ABSTRACT

NEBEL, AMBER Pharmacokinetics of Intravenous Carprofen in Canine Lactation. (Under the direction of Drs. Sara Lyle and Mark Papich).

Analgesia is an important component of veterinary care and fulfills the veterinarian’s oath to relieve suffering and discomfort. One of the most common drugs for this purpose in dogs are the nonsteroidal anti-inflammatory drugs (NSAIDs), particularly carprofen (Rimadyl®). The Precautions section of the official FDA approved label for carprofen states, “The safe use of Rimadyl® in animals less than 6 weeks of age, pregnant dogs, dogs used for breeding purposes, or in lactating bitches has not been established…”, thereby withholding adequate pain treatment for dogs having recently whelped and are lactating. Pharmacokinetic studies are available to characterize the disposition in dogs (Messenger, et al, 2015; US Pharmacopeial Convention, 2007; McKellar, et al, 1990); however, the disposition of carprofen in lactating dogs has not been examined fully, questioning the use and safety of carprofen in this patient population. The extent to which lactation changes the carprofen pharmacokinetics is unknown, nor is the extent to which carprofen concentrations are transferred to milk of lactating bitches or to their neonates. We hypothesized that after a single intravenous dose of carprofen to bitches, concentrations in milk are low and not high enough to produce harmful exposure in puppies. We tested our hypothesis in healthy, adult lactating bitches (n=4) using pharmacokinetic methods, and nonchiral and chiral specific assays measuring total, R-, and S-enantiomer carprofen concentrations with high performance liquid chromatography (HPLC) in maternal plasma, milk, and neonatal plasma samples. CMAX, elimination half-life, and clearance for maternal plasma were 9.09 µg/mL and 7.3 µg/mL (R-, S+), 6.82 and 6.22 hours (R-, S+), and a higher clearance rate of 95.81 mL/hr/kg (R-) and 73.87 mL/hr/kg (S+) than previously reported. This study confirmed that the approved dose is appropriate for perioperative and chronic pain in lactating
dogs and is safe with reduced exposure to nursing pups (<10% maternal dosage and a milk:plasma <1). This study also suggests that the pharmacokinetics of carprofen in lactating bitches may be different from their nonlactating counterparts. However, further pharmacokinetic studies with more dogs will be needed to confirm this and decrease the variation seen in our small population.
Pharmacokinetics of Intravenous Carprofen in Canine Lactation

by
Dr. Amber Marie Nebel

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North Carolina State University
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APPROVED BY:

Dr. Sara Lyle
Committee Co-Chair

Dr. Mark Papich
Committee Co-Chair

Dr. Kristen Messenger
DEDICATION

I would like to dedicate this work to all the lactating dogs that helped with my research, and all the future patients that I will have in my career as a reproductive veterinarian.
BIOGRAPHY

Dr. Amber Nebel is a third-year comparative theriogenology resident at NCSU. She has an interest in equine and canine reproductive pathologies as well as reproductive pharmacology. She is currently doing research in the safety of carprofen, or Rimadyl, in lactating bitches and how it affects neonates with the goal of proving that it is safe to give in this patient population. During her residency, she pursued a Master’s degree in Comparative Biomedical Science to answer this research question which has been unanswered previously.
ACKNOWLEDGMENTS

I would like to acknowledge my friends, family, and fiancé who have helped me problem solve logistics in my research design, made sure I didn’t starve, and helped assist with lodging while travelling for samples. I also want to thank all the owners and alumni of the NCSU College of Veterinary Medicine who have opened their homes and enrolled their dogs in my study. This study would not be complete without the wonderful dogs and litters I was able to work with and love on during my time with them. Lastly, I want to thank my committee who has been flexible in meeting during odd times while navigating my clinical schedule for my residency and have given their time and expertise while I learned the HPLC system and helped trouble shoot any issues that I have had.

I would also like to acknowledge the NCSU CVM Research Office for funding this research with the NCSU CVM Intramural Grant.
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CHAPTER 1

Literature Review

Research in human lactation

Lactation is a natural phenomenon allowing production of nutrition to neonates from their mothers. However, its physiology can cause some difficulties when studying drug passage of medications and subsequent neonatal exposure. Research in human lactation supported by the World Health Organization (WHO) and done by pioneers Jane Alcorn, PO Anderson, and many others for the past two decades have helped answer many questions on neonatal safety for specific medications as well as developing models for drug passage in research. The World Health Organization has recommended restrictions on human infant dosage to less than 10% of weight-adjusted maternal dose (Ling & Alcorn, 2010).

The majority of research in human lactation has been directed at determining changes in the stage of lactation, how it effects drug passage and neonatal exposure as a sequela, as well as transporter proteins in the mammary epithelium. Regarding stage of lactation, Anderson et al (2015, 2016) found the properties and stage of lactation to be highly important when it comes to estimating infant exposure dose. Lactation has two main phases: colostrual phase and lactogenesis II where the bulk volume of milk is produced. The colostral phase, as it would seem, is where and when colostrum is produced. Colostrum is the first milk that consists of a high concentration of protective antibodies or immunoglobulins and initially has a lower volume (approximately 10% of the volume of day seven lactation in human mothers). This stage of lactogenesis requires large pores to develop between mammary epithelial cells and allow passage of antibodies, other large proteins, and leukocytes from the plasma into the mammary gland. In humans, colostrum is produced for the first 3-4 days with gradual closure of these spaces 1-2 weeks after the transition
into lactogenesis II. Once these pores close and form tight junctions that are normally present, molecules gain access through the mammary epithelial barrier by passive diffusion or active transport proteins. Passive diffusion is dependent on a concentration gradient of unbound, nonionized drug as well as a high volume of distribution or lipophilicity. The protective barrier formed by the tight junctions can be compromised in certain pathologic conditions such as mastitis where the local inflammation weakens the tight junctions causing them to leak. This then begs the question, are infants more at risk to being exposed to drugs in early lactation (colostral phase) and in times of inflammation (mastitis) compared to mid- or late-stage lactation?

Physiological properties of breast milk and pharmacological properties of drugs have a huge impact on drug excretion and neonatal exposure. Lipophilicity was briefly discussed in terms of volume of distribution out of the plasma and into the body tissue, but lipophilicity also increases the chance of concentrating a drug in milk, as it contains 3-5% emulsified fat which is higher than plasma. Another important physiological property is pH and its logarithmic partner, pKa, as is described in the Henderson-Hasselbach equation:

\[ pH = pKa + \log\left(\frac{\text{ionized drug concentration}}{\text{un-ionized drug concentration}}\right) \]  

(Equation 1),

where pH is the “potential of hydrogen” and is a scale denoting acid and base status in aqueous solutions and pKa is the acid dissociation constant measuring strength of acidity. The pH of milk ranges from 6.7 to 7.3 with a mean of 7.03 (Anderson et al., 2016); it was found that hindmilk is slightly more basic than foremilk. The pH of milk and pKa of pharmaceuticals is important as it interplays with the pH partition theory or “ion trapping” since milk is a weak acid. Ion trapping in terms of milk means pharmaceuticals that are weak bases with a pKa > 8 tend to concentrate and become “trapped” in milk compared to ionized weak acids that tend to stay in the plasma.
Another property that correlates to drug excretion in milk is protein binding. The major proteins responsible for drug binding in the plasma and milk respectively are albumin and casein. Studies have found that pharmaceuticals with greater than 85% protein binding in the plasma have a low risk of dissociating into the milk. Studies have shown that carprofen has a higher affinity to bind to albumin compared to casein proteins. There are a few exceptions to this rule of pharmacology such as diazepam and fluoxetine, a sedative and anxiolytic, due to active metabolites that have a long half-life (Anderson et al., 2016).

Numerous studies have been conducted regarding transporter proteins, or xenobiotics, which is an important field of research regarding drug interactions and diffusion. Most of the initial work in human lactation was done by Jane Alcorn and her lab, where they found five major classes of transporter proteins on the mammary epithelium: organic cation transporters (OCT), organic anion transports (OAT), peptide transporters (PEP), nucleoside and nuclease transporters (NT), and ATP-binding cassettes (ABC). One transporter protein in the ABC family is the p-glycoprotein MDR1, or multidrug resistant proteins 1, 2, and 5, which are expressed in mammary glands, the placenta, and at the blood-brain-barrier which can cause increase in drug concentrations such as ivermectin. Another major xenobiotic to mention is of the peptide transporter family, PEP 1 and 2, for which β-lactam antibiotics, angiotensin converters, enzyme-inhibitors, and valacyclovir act as substrates (Ito & Alcorn, 2003). Alcorn subsequently found rat models that pharmaceuticals which act as substrates for xenobiotics pose an increased risk of exposure to neonates by increasing the milk:plasma concentration ratio above 1.

Previous research in human medicine has culminated into the development of the LactMed database (NCBI, 2011) which serves as a vital resource to medical professionals when prescribing medication to nursing mothers. Unfortunately, research in this patient population in
veterinary medicine has been lacking in canine and feline companion species, but less so in species historically deemed for food production such as cattle. In most cases, information from the LactMed database is extrapolated to these animal species if no research data or studies are available.

Pharmacokinetic modeling for lactation

The classic pharmacokinetic approach to studying and measuring drug excretion in milk is to administer a dose of a medication, either as a clinical or prospective study and measure several milk samples consisting of both the foremilk and hindmilk at various timepoints usually over the span of 72 hours. The samples are then analyzed for drug concentration and plotted on a graph against time allowing for the area-under-the-curve (AUC) to be measured via the trapezoidal rule. If the quantity of milk ingested is known, one can calculate the infant daily dose as well. Typically the quantity of milk is unknown, as stripping the whole volume from the mammary gland at feeding is viewed as unethical in some organizations. To help combat this problem, the WHO has published estimates of infant ingestion per day for various countries.

Another study design to estimate infant exposure is the “relative infant dose” (RID) which is the ratio of the infant’s daily dose to the mother’s daily dose. The WHO recommends the RID should be less than 10%, with medications greater than 25% avoided in nursing mothers. Limitations found with solely utilizing RID for drug safety are that it doesn’t account for dosing ranges for medications, neonatal metabolism and clearance, and active drug metabolites which may have a higher affinity to concentrate into milk.

A third method for modeling drug passage into breast milk is the milk:plasma (M/P) ratio. This equation takes the average milk concentration divided by the average maternal
plasma concentration. Once the M/P is known, the actual or predicted milk concentration is calculated using the M/P ratio and the maternal plasma concentration. Studies in human medicine have reported using plasma concentrations from nonlactating subjects for calculating the M/P as most drug pharmacokinetics are not statistically different between lactating and non-lactating women. An early pitfall with relying on predicted M/P in pharmacokinetic studies was high variation which did not account for lactational stage, route of administration, and ion trapping as previously mentioned. To overcome this limitation, researchers have started to calculate the M/P utilizing the AUC of milk and plasma instead of the average concentration which is the current accepted method in calculating M/P (Anderson & Sauberan, 2016). Milk to plasma ratios can be predicted from physiochemical properties using two different methods: phase-distribution modeling and log-transformed phase-distribution modeling (a revision of the original phase-distribution model). Both models incorporate physiochemical properties such as protein binding, the drug’s pKa, and octanol/water partition coefficient. The original phase-distribution model was more accurate in predicting the M/P for acidic drugs leading to the revision and development of the log-transformed phase-distribution model to predict the M/P for basic drugs.

An alternative method to predicting M/P is the quantitative structure-activity relationship (QSAR) which correlates chemical and molecular properties of the drug to previous published M/P ratios and was first used in 1985. The QSAR parameters are incorporated into a computer program that will predict the M/P ratio. This model is not as accurate as the phase-distribution methodology as its accuracy relies on the parameters put into the algorithm.

The most direct method of measuring infant exposure is measuring infant plasma and calculating infant dose from the infant/maternal plasma concentration. The infant/maternal plasma concentration is used by the American Academy of Pediatrics when assessing drug safety
in nursing mothers with prescribing drugs that have an infant concentration less than 10% of the maternal concentration.

There are two main types of pharmacokinetic modeling to analyze milk concentration data: population-based pharmacokinetic modeling and physiologically based pharmacokinetic modeling. Population-based modeling uses a sparse sampling technique and can incorporate both plasma and milk samples. Simulations often use population-based pharmacokinetic modeling to estimate expected variability of drug passage. This type of modeling is generally recommended by regulatory authorities to assess drug safety and efficacy (Sun et al., 1999; Ette & Williams, 2000). In contrast, physiologically based pharmacokinetic modeling is used to simulate breastmilk exposure to environmental chemicals. This modeling was successfully used to simulate codeine and morphine toxicity in breastmilk of a nursing mother who was a supermetabolizer under varying conditions of cytochrome p-450 enzymes to explain the cause of death in an infant. For the purpose of our study, a population-based pharmacokinetic model was used.

Carprofen research in veterinary species

A class of medication that is vital to any medical profession is analgesic drugs that provide pain relief and can serve as an anti-inflammatory agent. One of the most common analgesic medications used in veterinary medicine is carprofen. Carprofen, a preferential COX-2 (PTGS2; cyclooxygenase-2 or prostaglandin synthase-2 enzyme) inhibitor introduced in 1997, is approved for perioperative and chronic pain management in dogs (US Pharmacopeial Convention, 2007; KuKanich et al., 2012; Ricketts et al., 1998; Wilson et al., 2004). Carprofen is a racemic NSAID (nonsteroidal anti-inflammatory drug) that has anti-inflammatory, analgesic,
and anti-pyretic properties. Carprofen consists of two enantiomers R(-) and S(+) with the S(+) enantiomer having greater anti-inflammatory properties compared to the R(-) enantiomer. It has a low volume of distribution making it is less likely to diffuse out of the intravascular space into the body tissue even in inflammatory conditions (Messenger et al., 2014). Carprofen, like other NSAIDs is metabolized by the liver via phase II glucuronidation and is excreted via the gastrointestinal and urinary tracts. No differences in breed of dogs have been discovered, but studies have shown variation of rates of metabolism in research Beagles. This variation in metabolism is attributed to differences in cytochrome p-450 enzymes (Paulson et al., 1999; Fleisher et al., 2008; Jeunesse et al., 2013; Messenger & Papich, 2015) and polymorphisms similar to human medicine (Martinez et al., 2004; Kirchheiner & Brockmoller, 2005; Ali et al., 2009) but to a smaller extent as not enough studies have been done in this field. A difference between carprofen and other NSAIDs is that it undergoes enterohepatic recirculation, allowing it to be re-excreted into the gastrointestinal tract which may be the reason for some observed adverse side effects. Carprofen ordinarily has a good safety profile (KuKanich et al., 2012; Raekallio et al., 2006), but can produce adverse effects common to all NSAIDs, such as kidney, liver, and gastrointestinal injury (KuKanich et al., 2012). An adverse reaction specific to dogs is an idiosyncratic acute hepatopathy, with an incidence ranging from <0.05% to 1.6% (MacPhail et al., 1998; Hickford et al., 2001). The FDA labelled dose in the United States is either 4.4 mg/kg once daily or 2.2 mg/kg twice daily, administered orally or subcutaneously (Rimadyl® package insert, 2013).

Pharmacokinetics for carprofen have been studied in normal, healthy, nonlactating dogs and show the R(-) enantiomer has a greater AUC and slower clearance compared to its counterpart the S(+) enantiomer (McKellar et al., 1994; Priymenko et al., 1998): however, based
on an enantiomer specific *in vitro* assay in dogs, the S(+) enantiomer has greater anti-inflammatory function/activity in dogs which, is the opposite to what is seen in other species (Lees et al., 2004). Due to these nuances, most pharmacokinetic studies utilize enantiospecific assays rather than total (R + S) carprofen. In this study, both reporting methods are used (McKellar et al., 1990; Lascelles et al., 1998; Clark et al., 2003; Schmitt et al., 1990; McKellar et al., 1994; Priymenko et al., 1998; Lipscomb et al., 2002).

Carprofen has been used anecdotally in bitches undergoing cesarean sections as a single subcutaneous dose without adverse effects reported in either bitches or neonates (Escabor & Kolster, 2016; Ferrari et al., 2022). Following cesarean section, some of these bitches experience pain or have pre-existing problems (e.g., osteoarthritis) that warrant analgesia. The Precautions section of the FDA approved label for carprofen states, “[T]he safe use of Rimadyl® in animals less than 6 weeks of age, pregnant dogs, dogs used for breeding purposes, or in lactating bitches has not been established”. This labeling may result in, inadequate pain treatment and control in dogs having recently whelped or are lactating.

Recent research in human medicine has found active milk transporter proteins to be responsible for high levels of certain antibiotics in milk. To date, no transporter proteins for NSAIDs have been described in humans (Ling & Alcorn, 2010; Raekallio et al., 2006). Studies in women also show that medications with greater than 85% protein binding are less likely to pass into breast milk (Anderson & Sauberan, 2016; Begg & Atkinson, 1993; Atkinson & Begg, 1990). Carprofen is 99% protein-bound in plasma and is a weak acid (pKa = 4.3) (Schmitt & Geuntert, 1990); therefore, it is less likely to pass from the plasma to milk and is not a candidate for ion trapping via the pH-partition theory. Carprofen was initially evaluated for milk concentrations in cattle in 1991 (Lohuis et al., 1991) to establish withdrawal times for treatment.
of mastitis. Recently, Ferrari et al. (2022) compared the maternal plasma and milk samples in dogs receiving a 5-day course of carprofen following a c-section. The dogs were assigned to three groups - “normal” (88), “mastitis” (4), and “generalized inflammation” (8), where milk samples were collected once a day 2-5 hours after administration. Dogs with mastitis had higher concentrations of carprofen in the milk compared to the normal and generalized inflammation groups. Maternal and neonatal plasma concentrations were estimated from predictive modeling with 100% systemic absorption estimated for neonates. Predicted expected neonatal exposure was <1 (milk:plasma ratio) in all three groups. These authors concluded that carprofen was safe to administer to lactating bitches; however, no pharmacokinetics were performed outside of simulations.

Hypotheses

Our hypothesis is based on previous work performed with other NSAIDs in people and other animals and recent work reported regarding carprofen concentrations in canine milk. Minimal data is available regarding milk concentrations for carprofen in dogs (Ferrari et al., 2022), but no pharmacokinetic studies have been done to our knowledge with direct measurements of neonatal plasma concentrations.

The purpose of our prospective study was to establish a pharmacokinetic curve for plasma carprofen levels in lactating bitches, measure concentrations in canine milk of treated bitches, and measure concentrations in neonatal plasma. We predict that a single intravenous dose of carprofen in bitches will fail to produce milk concentrations in lactating bitches that exceed the limit of quantification in milk (Anderson & Sauberan, 2016). Lastly neonates nursing
from mothers after administration of a single intravenous does of carprofen will not have detectable levels of carprofen in their plasma.


CHAPTER 2

Material and Methods

Exclusion/inclusion criteria

A total of seven dogs were enrolled in the study and recruited from client-owned kennels with approval via consent form and a factual sheet on carprofen provided from the manufacturer. The ages of the dogs were 2.5-6 (average 4.34) years of age and weighing between 8.91 and 27.27 kgs (average 21.03 kgs). Litters ranged from 16-39 days of age (average 28 days); all puppies weighed more than 300 grams. Dogs and litters consisted of two breeds: American foxhound and Cavalier King Charles spaniel. Enrollment criteria were: (a) healthy based on physical examination, (b) uneventful whelping or delivery, (c) at least 14 days post-partum and (d) had no exposure to NSAIDs in the past 30 days prior to enrollment. Physical examinations of the dams and visual assessment of the puppies were performed at the start of the study prior to enrollment to determine whether they were healthy enough for the study. All dogs were housed in their normal homes with their owners and all samples were taken on-site without transportation of any animal. All seven dogs were deemed healthy prior to enrollment and had no prior exposure to NSAIDs for at least thirty days prior to sampling. A total of two dogs were excluded shortly after samples were started due to a case of unresolved mastitis present at milk collection and difficulty in venous access for sampling. A total of four complete sets of samples were collected (only the seventh dam’s plasma was successfully collected due to litter cooperation and lower milk volume at expression). The study and experimental design were reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University.
Experimental design

Each dog received a single intravenous dose of carprofen at the FDA approved dose of 4.4 mg/kg between 14-39 days post-partum after blank plasma and milk samples were collected from each dam (time=0). Although an oral or subcutaneous route is labelled for dogs in the US, we chose an intravenous route (extra-labelled use) because (a) this was proven safe and efficacious from past studies and in the UK, (b) the intravenous dose ensures 100% bioavailability, which is important for pharmacokinetic calculations, and (c) our aim is to calculate transfer of drug from plasma to milk, which can be measured regardless of dose route. A single milk and plasma sample prior to treatment administration was collected and measured to serve as a control for each female, and then a series of milk and simultaneous plasma samples were collected and measured following administration at predetermined time points: 0, 0.5, 1, 1.5, 2, 8, 12, 24, 36, 48, and 72 hours (Messenger et al., 2015).

Repeated blood sampling can be unsafe in young puppies, so a sparse sampling strategy was used for puppies in the same litters who were at least 14-days-old. This involved taking a minimum of 0.17-mL (calculated for less than 10% blood loss over the entire collection period for a puppy weighing 300 grams) from at least three puppies within the same litter and pooling the samples together for each time point. The pharmacokinetic analysis performed used a naïve averaged data approach on a rotational basis to minimize the handling and venipuncture of each neonate (Ette & Williams, 2004). Milk was collected via manual expression into sterile 2-mL cryovials. Blood was collected via venipuncture into sterile 2-mL heparinized tubes at a maximum of four attempts per dam or puppy. The samples were immediately placed on ice following collection, centrifuged within 30-90 minutes of acquisition, and stored at -80 °C until analysis.
High performance liquid chromatography (HPLC) was performed on all samples using validated methods previously developed in our laboratory (Messenger et al., 2015). Maternal plasma, milk, and pooled neonatal plasma samples were analyzed via HPLC to determine concentrations of carprofen in plasma and milk using methods modified from previous studies. The HPLC system consists of a four-solvent delivery system, an autosampler, and fluorescent detection with an emission wavelength set at 310 nm and detection wavelength set at 375 nm (Agilent 1100 Series: Agilent Technologies, Wilmington, DE, USA) (Messenger et al., 2015). The chromatogram was integrated with the associated computer program supplied by Agilent Technologies. Two different columns were used for analysis. All maternal plasma was analyzed with a chiral column (Ultron ES-OVM) allowing separation of the R- and S-enantiomers with the R-enantiomer coming off the column first (Figure 2.1) and all milk and neonatal samples were analyzed with a Zorbax Eclipse XDB-C18 column (Figure 2.2, 2.3). The chiral column was maintained at a constant temperature of 40 °C and below pressures of 200 bar or 3000 psi per manufacturer directions with a runtime of 25-30 minutes. The mobile phase for the chiral column was set at 85% 0.02 M potassium phosphate in water and 15% acetonitrile at a flow rate of 1 mL/min. The mobile phase for the Eclipse C18 column was set to 55% 0.02 M potassium phosphate in water and 45% acetonitrile at a flow rate of 1 mL/min with a runtime of 12 minutes. The mobile phase differed between the two columns to maximize separation of peaks and peak height respectively. The HPLC system was allowed to warm up and equilibrate for at least thirty minutes before any samples were run.

The concentrations measured for maternal and neonatal plasma were then averaged at each timepoint and graphed on non-logarithmic and logarithmic scale against time using the
FigSys software (BIOSOFT, Acropolis Computers Ltd). One graph included only the maternal plasma samples showing the concentration curves for the both the R(-) and S(+) enantiomer against time, and another graph was performed comparing the average maternal concentrations against the average neonatal concentrations.

**Calibration Standards**

A stock solution of carprofen was prepared using a racemic reference standard from the US Pharmacopeial Convention and dissolved in methanol to 1000 μg/mL. This was stored at refrigeration temperature in a closed container and used to develop standards and calibration samples for all three sampling groups (Xu et al., 2021). The calibration curve for the maternal plasma consisted of six standards ranging from 0.1-50 μg/mL and five standards ranging from 0.05-1 μg/mL for both milk and neonatal plasma. Calibration curves were accepted if the linear coefficient of determination ($r^2$) was ≥ 0.99 (Figures 2.4-2.6). The limit of detection was set at 0.001 μg/mL and the limit of quantification was set at 0.003 μg/mL. All calibration curves were accepted with linear coefficient of determinations ($r^2$) ≥ 0.99.

**Sample preparation**

Preparation for all plasma, milk, calibration, and blank samples were identical aside from volume of sample and 4% phosphoric acid due to sample volume available. Cryovials were thawed the day of preparation, and sample volumes up to 500 μL were added to pre-labelled microcentrifuge tubes (volume range was 25-500 μL). The samples were diluted in a 1:1 ratio with an equivalent volume of 4% phosphoric acid in water and then centrifuged for 5 minutes at 15000 rpm to aid in protein breakdown. The mixed samples (plasma or milk with phosphoric
acid aside from the protein film forming a clear to slightly cloudy solution) were loaded into labelled preconditioned solid-phase extraction cartridges (Oasis Prime HLB 1 mL; Waters Corporation, Miliford, MA, USA) on a manifold. Vacuum pressure was applied to pull samples through the cartridge at a rate of 1 mL/min equating to -5 mmHg, and the cartridges were washed with 1 mL of a 95% water and 5% methanol solution. After washing, the samples were eluted with 1 mL of a 90% acetonitrile and 10% methanol solution into labelled glass tubes via vacuum pressure. The eluates were then placed into an evaporator with water at 40 °C and 20 psi for 25 minutes. After evaporation, the dried residue was reconstituted with 250 μL of the mobile phase associated with the specific column. The samples were vortexed briefly and 200 μL were transferred to the pre-labelled amber injection vial with a 250-μL insert for analysis. An injection volume of 20 μL was used for analysis for both plasma and milk samples. Samples were run with a single standard from the stock solution for quality-control at the beginning and middle of each sequence following the calibration curve. Standards were run before any research samples to ensure separation of peaks and peak height was appropriate; they were stored at refrigeration temperatures with parafilm to avoid evaporation (US Pharmacopeial Convention, 2007; Xu et al., 2021). Laboratory preparation of samples were conducted in accordance with previous work, the laboratory manual, and standard operating procedures. Maternal and neonatal plasma carprofen concentrations were plotted against time using the FigSys graphing software.

**Pharmacokinetics**

For pharmacokinetic analysis, drug concentrations were analyzed using our pharmacokinetic software (Phoenix® WinNonlin™ and NLME®, Certara, St. Louis MO). Pharmacokinetic models were fit to this data to determine important pharmacokinetic
parameters. Pharmacokinetic curves for maternal plasma drug concentrations were graphed for at each timepoint up to 24 hours as they fell under the level of detection afterwards and plotted against concentration in a two-compartment model for each dog. Analysis of pharmacokinetic modeling was performed, and it was decided to exclude Dog #4 due to minimal samples taken in the first 24 hours requiring a large percentage of extrapolation (35% with <20% being acceptable). Due to only four litter samples and one milk sample being above the limit of quantification, there was not enough data to do a pharmacokinetic analysis on canine milk or neonatal plasma. Data analysis was done using a weighting factor of 1/(predicted Y)^2, where Y is the plasma concentration. Best fit was then determined on visual analysis of goodness-of-fit, residual plot, and the Aikake’s information criteria (AIC). Then pharmacokinetic parameters were calculated from this data (Table 2.1-2.3).
Table 2.1 Statistical pharmacokinetic parameters for carprofen R(-) and S(+) enantiomers of maternal plasma in a 2-compartment model.

A, B: Y-axis intercepts for the phases of distribution and elimination; α, β, fractional elimination rate constants; k1, k2: rate constant for the distribution and elimination, respectively; k10, k12, k21: elimination and compartmental rate constants; MRT: mean residence time; k10 HL: elimination half-life; α-HL: distribution half-life; β-HL: terminal half-life; AUC, area-under-the-curve; V1, volume of the central compartment; Vss: volume of distribution at steady state; Cl, total plasma clearance.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>R(-) enantiomer</th>
<th>Median</th>
<th>Range</th>
<th>S(+) enantiomer</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>µg/mL</td>
<td>7.7</td>
<td>(3.3-374.5)</td>
<td></td>
<td>6.0</td>
<td>(2.9-12.8)</td>
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<td>(0.8-11.3)</td>
<td>0.7</td>
<td>(0.4-1.3)</td>
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</tr>
<tr>
<td>B</td>
<td>µg/mL</td>
<td>1.4</td>
<td>(0.3-3.1)</td>
<td>3.0</td>
<td>(1.3-3.1)</td>
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</tr>
<tr>
<td>β</td>
<td>1/hr</td>
<td>0.1</td>
<td>(0.1-0.2)</td>
<td>0.1</td>
<td>(0.1-0.1)</td>
<td></td>
<td></td>
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<tr>
<td>αHL</td>
<td>hr</td>
<td>0.6</td>
<td>(0.1-0.8)</td>
<td>1.0</td>
<td>(0.5-1.6)</td>
<td></td>
<td></td>
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<tr>
<td>βHL</td>
<td>hr</td>
<td>6.8</td>
<td>(4.0-12.6)</td>
<td>6.2</td>
<td>(5.2-6.8)</td>
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<td></td>
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<tr>
<td>AUC</td>
<td>hr*µg/mL</td>
<td>23.0</td>
<td>(7.5-51.5)</td>
<td>29.8</td>
<td>(15.9-47.9)</td>
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<td></td>
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<tr>
<td>AUCMC</td>
<td>hr*µg/mL</td>
<td>106.5</td>
<td>(86.4-147.2)</td>
<td>192.3</td>
<td>(105.4-311.7)</td>
<td></td>
<td></td>
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<tr>
<td>CL</td>
<td>mL/hr/kg</td>
<td>95.8</td>
<td>(43.0-292.9)</td>
<td>73.9</td>
<td>(46.0-138.7)</td>
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<td></td>
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<tr>
<td>CLD2</td>
<td>mL/hr/kg</td>
<td>80.6</td>
<td>(22.1-363)</td>
<td>33.4</td>
<td>(29.3-201.9)</td>
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<td></td>
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<tr>
<td>CMAX</td>
<td>µg/mL</td>
<td>9.1</td>
<td>(3.6-377.6)</td>
<td>7.3</td>
<td>(5.8-15.7)</td>
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<tr>
<td>k10</td>
<td>1/hr</td>
<td>0.5</td>
<td>(0.4-7.4)</td>
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<td>(0.2-0.5)</td>
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<td>k10HL</td>
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<td>1.4</td>
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<td>2.1</td>
<td>(1.5-3.6)</td>
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<tr>
<td>k12</td>
<td>1/hr</td>
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<td>(0.3-3.8)</td>
<td>0.2</td>
<td>(0.1-0.7)</td>
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<tr>
<td>k21</td>
<td>1/hr</td>
<td>0.2</td>
<td>(0.1-0.3)</td>
<td>0.3</td>
<td>(0.2-0.3)</td>
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<tr>
<td>MRT</td>
<td>hr</td>
<td>6.4</td>
<td>(2.1-11.5)</td>
<td>6.5</td>
<td>(6.5-6.7)</td>
<td></td>
<td></td>
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<tr>
<td>V1</td>
<td>mL/kg</td>
<td>242.1</td>
<td>(5.8-611.8)</td>
<td>301.3</td>
<td>(139.8-378.0)</td>
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<td></td>
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<tr>
<td>V2</td>
<td>mL/kg</td>
<td>371.9</td>
<td>(83.8-2756.6)</td>
<td>159.6</td>
<td>(98.9-620.9)</td>
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<tr>
<td>Vss</td>
<td>mL/kg</td>
<td>614.0</td>
<td>(89.7-3368.4)</td>
<td>476.9</td>
<td>(299.4-922.2)</td>
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</tbody>
</table>
Table 2.2 Pharmacokinetic parameters of maternal plasma carprofen R(-) enantiomers per dog.

A, B: Y-axis intercepts for the phases of distribution and elimination; α, β, fractional elimination rate constants; k1, k2: rate constant for the distribution and elimination, respectively; k10, k12, k21: elimination and compartmental rate constants; MRT: mean residence time; k10 HL: elimination half-life; α-HL: distribution half-life; β-HL: terminal half-life; AUC, area-under-the-curve; Vss: volume of distribution at steady state; Cl, total plasma clearance.

<table>
<thead>
<tr>
<th>Dog</th>
<th>αHL (hr)</th>
<th>βHL (hr)</th>
<th>AUC (hr*ug/mL)</th>
<th>AUMC (hr<em>hr</em>ug/mL)</th>
<th>CL (mL/hr/kg)</th>
<th>CLD2 (mL/hr/kg)</th>
<th>Cmax (ug/mL)</th>
<th>K10 (1/hr)</th>
<th>K10_HL (hr)</th>
<th>K12 (1/hr)</th>
<th>K21 (1/hr)</th>
<th>MRT (hr)</th>
<th>Vss (mL/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>12.63</td>
<td>7.51</td>
<td>86.38</td>
<td>292.9</td>
<td>363.02</td>
<td>3.6</td>
<td>0.48</td>
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<td>0.59</td>
<td>0.13</td>
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<td>3368.43</td>
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<tr>
<td>2</td>
<td>0.06</td>
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<td>43.03</td>
<td>22.08</td>
<td>377.57</td>
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<td>0.09</td>
<td>3.79</td>
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<td>89.65</td>
</tr>
<tr>
<td>3</td>
<td>0.82</td>
<td>6.82</td>
<td>22.96</td>
<td>147.15</td>
<td>95.81</td>
<td>80.55</td>
<td>9.09</td>
<td>0.4</td>
<td>1.75</td>
<td>0.33</td>
<td>0.22</td>
<td>6.41</td>
<td>614.01</td>
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</table>
Table 2.3 Pharmacokinetic parameters of maternal plasma carprofen S(+) enantiomers per dog.

A, B: Y-axis intercepts for the phases of distribution and elimination; α, β, fractional elimination rate constants; k1, k2: rate constant for the distribution and elimination, respectively; k10, k12, k21: elimination and compartmental rate constants; MRT: mean residence time; k10 HL: elimination half-life; α-HL: distribution half-life; β-HL: terminal half-life; AUC, area-under-the-curve; Vss: volume of distribution at steady state; Cl, total plasma clearance.

<table>
<thead>
<tr>
<th>Dog</th>
<th>αHL (hr)</th>
<th>βHL (hr)</th>
<th>AUC (hr*ug/mL)</th>
<th>AUMC (hr<em>hr</em>ug/mL)</th>
<th>CL (mL/hr/kg)</th>
<th>CLD2 (mL/hr/kg)</th>
<th>Cmax (ug/mL)</th>
<th>K10 (1/hr)</th>
<th>K10_HL (hr)</th>
<th>K12 (1/hr)</th>
<th>K21 (1/hr)</th>
<th>MRT (hr)</th>
<th>Vss (mL/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.52</td>
<td>6.22</td>
<td>15.86</td>
<td>105.42</td>
<td>138.73</td>
<td>201.91</td>
<td>7.3</td>
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<td>0.33</td>
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<td>922.19</td>
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<tr>
<td>2</td>
<td>1.59</td>
<td>5.23</td>
<td>29.78</td>
<td>192.28</td>
<td>73.87</td>
<td>29.31</td>
<td>5.82</td>
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<td>0.3</td>
<td>6.46</td>
<td>476.86</td>
</tr>
<tr>
<td>3</td>
<td>1.03</td>
<td>6.8</td>
<td>47.85</td>
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<td>45.97</td>
<td>33.38</td>
<td>15.74</td>
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<td>0.24</td>
<td>0.21</td>
<td>6.51</td>
<td>299.41</td>
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</tbody>
</table>
Figure 2.1 Chiral Chromatogram of Maternal Plasma #2 at 30 minutes

Figure 2.2 Nonchiral Chromatogram of Maternal Milk #1 at 12 hours
Figure 2.3 Nonchiral Chromatogram of Litter Plasma #1 at 30 minutes.

Figure 2.4 Calibration Curve for R-enantiomer The curve consists of external standards 0.05, 0.25, 0.5, 2.5, 5, and 25 μg/mL.
Figure 2.5 Calibration Curve for S-enantiomer The curve consists of external standards 0.05, 0.25, 0.5, 2.5, 5, and 25 μg/mL.

Figure 2.6 Calibration Curve for Milk Calibration curve consisted of external standards of 0.05, 0.1, 0.5, and 1 μg/mL.
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https://doi.org/10.1111/jvp.12233


CHAPTER 3

Results

Introduction

As mentioned, complete samples from all three test groups were collected from four out of seven dogs with some samples missing due to either inability to express milk at that timepoint or inadequate volume of plasma recovered after centrifugation and thawing. Of these four dogs, pharmacokinetic analysis was performed and it was later decided to exclude Dog #4 in the modeling. None of the dogs experienced an adverse reaction to the carprofen.

High performance liquid chromatography

For the HPLC assays, all maternal samples showed that carprofen levels dropped below the limit of detection by 24 hours which was expected for the dosing administered (24-hour dosing at 4.4 mg/kg) and was comparable to previous studies in healthy dogs (Lascelles et al., 1998; Lascelles et al, 2004; Clark et al., 2003; Lipscomb et al., 2002). An example of one of the chromatographs is shown in Figure 3.1. Post-carprofen administration concentrations ranged from 0 μg/mL to 6.11 μg/mL for the R(-) enantiomer and 0 μg/mL to 10.85 μg/mL for the S(+) enantiomer.

Most of the neonatal samples were below the limit of detection for most time points with only 17 out of 35 samples being above the limit of detection (0.001 μg/mL). Out of these 17 samples however, only four were above the limit of quantification (0.003 μg/mL). The majority were in Litter #1 (0.5 (Figure 2.3), 1.5, and 12 hours post maternal treatment) and the last was at the first time point for Litter #2 (0.5 hours post maternal treatment). When we analyzed the milk samples, carprofen was able to be measured above the limit of quantification and detection at a
single timepoint (12 hours) in Dam #1 (Figure 2.2). The average plasma concentrations for each timepoint among the dams were plotted on a logarithmic and nonlogarithmic scale with separation of the R- and S-enantiomer (Figure 3.1) on FigSys P software. The neonatal plasma samples were also averaged for each timepoint and plotted on a logarithmic scale against the plasma concentrations of the dams for comparison with the single milk sample (Figure 3.2). None of the neonatal plasma concentrations at any time point were more than 10% of the total maternal plasma concentrations.

**Pharmacokinetics**

For pharmacokinetic analysis, a total of 21 concentration-time samples (time 0.5-24 hours) from 3 dogs were included into population pharmacokinetic modeling for both R- and S-enantiomer of carprofen. The Phoenix software was used in a 2-compartment modeling system based on the route of administration being intravenous and utilizing the AIC where an AIC < 0 is desirable. Pharmacokinetic analysis was done only for the maternal data (Figures 4.1-4.4) due to so few timepoints above the limit of quantification for both neonatal plasma and milk samples. Observed versus predicted plots were analyzed and all were accepted with a minimum of three data points by visual inspection. Parameters for both the R- and S-enantiomer were analyzed, and it was decided to remove Dog #4 from the model due to a high percentage of extrapolation of 35% (<20% being acceptable).

Enantiomer-specific primary parameter estimates for total (bound and unbound carprofen) are shown in Table 1.2 and 1.3 and were based on the equation:

\[ C_t = Ae^{-\alpha t} + Be^{-\beta t} \] (Equation 2),
where A and B are the coefficients (µg/mL), α (distribution rate constant), and β (terminal elimination rate constant) are exponents (per hour), and C\textsubscript{t,p} is the plasma concentration (µg/mL) of carprofen at time t. Secondary parameter estimates were obtained using standard compartmental equations (Gibaldi & Perrier, 1982) and are shown in Table 1.1. The following parameter estimates for the total drug concentrations were different for the R(-) enantiomer than previously reported (Messenger et al, 2015, Schmitt & Guentert, 1990): A, B, AUC, C\textsubscript{MAX}, Cl, mean residence time (MRT), k\textsubscript{12}, k\textsubscript{10HL}, k\textsubscript{21}, V1, and V\textsubscript{ss}. The following parameter estimates for the total drug concentrations were different for the S(+) enantiomer than previously reported: A, B, α, αHL, AUC, C\textsubscript{MAX}, Cl, mean residence time (MRT), k\textsubscript{12}, k\textsubscript{10HL}, k\textsubscript{21}, V1, and V\textsubscript{ss}. Where A is the Y-axis intercept for α (fractional elimination rate constant), B is the Y-axis intercept for β (fractional elimination rate constant), and AUC is the area-under-the-curve using the linear trapezoidal rule for ascending values and logarithmic trapezoidal rule for descending values. C\textsubscript{MAX} is the maximum concentration. Cl is plasma clearance of carprofen, k\textsubscript{12} and k\textsubscript{21} are compartmental rate constants between the intravascular to extravascular space (k\textsubscript{12}) and vice versa (k\textsubscript{21}), and k\textsubscript{10HL} is the elimination half-life. Lastly, V1 is the central compartment (intravascular space) and V\textsubscript{ss} is the volume of distribution between the central and secondary compartment (extravascular space) at steady state.
Figure 3.1 Maternal Carprofen Concentrations in Dogs Maternal carprofen concentrations in plasma averaged for each timepoint and placed on a nonlogarithmic scale. The S-enantiomer concentration is in red circles and R-enantiomer concentration in blue squares.
Figure 3.2 Comparative Carprofen Concentrations in Dogs: Comparison of maternal carprofen concentrations in plasma averaged for each timepoint out to 24-hours on a logarithmic scale broken into R- and S-enantiomers concentrations, total carprofen concentrations in neonatal plasma, and the single milk sample above LOQ. The litters were also averaged together for each timepoint. S-enantiomer concentration in red circles, R-enantiomer concentration in blue squares, neonatal plasma concentration in green diamonds, and milk as a black triangle.
Figure 3.3 Pharmacokinetics of Carprofen in Plasma Dog #1 A two-compartment pharmacokinetic modeling system on a nonlogarithmic scale was used to graph the R-enantiomer concentration of carprofen (black circles) compared to the S-enantiomer concentration of carprofen (purple squares) against time.
Figure 3.4 Pharmacokinetics of Carprofen in Plasma Dog #2 A two-compartment pharmacokinetic modeling system on a nonlogarithmic scale was used to graph the R-enantiomer concentration of carprofen (black circles) compared to the S-enantiomer concentration of carprofen (purple squares) against time.
Figure 3.5 Pharmacokinetics of Carprofen in Plasma Dog #3 A two-compartment pharmacokinetic modeling system on a nonlogarithmic scale was used to graph the R-enantiomer concentration of carprofen (black circles) compared to the S-enantiomer concentration of carprofen (purple squares) against time.
REFERENCES


CHAPTER 4

Discussion

Carprofen is one of the most commonly prescribed and administered analgesics for a variety of indications such as short and long-term pain management for perioperative use, mastitis, metritis, and osteoarthritis, to name a few. Despite its wide-spread use in veterinary medicine, this is the first study to describe population pharmacokinetics of intravenous carprofen in the plasma of lactating bitches, measure carprofen concentrations in milk under an intense sampling schedule, and measure carprofen concentrations in neonatal plasma. Even though we were unable to analyze pharmacokinetics for all testing groups, this study provides support that carprofen is safe to administer in lactating bitches due to low observed milk concentrations and negligible risk for neonatal exposure.

Maternal plasma concentrations

The concentrations of carprofen in the maternal plasma of lactating dogs were different than that previously published for non-lactating dogs (McKellar, et al, 1994; Schmitt & Guentert, 1990). The difference in concentrations for each enantiomer is possibly due to higher plasma volume and underlying physiological inflammation associated with uterine involution. Changes in plasma volume have been documented to increase by 23% in lactation for sheep and 45% in women who are pregnant. Unfortunately, there have been no specific studies done in canine pregnancy to quantify this change, but an increase in plasma volume would dilute carprofen concentrations and explain our observations. Another potential consequence of an increased plasma volume is dilution of albumin which could impact protein bound drugs such as carprofen. The effect on protein binding was not investigated in this study, but would be recommended for
future work. The process of uterine involution following parturition takes about 86-87 days for completion and is characterized by uterine gland regression and uterine luminal epithelium degeneration. This physiological inflammatory response is predominately characterized by lymphocytes at this time but earlier on has a neutrophilic component as well. Unbound and active carprofen was recently shown to not gravitate towards local areas of inflammation (Messenger, et al., 2015), but this process could be a possibility in our study population with the site of inflammation being larger (the uterus) than. However, this was not investigated in our current study and cannot be confirmed at this time. Our study found multiple differences in the pharmacokinetic parameter estimates compared to previous studies. The maximum concentration measured lower than previous studies (McKellar et al., 1990; Clark et al, 2003) at 9.086 μg/mL and 7.303 μg/mL for R(-) enantiomer and S(+) enantiomer respectively. The time for maximum concentration (T_{MAX}) was similar as well to other reports for oral administration (Clark et al, 2003) at 30-90 minutes.

One difference seen in our population was a higher clearance rate 95.812 mL/hr/kg and 73.866 mL/hr/kg (R-enantiomer, S-enantiomer). This may be due to physiological changes such as an increase in plasma volume, decrease in vascular resistance, increase in renal blood flow, and increase in hepatic metabolism that occurs in pregnancy and lactation. Estradiol and progesterone in pregnancy in women increases hepatic metabolism via xenobiotics as well as CY-P450 enzyme activity and increased hepatic blood flow (Jeong, 2010). We theorize these changes are similar in dogs and gradually normalize to the pre-pregnant state as uterine involution occurs which can be from 60-90 days after whelping (Johnston et al, 2001). This could be the reason why our lactation group had a faster clearance compared to nonlactating dogs (Messenger et al, 2015; Messenger & Papich, 2013; McKellar et al, 1990; Clark et al,
2003). Unfortunately, no previous studies have examined changes in drug metabolism for hepatic clearance in the dog to our knowledge. Pharmacokinetic studies of penicillin and benzylpenicillin in nonpregnant, pregnant, and lactating sheep have shown a 23% higher clearance during lactation, theorized to be due to an increase of plasma volume and blood flow (OuKessou & Benlamith, 1990). The increase in clearance rate in our study would explain other parameter changes seen such as a lower AUC for both enantiomers, lower MRT for both enantiomers, and lower rate constants from the intravascular space to the extravascular space ($k_{10}$) and its associated half-life ($k_{10HL}$). The terminal half-life, $\beta_{HL}$, was similar to other reports but was just slightly lower by 1 hour. It has been reported that clinical efficiency is met when 80% of the COX-2 receptors are inhibited (Lees et al, 2004). Since the S(+) enantiomer has the most anti-inflammatory action in the racemic mixture, enantiomer-specific in vitro studies have found a concentration above 2-3 $\mu$g/mL achieves the minimum of 80% inhibition (Messenger et al, 2015). In comparison our data revealed that 2 of the 3 dogs had concentrations fall below the 2-3 $\mu$g/mL between 8-24 hours post-administration, which may mean that pain control is not met for a full 24 hours in lactating bitches. However, there was substantial variation between each dog and a small sample size; therefore, more work will be needed before a conclusion can be drawn regarding the pharmacokinetics and the clinical control of pain.

Another main difference in our population was $V_1$ was 2-3 times higher compared to previous reports. This could be due to the residual increase in plasma volume from pregnancy or carprofen could have been drawn out into the extravascular space due to underlying uterine involution. As mentioned previously, there was a large variation between each dog, so even though previous physiological changes could have a role for the changes seen, more dogs would need to be assessed to fully examine the pharmacokinetics before reaching a conclusion.
Neonatal plasma concentrations

There were only four timepoints across two litters where carprofen concentrations were above the limit of quantification. We theorize that carprofen was not consistently measured above the limit of quantification in neonatal plasma due to variation in the volume of milk ingested between litters as they were allowed to nurse unrestricted and concentration levels in milk were low. Most of the samples that measured above the limit of quantification were from Litter #1. We believe this was because Dam #1 was the heaviest dog (23.3 kg) and therefore had the largest dose of carprofen administered allowing for more exposure to her litter. Of the four samples that had carprofen concentrations above the limit of quantification, all but one occurred within the first 90 minutes, when the most transference between compartments would be expected. When total concentrations of carprofen (averaged at each timepoint and for each timepoint) were compared between maternal plasma and neonatal plasma, neonatal plasma concentrations were less than 10% of maternal concentrations which is in line with the WHO recommendations for human infant exposure.

Milk concentrations

The lack of detection of carprofen in milk was expected due to the partition theory as well as carprofen’s high percentage of protein binding in the plasma (>98%). Carprofen also has a higher affinity for albumin (major protein in plasma) compared to casein (major protein in milk) which explains why very little carprofen was measured in the milk. It is interesting how the single milk sample above the limit of quantification was at the timepoint of 12 hours post-treatment in Dam #1 coinciding with Litter #1 which measured above the limit of quantification
at that timepoint as well. We do not have an explanation on why carprofen concentrations in the milk were not detectable at the other timepoints that associated with detectable or quantifiable levels in the neonatal plasma, as milk was the only source of carprofen to this test group.

Despite our small sample size, the findings of carprofen concentrations in milk were similar to the low levels of carprofen in milk found in the study done by Ferrari et al (2022) utilizing a five-day course of oral carprofen. This study showed the highest level of carprofen in their control group to be 0.7 μg/mL with the majority being less than 0.3 μg/mL. Ferrari et al’s study (2022) had slightly higher levels of carprofen in the milk when compared to our study, the highest level in milk measuring 0.102 μg/mL, most likely due to the stage of lactation. Ferrari’s study enrolled dogs in the beginning of their lactation which would involve a smaller volume of milk produced as well as colostrum. This stage in lactation would allow for larger-sized proteins to pass through the mammary gland barrier in the first 24-hours as well as a higher concentration due to the smaller volume of milk produced at this time. In comparison our study, dogs were enrolled dogs in mid-lactation where the mammary epithelial tight junctions should be intact and a larger volume of milk is produced diluting out the concentration of carprofen that was transferred from the plasma. In both studies however, even with the higher concentrations in early lactation, the milk:plasma ratio is still less than one, deeming it safe for neonates.

Limitations

Aside from the small sample size, other limitations to our study are the single administration of carprofen and in an off-label route than is typical in the US. As our study was designed to create a pharmacokinetic curve, we prioritized sampling over multiple timepoints after a single administration of carprofen versus a multiple-day treatment course, as the levels of
carprofen should behave the same way with each administration. Also, utilizing a single administration would be similar to a single dose of carprofen at the time of cesarean section which is the primary treatment group for analgesia. We decided to administer carprofen intravenously instead of the labelled route (subcutaneously or oral in the US) to increase bioavailability, calculate pharmacokinetics, and previous studies using an intravenous route did not see any adverse reactions (Messenger et al., 2015; Schmitt & Guentert, 1990). This route of administration is labelled for carprofen outside of the US. Unfortunately, we were unable to produce pharmacokinetic curves for both canine milk and neonatal plasma due to the few timepoints above the limit of quantification. However, we were able to calculate milk:plasma ratios based on concentration and not AUC for each timepoint which show that for all timepoints they are less than one. Since this study was not designed to gain FDA labelling for carprofen in lactating dogs, we did not run our samples in triplicates, ensure every calibration back calculated within 15%, and utilized the linear coefficient of determination to approve all calibration curves.

**Conclusion**

Despite these limitations, this is the first study to our knowledge that has measured carprofen in neonatal plasma as well as canine milk at multiple timepoints after maternal administration. Even though previous studies have measured carprofen in canine milk on a once daily sampling basis, none reported to have measured concentrations more frequently than that. All previous studies to our knowledge have used predicted modelling to estimate neonatal exposure and M/P ratios but have not directly measured neonatal plasma or M/P ratios. It is encouraging to see that many of the samples that were taken of canine milk and neonatal plasma were below the limit of detection as this supports our hypothesis that carprofen is a safe analgesic to give to nursing dogs.
Regarding the pharmacokinetics, it is difficult to draw conclusions from only three dogs with a high level of variation between each one. We would need data from approximately 8-10 more dogs in order to normalize data and reduce the variation before any conclusions can be drawn aside from there may be a difference between nonpregnant/nonlactating and lactating dogs.

We hope this study design will be used by others to undergo a more expansive study with sufficient power to gain the necessary pharmacokinetic data to answer the questions that we were not able to do in our study. This study and sampling design can also be used to expand our future knowledge of drug passage in canine lactation with other medications that are commonly prescribed such as antibiotics and anxiolytics. Also, more research is needed examining the physiologic changes that occur in lactation, and how these changes affect drug metabolism to better explain the high clearance rate seen in our study. This and future work will aid veterinarians to practice evidence-based medicine more efficiently and limit second-hand exposures that could be detrimental to the health of the puppies.
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