than the previous NCA report, which likely overestimated this value (22.06 h.μg/mL vs. 27.25 h.μg/mL). The elimination half-life of 4.42 hours was lower than the NCA report of 7.93 hours; however, it was much closer to previously reported in similar age piglets (4.82 and 5.15 hours (18)) and lower than that of mature swine (7.93 hours). The IC50 for flunixin utilizing cortisol as a biomarker was 0.06 μg/mL. There are no previous PK/PD models for NSAIDs utilizing cortisol as an outcome for comparison; however, this value does compare to values measured for various NSAIDs in other large animal species for inhibiting PGE2 production. PK/PD modeling of flunixin in piglets has been performed previously; however, that study utilized a kaolin-induced inflammation model and mechanical nociceptive threshold (MNT) testing. Based on MNTs, the estimated ED50 from that study was 6.6 mg/kg (18), differing quite significantly from the ED50 of 0.51 mg/kg estimated in the present study. Given the different model used (induced inflammation vs. surgical castration and tail-docking), different outcome used, and different route of administration of the drug (intravenous vs. intramuscular), it is difficult to make comparisons. The same difficulties would apply to the above discussion of ketoprofen as well. In the present study, simulation of the dose-effect relationship showed that a dose of 2.2 mg/kg corresponds to a drug response of 81.05% of the maximal possible response. However, given the

discrepancy between ED50 values, it would be pertinent to perform further research to ensure the most optimal dose is given.

This study does have some limitations worth consideration. The models utilized data from surrogate markers of the analgesic response (biomarkers more closely related to the anti-inflammatory and stress response), and therefore the actual analgesic response may be different. In addition, relatively few piglets (n=8) were included in these population analyses, so the inter-individual variability may not be optimally represented. Finally, the piglets in these studies were individually housed, which could influence the outcomes examined in this study compared to piglets housed together with the sow. However, a controlled environment was required to maintain the intravenous catheters and subcutaneous interstitial fluid probes. Further research would need to be conducted on-farm to determine whether the ED50 values still hold when more confounding factors are included.

The ultimate goal of the pop-PK/PD modeling was to ensure that the optimal dose regimen is given to piglets for pain mitigation at castration and tail-docking. This study shows that the currently marketed doses of ketoprofen (3.0 mg/kg) and flunixin (2.2 mg/kg) correspond to a drug response of 33.97% (ketoprofen-PGE2), 40.75% (ketoprofen-cortisol), and 81.05% (flunixin-cortisol) of the maximal possible responses. Given this information, flunixin may be the best NSAID to use in mitigating castration and tail-docking pain at the current label dose.

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CHAPTER 7

Summary and Future Directions

In the United States, piglets undergo painful husbandry procedures such as castration and tail-docking without analgesia. Currently, for commercial farms in the United States, there are limited feasible alternatives to these procedures, and thus effective analgesics are required to improve piglet welfare. However, several studies have assessed the effectiveness of NSAIDs to reduce pain associated with piglet castration and tail-docking, and there are conflicting results regarding their analgesic efficacy.[1–7] As there is limited knowledge regarding the optimal dose required to provide effective analgesic and anti-inflammatory effects, PK/PD modeling offers an approach allowing informed decisions regarding the optimal dose based on generated pharmacodynamic parameters (e.g., IC50 and ED50). Therefore, this research aimed to describe the pharmacokinetic and pharmacodynamic relationships of several NSAIDs at piglet processing. To achieve this goal, it was critical to 1) describe the pharmacokinetics of NSAIDs in piglets at the age at which routine processing occurs; 2) assess the effects of NSAIDs at piglet castration and tail-docking; 3) develop PK/PD models to determine key PD parameters helpful in determining an effective dose regimen.

The first two chapters focused on the pharmacokinetics of NSAIDs in neonatal piglets (Chapter 3) and weaned pigs (Chapter 4). Most dose recommendations for NSAIDs are based on studies conducted in older pigs and not neonatal piglets. Typically, neonates display reduced clearance of many drugs compared with older individuals primarily due to a greater body water content leading to a higher volume of distribution, a larger fraction of body mass consisting of highly perfused tissues, a lower plasma concentration of binding proteins (e.g., albumin and α-1 acid glycoprotein) and incomplete maturation of hepatic-enzymes systems.[8,9] To determine the

protein-unbound, pharmacologically active concentrations of NSAIDs and assess distribution to the interstitial fluid (ISF), an *in vivo* ultrafiltration technique was utilized. This was the first report of ultrafiltration for the collection of NSAIDs in piglets. As expected, the ratio of ISF maximal concentration to maximal plasma concentration was higher for neonatal piglets than weaned pigs, particularly for meloxicam (meloxicam 2% vs. 0.3%; flunixin 0.6% vs. 0.4%). In addition, the meloxicam and flunixin plasma half-lives were longer for piglets (4.46 hours and 7.93 hours, respectively) than weaned pigs (3.34 hours and 5.41 hours, respectively). There is a substantial interplay between pharmacokinetics and the resultant pharmacodynamic effects, and these reported differences in pharmacokinetics between neonatal and weaned pigs highlight the importance of performing pharmacokinetic studies in the population of interest before making dosing recommendations.

Given that pain is a complex and multidimensional phenomenon, it is vital to use a multimodal approach in assessing acute pain and inflammation. Chapter 5 assessed the efficacy of meloxicam, ketoprofen, and flunixin utilizing three different objective measures: plasma cortisol, ISF prostaglandin E2 (PGE2), and activity via accelerometry. This study was also the first to utilize an accelerometry methodology in assessing postoperative piglet pain. In addition, data supporting the use of NSAIDs to manage pain associated with castration and tail-docking is conflicted between studies; therefore, it was also essential to assess these three NSAIDs in direct comparison. The results presented in Chapter 5 show that flunixin (2.2 mg/kg) or ketoprofen (3.0 mg/kg) may be the most effective NSAIDs in alleviating pain associated with castration and tail-docking in piglets and that meloxicam (0.4 mg/kg) was not very effective at the European label dose despite its use in Europe and Canada.

Unsurprisingly, piglets that were castrated and tail-docked with no analgesia had elevated plasma cortisol and ISF PGE2 concentrations compared to sham-handled controls. These piglets also displayed significantly decreased activity levels and alterations in the 24-hour activity patterns. All three NSAIDs succeeded in reducing PGE2, but only flunixin maintained this inhibition beyond 24 hours post-dose. Flunixin also achieved the most significant reduction in plasma cortisol throughout the study period. Both flunixin and ketoprofen mitigated the effect of castration and tail-docking on piglet activity levels and patterns, as these piglets were closer to the sham-handled piglets. Finally, this study shows that the effects of handling piglets and administering an intramuscular injection are negligible, at least in a controlled setting with a single individual administering all injections. To summarize, flunixin (2.2 mg/kg) and ketoprofen (3.0 mg/kg) administered intramuscularly two hours before castration and tail docking were the most efficacious treatments when considering the effects on plasma cortisol, ISF PGE2, and activity. Meloxicam did not provide adequate analgesia, despite its current use in Europe and Canada.

The correlation between the administered dose and the drug concentration at the site of action contributes to the pharmacodynamic response. Thus, Chapter 6 presents a population-PK/PD approach utilizing data from Chapters 3 and 5 to ensure that the optimal dose is given to piglets for pain mitigation at castration and tail-docking. This study is the first to assess the PK/PD relationships directly in piglets that were surgically castrated and tail-docked. Previous studies have reported IC50 values associated with PGE2 reduction for NSAIDs in other species, but none have been reported for pigs, and this study was the first to report an IC50 value associated with cortisol reduction for NSAIDs in any species. The currently marketed doses of ketoprofen (3.0 mg/kg) and flunixin (2.2 mg/kg) correspond to a drug response of 33.97% (ketoprofen-PGE2), 40.75% (ketoprofen-cortisol), and 81.05% (flunixin-cortisol) of the maximal possible responses.

Given this information, flunixin may be the best NSAID to use in mitigating castration and tail-docking pain at the current label dose. However, these models utilized data from surrogate markers of the analgesic response (biomarkers more closely related to the anti-inflammatory and stress response), and therefore the actual analgesic response may be different. Future studies combining these objective markers with other measures of pain, or other methods that assess spontaneous pain-related behaviors, may provide more evidence to support an accurate estimate of drug response and optimal dose. Future PK/PD modeling efforts will also attempt to correlate the drug concentrations with the activity data presented in Chapter 5.

While the population-PK/PD approach utilized in Chapter 6 allows an insight into the interindividual variability, these piglets come from a small, homogenous group of healthy animals in a research environment. The piglets were also removed from the sow and individually housed. It is unknown is how the pharmacokinetics and pharmacodynamics are altered when piglets are raised in a commercial environment and whether this small group of piglets provides an accurate depiction of the population. While a controlled environment was required to maintain the intravenous catheters and subcutaneous ISF probes, future studies need to be conducted on-farm and with a larger sample size to determine whether the pharmacokinetics, pharmacodynamics, and interindividual variability are altered when more confounding factors are present. A large degree of inter-individual variability (124.08%) was associated with the cortisol IC50 following ketoprofen administration, and a larger population with inclusion of covariates (such as body weight, health status, litter, sow parity) could help elucidate the source of this variability.

The results provide evidence that flunixin may be the best NSAID to use at the current label dose. However, the piglets in these studies were administered a dose based on the individual bodyweight of each piglet. Realistically in a commercial setting, producers will administer a pre-

determined volume of NSAID independent of piglet body weight. Pharmacokinetic parameters such as Cmax and AUC may vary between low body weight and high body weight pigs due to administration of a standardized volume dose, resulting in inadequate dosing and reduced analgesic effect in high body weight piglets. Therefore, future studies should assess the efficacy of NSAIDs following administration of a standardized volume dose. An additional challenge in developing a feasible analgesic treatment that is appropriate for use in a commercial setting is the timing of the dose. Due to the delay in onset related to absorption and distribution of intramuscular NSAIDs, the trade-off in administering an intramuscular injection is that the piglets must be handled twice. In these studies, the NSAIDs were administered two hours before processing to allow distribution to the site of action. In a commercial setting, the time and labor involved in administering an NSAID two hours before processing may be prohibitive and result in a failure to apply these findings on commercial farms. However, if intramuscularly administered NSAIDs are given immediately before the procedure, immediate procedural pain is likely not adequately addressed. To address this, future studies will assess a novel, non-invasive, needleless delivery of NSAIDs, potentially providing more rapid and practical analgesia on-farm.

These future study approaches would collectively improve the quality of evidence to support the use of NSAIDs in reducing pain and inflammation associated with piglet processing. This knowledge would allow the development of a rational, scientifically-based recommendation for piglet analgesia and improve piglet welfare.

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APPENDIX

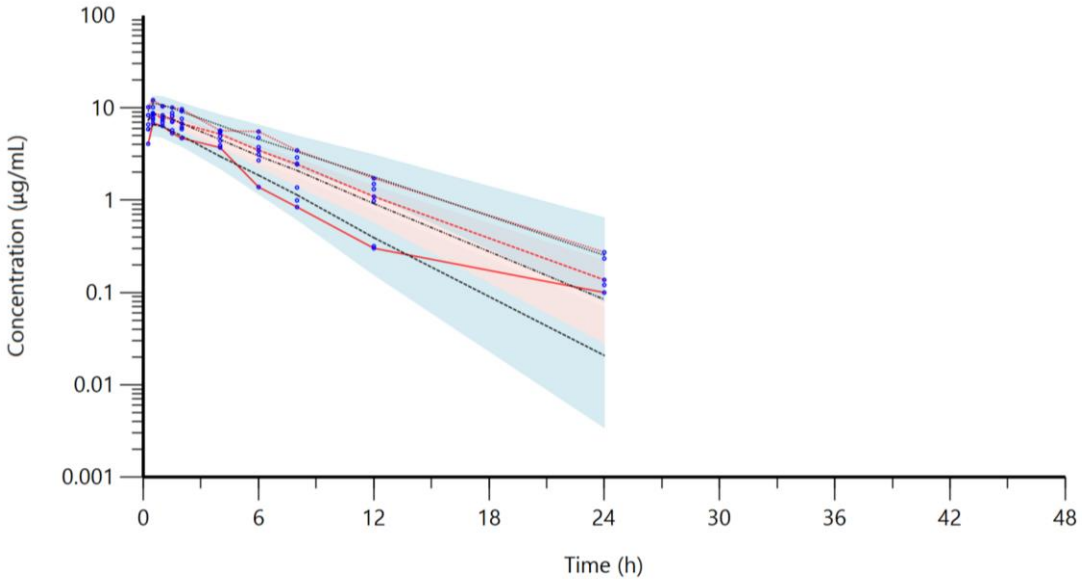

Figure 6.S1. Visual predictive check (VPC) of the final model for ketoprofen in plasma (using 300 replicates). *Observed plasma concentrations are depicted by the open circles. The 5th, 50th and 95th percentiles of the observed concentrations are represented by the red lines. The 5th, 50th and 95th percentiles of the predicted concentrations are represented by the black dashed lines. The 95% confidence intervals (CI) for the predicted 5th and 95th percentiles are represented by the blue shaded regions. The 95% CI for the predicted 50th percentile is represented by the red shaded region.*

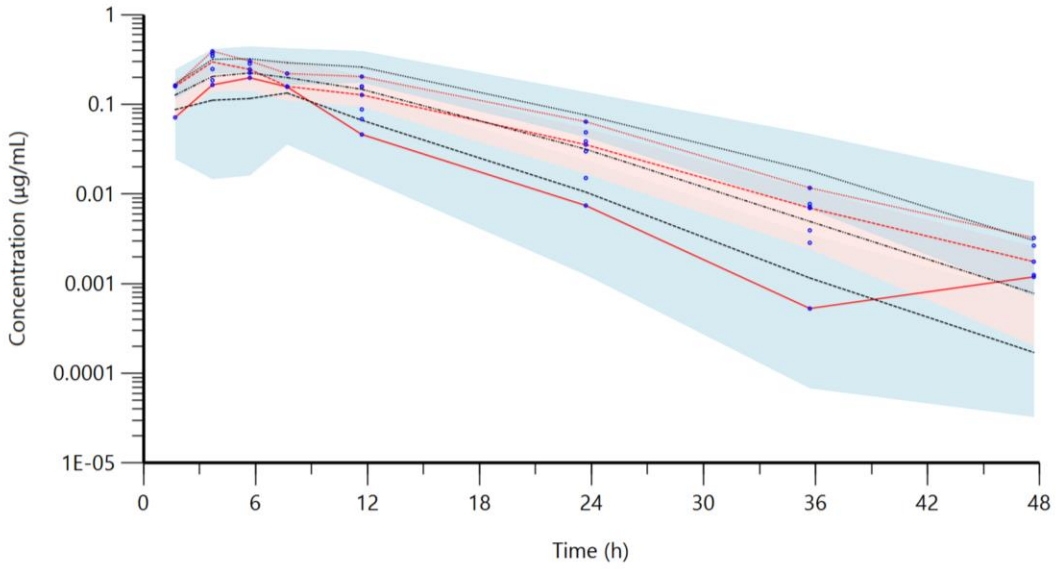

Figure 6.S2. Visual predictive check (VPC) of the final model for ketoprofen in interstitial fluid (using 300 replicates). *Observed interstitial fluid concentrations are depicted by the open circles. The 5th, 50th and 95th percentiles of the observed concentrations are represented by the red lines. The 5th, 50th and 95th percentiles of the predicted concentrations are represented by the black dashed lines. The 95% confidence intervals (CI) for the predicted 5th and 95th percentiles are represented by the blue shaded regions. The 95% CI for the predicted 50th percentile is represented by the red shaded region.*

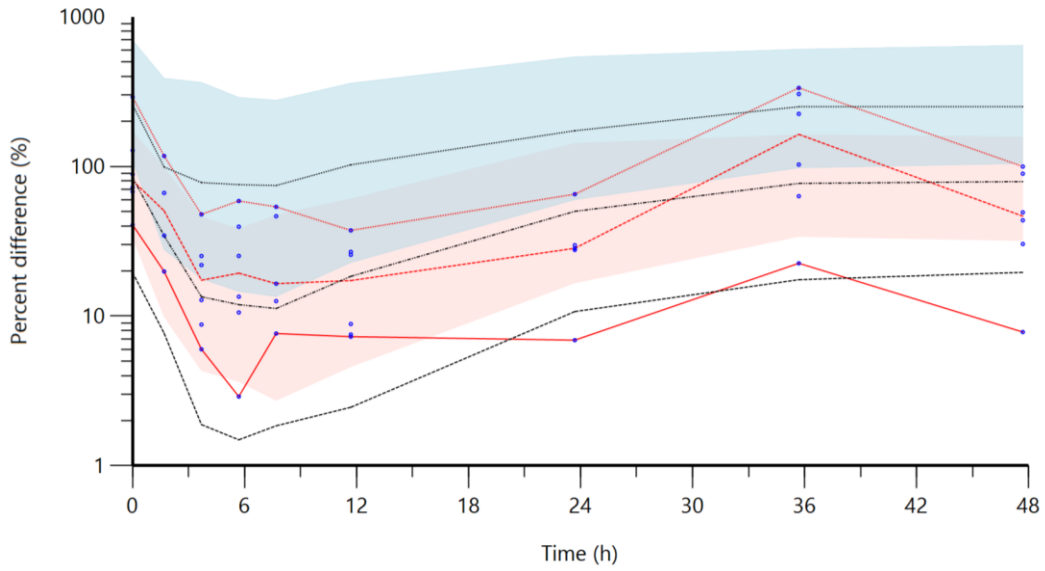

Figure 6.S3. Visual predictive check (VPC) of the final model for percent difference in PGE2 following administration of ketoprofen, compared to untreated, castrated and tail docked control piglets (using 300 replicates). *Observed PGE2 percentages are depicted by the open circles. The 5th, 50th and 95th percentiles of the observed concentrations are represented by the red lines. The 5th, 50th and 95th percentiles of the predicted concentrations are represented by the black dashed lines. The 95% confidence intervals (CI) for the predicted 5th and 95th percentiles are represented by the blue shaded regions. The 95% CI for the predicted 50th percentile is represented by the red shaded region.*

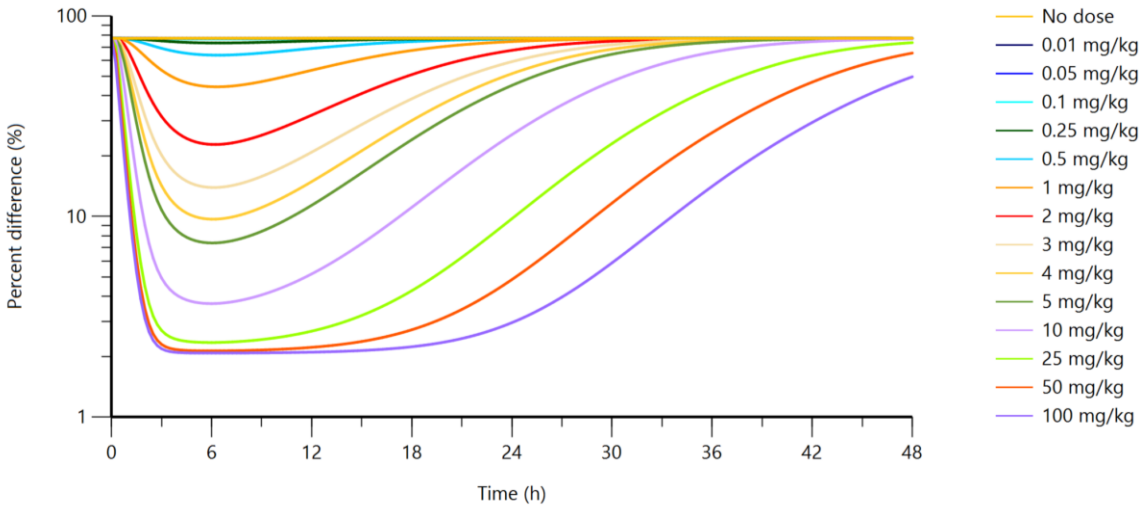

Figure 6.S4. Simulated time profile of PGE2 for 15 single intramuscular dose administrations of ketoprofen to piglets undergoing castration and tail-docking.

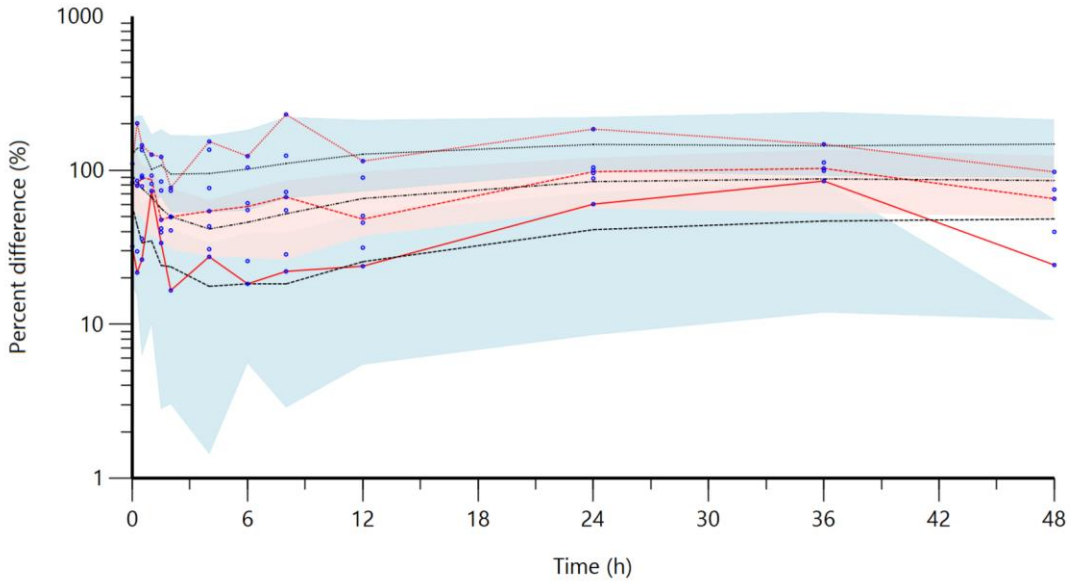

Figure 6.S5. Visual predictive check (VPC) of the final model for percent difference in cortisol following administration of ketoprofen, compared to untreated, castrated and tail docked control piglets (using 300 replicates). *Observed cortisol percentages are depicted by the open circles. The 5th, 50th and 95th percentiles of the observed concentrations are represented by the red lines. The 5th, 50th and 95th percentiles of the predicted concentrations are represented by the black dashed lines. The 95% confidence intervals (CI) for the predicted 5th and 95th percentiles are represented by the blue shaded regions. The 95% CI for the predicted 50th percentile is represented by the red shaded region.*

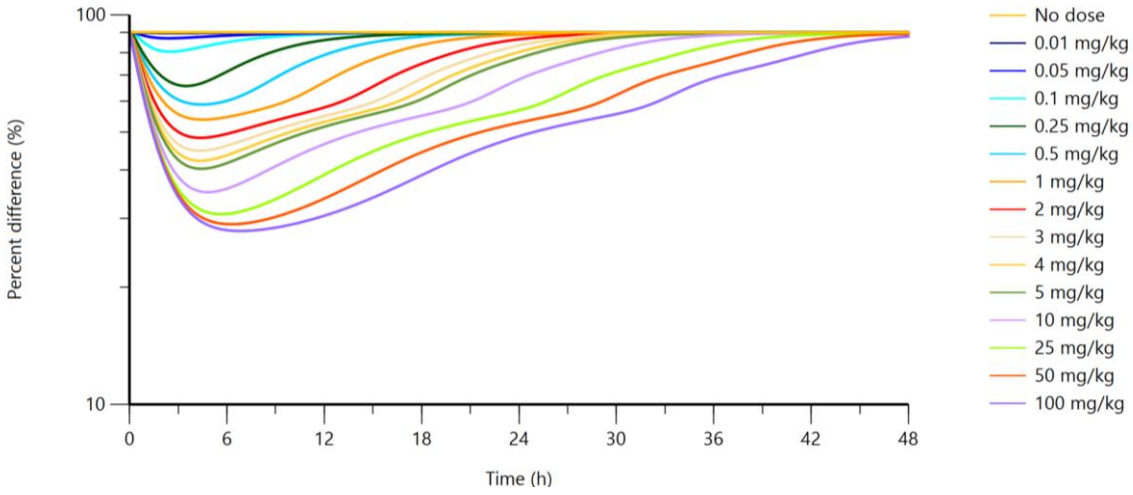

Figure 6.S6. Simulated time profile of cortisol for 15 single intramuscular dose administrations of ketoprofen to piglets undergoing castration and tail-docking.

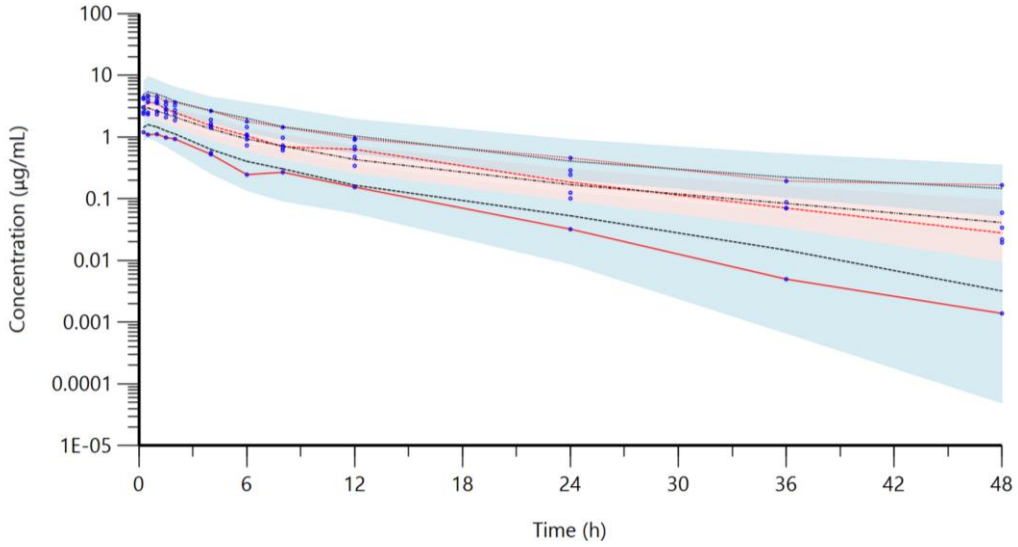

Figure 6.S7. Visual predictive check (VPC) of the final model for flunixin in plasma (using 300 replicates). *Observed plasma concentrations are depicted by the open circles. The 5th, 50th and 95th percentiles of the observed concentrations are represented by the red lines. The 5th, 50th and 95th percentiles of the predicted concentrations are represented by the black dashed lines. The 95% confidence intervals (CI) for the predicted 5th and 95th percentiles are represented by the blue shaded regions. The 95% CI for the predicted 50th percentile is represented by the red shaded region.*

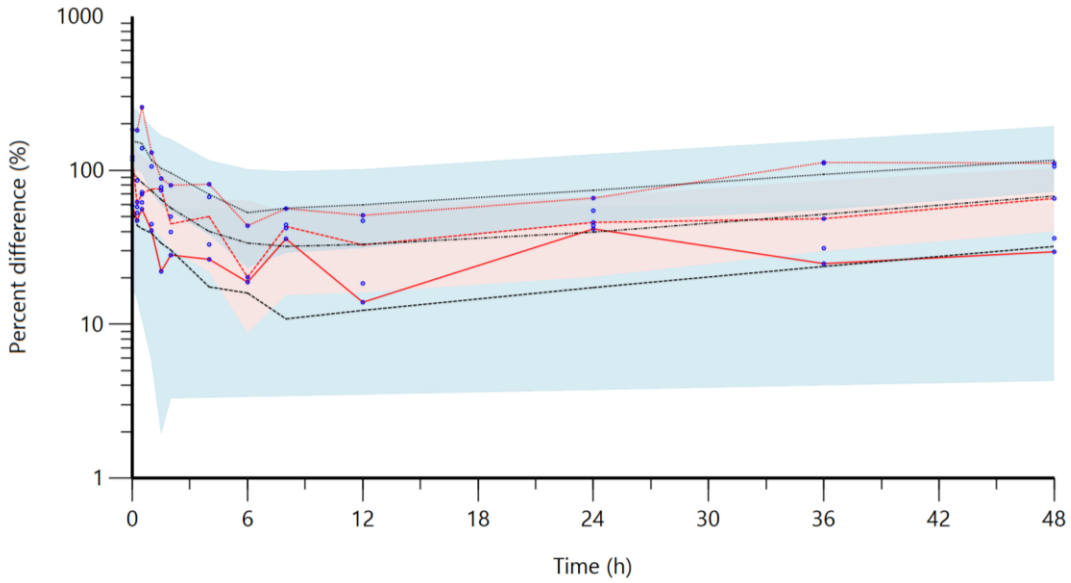

Figure 6.S8. Visual predictive check (VPC) of the final model for percent difference in cortisol following administration of flunixin, compared to untreated, castrated and tail docked control piglets (using 300 replicates). *Observed cortisol percentages are depicted by the open circles. The 5th, 50th and 95th percentiles of the observed concentrations are represented by the red lines. The 5th, 50th and 95th percentiles of the predicted concentrations are represented by the black dashed lines. The 95% confidence intervals (CI) for the predicted 5th and 95th percentiles are represented by the blue shaded regions. The 95% CI for the predicted 50th percentile is represented by the red shaded region.*

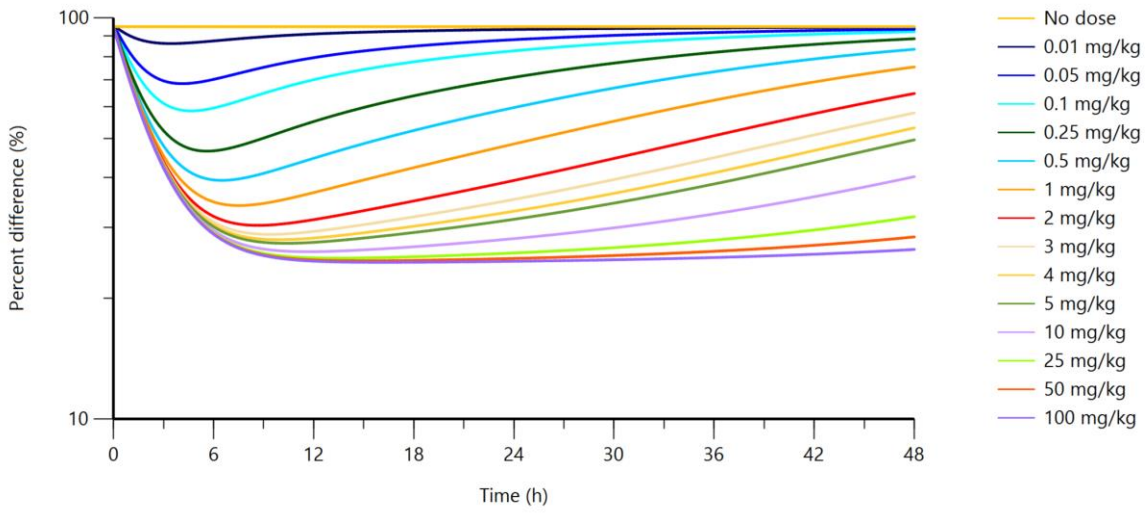

Figure 6.S9. Simulated time profile of cortisol for 15 single intramuscular dose administrations of flunixin to piglets undergoing castration and tail-docking.