MESSENGER, KRISTEN. The Role of Drug and Patient Factors in the Pharmacokinetics of the Nonsteroidal Anti-inflammatory Drug Carprofen in Dogs (Under the direction of Mark Papich).

Nonsteroidal anti-inflammatory drugs (NSAIDs), including carprofen, are among the most commonly administered analgesic drugs in veterinary medicine. However, there are serious adverse events associated with carprofen administration that are still unexplainable and unpredictable. We propose that differences in carprofen disposition, or pharmacokinetics, may result in adverse effects or lack of efficacy in some dogs. Despite the widespread use of carprofen in dogs and numerous published reviews, there continues to be speculation of the factors that could influence carprofen pharmacokinetics which could predispose an animal to developing adverse drug effects.

In the first study, we examine the effects of protein binding drug interactions on the pharmacokinetics of intravenously administered carprofen in healthy Hound dogs, using a second highly protein bound drug, cefovecin, to cause a drug displacement interaction. An additional objective was to examine whether there were differences in protein binding between enantiomers of carprofen, which might account for differences in drug efficacy. Although minor significant differences in in vivo pharmacokinetics were detected, the overall effects were very small and we concluded that there would be no clinical significance of this interaction. Also, although protein binding was statistically different between enantiomers, the difference was so small that it is unlikely to account for any differences in drug pharmacodynamics in dogs.
In the second study, we examined the population pharmacokinetics of carprofen in clinical patients, and made comparisons with data obtained from healthy research dogs. We assessed the effects of population differences, such as breed, gender, age, weight, and health status on carprofen distribution and clearance using nonlinear mixed effects modeling. Osteoarthritis was a significant covariate in the analysis on Cl/F for the S(+) enantiomer, resulting in a 27% lower Cl/F in dogs having osteoarthritis. Additionally, healthy Beagle dogs exhibited different pharmacokinetics from other healthy research dogs (Hounds) and the clinical population of dogs. The current clinical dosing regimens of carprofen do not need alteration at this time, as no dogs in the study exhibited significant adverse events that were linked to pharmacokinetic differences. However, these findings are important because healthy Beagle dogs are frequently used to in studies to define clinical dosing regimens, and may not not reflect the intended clinical population.

The final project focused on the effects of inflammation on the disposition of carprofen in healthy Beagle dogs using in vivo ultrafiltration- a minimally invasive and pharmacologically relevant technique to simultaneously collect unbound (active) carprofen concentrations and biomarkers of drug efficacy directly from sites of action. We discovered that carprofen distributes relatively evenly to both normal and inflamed tissues, which is contrary to most hypotheses that NSAIDs distribute preferentially to sites of action and spare “normal” tissues. We also noted that plasma drug concentrations of carprofen do not reflect the concentrations at tissue sites, further emphasizing the importance of study designs integrating effect-site drug collection.

Overall, these studies demonstrate that plasma and tissue pharmacokinetics of carprofen in dogs are minimally influenced by factors such as protein binding interactions or
inflammation, and in vivo ultrafiltration appears to be a novel technique to study the pharmacokinetics and pharmacodynamics of anti-inflammatory drugs directly at tissue sites. Additionally, there appear to be very few patient-specific factors that influence the pharmacokinetics of carprofen in dogs, which is perhaps a testament to this drug’s overall safety in this species. The lower apparent clearance of the active enantiomer of carprofen in dogs with osteoarthritis was an interesting finding, which leads to further questions as to the cause of this finding. Lastly, the lower apparent clearance in the overall clinical population, as compared to healthy research dogs, such as Beagles, does raise many questions as to whether there might be breed-specific differences in carprofen metabolism, but also whether the widespread use of Beagle dogs in preclinical drug safety and efficacy studies is representative of other dog breeds.
The Role of Drug and Patient Factors in the Pharmacokinetics of the Nonsteroidal Anti-
inflammatory Drug Carprofen in Dogs.

by
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DEDICATION

To my family for their continuous love and support.
BIOGRAPHY

Kristen Messenger is originally from Charlotte, North Carolina. She has spent the majority of her educational career at North Carolina State University and the College of Veterinary Medicine. She received board certification in Veterinary Anesthesia and Analgesia in 2011, and board certification in Veterinary Clinical Pharmacology in 2014 under the mentorship of her PhD advisor, Dr. Mark Papich. Currently she is a Lecturer in Anesthesiology at the College of Veterinary Medicine, and divides her time between clinical and didactic teaching and research. Her research interests are in pain management, and include the pharmacokinetics and pharmacodynamics of analgesic drugs in large and small animals.
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<td>Alpha-1 acid glycoprotein</td>
</tr>
<tr>
<td>ADE</td>
<td>Adverse drug event</td>
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<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>ALKP</td>
<td>Alkaline phosphatase</td>
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<td>AUC</td>
<td>Area under the time-concentration curve</td>
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<td>Description</td>
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</tr>
<tr>
<td>UF</td>
<td>Ultrafiltration</td>
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1. INTRODUCTION

Carprofen is a nonsteroidal anti-inflammatory drug approved in the United States for the treatment of pain and inflammation associated with osteoarthritis and surgery. It is commonly used across the world as an analgesic in dogs and other animals, and exerts its major effects via the inhibition of cyclo-oxygenase (COX) enzymes in order to decrease pro-inflammatory mediator production. Clinical studies have documented the effectiveness of carprofen for providing analgesia to dogs, but there have also been numerous adverse events reported following carprofen administration. These events include vomiting, diarrhea, anorexia, gastrointestinal ulceration, kidney injury, liver injury, and death. Additionally, some dogs do not respond to treatment, i.e., they are still painful or continue to have decreased mobility despite carprofen administration. The overall purpose of these studies was to determine if there are differences in the pharmacokinetics of carprofen that might explain why some dogs develop adverse side effects following administration, or fail to respond to the analgesic effects of carprofen.

First we determined whether protein-binding drug interactions would result in relevant changes in the pharmacokinetics of carprofen, as it is highly protein-bound at > 99%. In this series of experiments, we determined the enantiospecific protein binding of carprofen in canine plasma, we confirmed that a drug displacement interaction occurs with the highly (>98%) protein-bound antimicrobial cefovecin in vitro, and we tested the interaction in vivo in healthy Hound dogs. We found that a minor interaction does occur in vivo, but it occurred for the R(-) enantiomer, which exhibits far less anti-inflammatory activity than the active S(+) enantiomer. We concluded that a clinically relevant protein drug-displacement interaction does not occur in vivo and that the two drugs tested could be safely co-administered in dogs.
Next we questioned whether or not the previous pharmacokinetic studies on carprofen would be representative of the pharmacokinetics in the intended treatment population, as the majority of previous studies were conducted in young, otherwise healthy Beagle dogs. We conducted a population pharmacokinetic study as a clinical trial, enrolling dogs of all breeds and ages which were prescribed carprofen by a veterinarian to treat a painful and/or inflammatory condition such as osteoarthritis. We used non-linear mixed effects modeling to determine population parameter estimates for orally administered carprofen using two different dosing regimens in dogs, as well as testing a series of covariates such as age, weight, breed, gender, disease status, and kidney and liver health as possible factors that may significantly affect carprofen parameter estimates in clinical patients. We found that dogs with a history of osteoarthritis have a lower apparent clearance compared with the rest of the clinical population. Additionally, estimates for clearance in this entire clinical population of dogs studied were lower than previously reported estimates for carprofen clearance in healthy Beagle research dogs. The cause for changes in clearance associated with osteoarthritis are undetermined. We also found that the dosing regimen of once versus twice daily administration does not affect the overall exposure to carprofen; i.e. dogs receiving lower doses twice a day are exposed to the same plasma concentrations as dogs receiving higher doses once a day.

Finally, we determined the distribution of unbound (active) carprofen in inflamed and normal (control) tissue sites using an ultrafiltration technique. A previously established model of carrageenan-induced inflammation was studied in healthy Beagle dogs. Unbound carprofen concentrations were collected and quantified from saline control and inflamed tissue sites following subcutaneous carrageenan injection. Prostaglandin E₂, a pro-inflammatory biomarker, was quantified from tissue fluid samples in order to examine the anti-inflammatory
effects of carprofen at relevant sites. We found there to be no significant difference in unbound carprofen concentrations or pharmacokinetics parameters in inflamed tissue sites compared to control sites, and PGE$_2$ was significantly reduced in inflamed tissues as early as 2 hours following carprofen administration. We observed that the unbound concentrations of carprofen in the tissues was greater and occurred over a longer period of time than what could be predicted using plasma concentrations with corrections made for protein binding, emphasizing the need for pharmacokinetic studies with NSAIDs that examine drug concentrations directly at tissue sites rather than using plasma concentrations to infer tissue concentrations.

The findings in these studies above suggest that surprisingly few factors influence the pharmacokinetics of carprofen in dogs to an extent that may affect clinical efficacy. Although we assessed protein binding interactions with only one drug (cefovecin), the displacement interaction that occurred did not result in a major change in the total (bound + unbound) plasma pharmacokinetics and no dogs experienced adverse effects. The population study allowed us to evaluate patient-specific factors that might result in pharmacokinetic changes in individuals, but neither the pharmacokinetics nor plasma concentrations of carprofen varied widely across a diverse patient population. Even though clearance was lower in our clinical patients- and even lower (by about 25%) in dogs with osteoarthritis-compared to healthy research dogs, this difference did not increase carprofen exposure to a degree that produced adverse events in the dogs. Our tissue studies showed that plasma drug concentrations do not appear to predict activity in the tissue. Carprofen was collected from the tissue sites for far longer than we would have predicted based on plasma drug concentrations. Lastly, these studies have also raised new and important questions about differences in carprofen metabolism in specific dog
populations, such as Beagles, and also during inflammatory disease conditions such as osteoarthritis.
2. LITERATURE REVIEW

*Pain and inflammation*

The treatment of pain in humans and animals is extremely important in medicine. The sensation of pain is experienced by individuals to serve as a protective role, in order to prevent exposure to damaging stimuli. However, pain can become maladaptive, no longer serving to protect the individual and instead causing harm in and of itself. Although the consequences of untreated pain are better documented in human medicine, animals can experience similar outcomes including decreased immune function, which leads to delayed wound healing, and the development of chronic pain conditions which can be unresponsive to traditional analgesics (Brune & Patrignani, 2015). Pain is incredibly complex and is affected by many factors, one of which is inflammation. Inflammation results in pain sensation through the stimulation of tissue nociceptors by pro-inflammatory substances including prostaglandins, bradykinins, and cytokines (tumor necrosis factor and interleukins) (Muir & Woolf, 2001; Svensson & Yaksh, 2002; Latremoliere & Woolf, 2009). Peripheral nociceptor activation and sensitization is the first step in the pain pathway, ultimately leading to the sensory component of pain in the brain. Long term sensitization of peripheral nociceptors can lead to changes in neuronal growth and processing in the spinal cord, ultimately resulting in chronic, debilitating pain that is non-responsive to therapy (Millan, 1999; Muir & Woolf, 2001; Svensson & Yaksh, 2002; Latremoliere & Woolf, 2009). Preventing this devastating sequela is of critical importance. Inflammation and inflammatory pain can often be effectively treated with nonsteroidal anti-inflammatory drugs (NSAIDs) because they inhibit the generation of pro-inflammatory mediators by inhibiting cyclooxygenase enzymes (Figure 2.1). This important class of drugs will be discussed in great detail throughout the following review.
The important roles of cyclooxygenase in homeostasis and injury

Cyclooxygenase is a major enzyme in the arachidonic acid cascade and interacts with arachidonic acid to generate unstable intermediary compounds that ultimately result in eicosanoid production (Figure 2.1). Currently 3 isoforms of COX have been identified: COX-1, -2, and -3.

Cyclooxygenase 1 is generally considered to be a constitutive enzyme that is found in tissues throughout the body and maintains homeostatic functions through regulation of various
prostaglandins (PGs) and thromboxanes (TXs), whereas COX-2 is classified as “inducible,” meaning it is up-regulated during states of tissue injury, and is therefore responsible for the pain and inflammation associated with tissue damage (Vane et al., 1998).

The COX-1 isoform is found at high levels in the endothelium, gastric mucosa, platelets, monocytes, and kidneys (Ellis et al., 1976; Simmons et al., 2004; Wilson et al., 2004). In these cells and tissues, it is an important enzyme for the generation of cytoprotective prostaglandins, which include prostaglandin-D2 (PGD2), -E2 (PGE2), -F2α (PGF2α), -I2 (prostacyclin, PGI2), and thromboxane A2 (TXA2) (Figure 2.1). Each of these substances then exerts various effects through interactions with G-protein coupled receptors, including the 4 subtypes of the PG receptor: E2, E3, E4, and the prostacyclin (IP2) receptors (Svensson & Yaksh, 2002; Simmons et al., 2004).

The COX enzymes, in particular COX-1, are involved in generating prostaglandins involved in homeostasis and repair of the gastrointestinal tract. Cyclooxygenase-1 induced PGE2 and PGI2 function to maintain healthy gastrointestinal mucosa and assist in tissue repair following injury (Robert et al., 1979; Robert et al., 1983; Vane et al., 1998; Simmons et al., 2004). The cytoprotective effects of these prostaglandins in the gastrointestinal tract include the following: inhibition of acid secretion, maintenance and enhancement of local mucosal blood flow, stimulation of mucus and bicarbonate secretion, and stimulation of epithelial cell growth (Robert et al., 1967; Main & Whittle, 1973; Bolton et al., 1978; Johansson & Kollberg, 1979; Wright et al., 1990; Wallace, 2008).

Both COX-1 and -2 (which will be discussed in more detail later) are found in the kidneys, generating local PGI2 and PGE2 that are important for the regulation of renal blood flow during compromised states such as hypovolemia or hypotension. These prostaglandins
thereby serve a protective role under these conditions (Vane et al., 1998). Prostaglandin E$_2$ also regulates electrolyte excretion and reabsorption and renin release through COX expression in the macula densa and loop of Henle (Simmons et al., 2004; Peti-Peterdi & Harris, 2010). The homeostatic mechanisms of PGs in the kidney are blocked when NSAIDs are administered, which has resulted in serious kidney injury (Cheng & Harris, 2005).

Vascular tone and platelet aggregation are also regulated by PGs, specifically TXA$_2$ and PGI$_2$. COX-1, but not COX-2 is found in the platelet and produces TXA$_2$. Thromboxane A$_2$ is pro-thrombotic (results in clotting), while PGI$_2$, produced by COX-2 in endothelial cells is anti-thrombotic and vasodilatory (Ellis et al., 1976; Needleman et al., 1976; Moncada et al., 1976). In humans, the vascular events, such as myocardial infarction, that occur following COX-2 selective NSAID administration (i.e. rofecoxib) have led to serious adverse events, including death (Antman et al., 2007; Brune & Patrignani, 2015). The mechanism of this effect is via inhibition of COX-2, reducing the formation of vasodilatory PGI$_2$, while maintaining TXA$_2$ (and therefore pro-thrombotic activity) through the COX-1 pathway (Funk & Fitzgerald, 2007).

The inducible form of the COX enzyme, COX-2, was discovered in the early 1990’s (Xie et al., 1991). This isoform is inducible by pro-inflammatory mediators such as interleukin-1 and tumor necrosis factor-alpha. Cyclooxygenase-2 is located in many cells and tissues throughout the body, including those expressing COX-1 listed above. In the gastrointestinal tract, COX-2 serves an important role in the healing of gastrointestinal injuries such as ulceration (Mizuno et al., 1997; Gretzer et al., 1998). Although traditionally considered “inducible,” it is constitutively expressed in the brain and spinal cord, where it is likely
involved in neuronal transmission, central nervous system function, and modulating signs of inflammation such as fever (Vane et al., 1998; Simmons et al., 2004).

In inflammatory pathology, COX-2 is upregulated in response to stimuli from pro-inflammatory cytokines and chemokines (TNF-alpha, interleukin (IL)-1 beta, and IL-2) released by leukocytes (neutrophils, macrophages), and attracts additional cells and pro-inflammatory mediators to sites of tissue injury (Simmons et al., 2004). Both acute and chronic injury result in increased COX-2 activity, leading to increased pro-inflammatory mediator generation, continued activation of peripheral nociceptors and spinal neurons, and ultimately maladaptive tissue injury and pain. Chronic inflammation, such as in arthritis, exemplifies how inflammatory processes can cease to be protective, and even result in harm to the individual (Vane et al., 1998; Latremoliere & Woolf, 2009). Prostaglandins are believed to play a major role in the development of hyperalgesia (an exaggerated, abnormal response to nociceptive input) increased though modulation of pain pathways in the dorsal horn of the spinal cord, in fact, upregulation of COX-2 in the spinal cord occurs following peripheral injury (Vane et al., 1998; Yaksh et al., 2001; Svensson & Yaksh, 2002).

Cyclooxygenase-3 was the most recent enzyme to be described, and is considered to be a splice-variant of COX-1 (Chandrasekharan et al., 2002). To date, this enzyme has been identified in the central nervous system of the dog, mouse, and human (Hersh et al., 2005). Its role as a target for analgesics has been questioned, although it may explain the mechanism of action of certain analgesic drugs such as acetaminophen which has little effects on COX-1 or -2.
Nonsteroidal anti-inflammatory drug pharmacology

Nonsteroidal anti-inflammatory drugs are one of the most widely used classes of analgesics in both human and veterinary medicine (Wolfe et al., 1999; Svensson & Yaksh, 2002; Lees et al., 2004a; Lascelles et al., 2006; Silber et al., 2010). These drugs are used to treat a variety of conditions, including pain and inflammation associated with osteoarthritis and surgery. Approximately 1 in every 5 senior-aged dogs is diagnosed with osteoarthritis, therefore NSAIDs play an important role in veterinary pain management (Johnston, 1997) Nonsteroidal anti-inflammatory drugs exert their analgesic and anti-inflammatory effects via the inhibition of the COX enzymes, and ultimately decrease the production of the eicosanoid inflammatory mediators such as PGs and TXs (Vane et al., 1998). Both therapeutic and toxic effects related to NSAID use can be linked to the inhibition of COX enzymes.

Classification of NSAIDs based on COX inhibition

Two broad classifications of NSAIDs are the traditional non-selective COX inhibitor drugs that inhibit both COX-1 and -2, and the selective COX-2 drugs. These drugs vary in their chemical structure, which accounts for the selectivity for COX enzymes. Examples of non-selective COX inhibitors include aspirin, indomethacin, and phenylbutazone. Selective COX-2 inhibitor drugs inhibit the COX-2 enzyme at much lower concentrations than they inhibit COX-1, and are therefore called “COX-1 sparing;” Examples of these drugs include deracoxib and robenacoxib. Some NSAIDs are classified or referred to as “COX-2 preferential” which means that they tend to inhibit COX-2 enzymes at lower doses than they do for COX-1, but there is still some degree of dual inhibition, and include drugs such as carprofen and meloxicam (Curry et al., 2005). Classification of COX selectivity for these drugs are typically based on in vitro assays, many of which have important limitations that do not translate to in vivo
situations. For example, cell cultures have been used to assess COX-2 activity through inhibition of PGE₂ stimulated by lipopolysaccharide (LPS), however these systems do not take into account factors such as drug protein binding or partitioning into other components of blood such as red blood cells (Ricketts et al., 1998; Lees et al., 2004b). Ex vivo studies utilizing whole blood assays have been recommended by some authors to assess the COX selectivity of NSAIDs, as these assays are easy to perform and allow for screening of multiple drugs without having to perform costly and lengthy in vivo experiments (Brideau et al., 1996). However, these assays can have large variability and do not represent COX inhibition in target sites (i.e., inflamed joints), therefore a more ideal model would be in vivo studies (Giuliano & Warner, 1999; Blain et al., 2002; Khan et al., 2002; Lees et al., 2004b).

The selective COX-2 inhibitors were originally developed with hopes to improve the safety profile of traditional non-selective NSAIDs. Many of the adverse effects, such as gastrointestinal bleeding and ulceration, were believed to be due to inhibition of protective PGs and TXs synthesized by COX-1 (Wolfe et al., 1999; Simmons et al., 2004). Despite attempts to improve the overall safety profile of NSAIDs by developing these selective COX-2 inhibitors, they are still associated with many adverse events, most likely secondary to inhibition of the constitutive and inducible eicosanoids when damage to the gastrointestinal mucosa occurs (Mizuno et al., 1997; Gretzer et al., 1998; Lascelles et al., 2005; Wooten et al., 2009; Monteiro-Steagall et al., 2013; Hunt et al., 2015).

**General pharmacokinetics and pharmacological properties of NSAIDs**

The pharmacokinetics of NSAIDs have many similarities despite different chemical classes of these drugs. Similar pharmacokinetic characteristics include a low volume of distribution (0.1-0.2 L/kg), which is secondary to very high plasma protein binding- generally
greater than 99%. They tend to exhibit good-to-excellent bioavailability when administered via extravascular routes (Schmidt & Guentert, 1990; Lees et al., 2004a; Papich & Martinez, 2015). There are exceptions to these general features, such as mavacoxib which has a higher volume of distribution (1.6 L/kg) than most NSAIDs; this NSAID is also very unique in that it exhibits a prolonged plasma half-life of approximately 17 days (Cox et al., 2010). While most NSAIDs have high oral bioavailability, for some drugs, in particular robenacoxib, absorption and bioavailability are affected by the presence food (Jung et al., 2009). Nonsteroidal anti-inflammatory drugs are metabolized extensively in the liver and are largely excreted via the gastrointestinal or urinary tracts (Curry et al., 2005). Certain NSAIDs, including carprofen, undergo enterohepatic recirculation, which may play a role in gastrointestinal tract injury (Reuter et al., 1997; Priymenko et al., 1998). There is very little renal elimination of unchanged (i.e., not metabolized) NSAIDs in both humans and veterinary species (Lees et al., 2004a).

The use of NSAIDs in Dogs

Nonsteroidal anti-inflammatory drugs are a pillar of analgesic therapy in dogs diagnosed with a variety of painful and inflammatory conditions, both acute and chronic in nature. Their use in veterinary medicine dates back to the 1800’s, long before the discovery of the mechanism of action of aspirin by Vane in 1971 (Vane, 1971; Lees et al., 2004a). Currently there are numerous NSAIDs approved for use in dogs across the world, all intended to treat pain and inflammation, the more common of which include carprofen, firocoxib, deracoxib, meloxicam, and mavacoxib (Cox et al., 2010; Deramaxx® package insert, 2011; Holloway et al., 2012; Previcox® package insert, 2013; Rimadyl® package insert, 2013; Metacam® package insert, 2014).
The chronic (i.e., > 28 days) use of NSAIDs in dogs is largely for the management of osteoarthritis (OA) and related pain. Chronic administration of an NSAID for this disease has multiple benefits, including reduced central sensitization (“wind-up”) and decreased joint damage and disease progression (Yaksh et al., 2001; Innes et al., 2010; Holloway et al., 2012). Despite perceived and reported benefits associated with chronic use of an NSAID, these drugs are associated with a variety of adverse effects (discussed below), and should be administered cautiously and with clinician oversight including rechecks and periodic urinalysis and serum chemistry analysis to assess kidney values and liver enzyme activities.

*General adverse effects of NSAIDs in Dogs*

Although the true prevalence of adverse drug events (ADEs) associated with NSAID use in dogs is unknown (Lascelles *et al.*, 2005; Innes *et al.*, 2010; Monteiro-Steagall *et al.*, 2013), in humans the ADE prevalence is reportedly low. For example, the incidence of clinically significant gastrointestinal side effects has been reported to range between 1 – 4%, however the economic impact of the adverse effects is significant (Rodriguez-Monguio *et al.*, 2003). The most commonly reported adverse effects following NSAID administration in dogs are similar to those in people, and include gastrointestinal disturbances (nausea, vomiting, and diarrhea). More worrisome are the serious adverse effects that can occur following NSAID administration, including gastrointestinal perforation (secondary to ulceration), renal and hepatic toxicity, and coagulopathy (MacPhail *et al.*, 1998; Lascelles *et al.*, 2005; Enberg *et al.*, 2006; Monteiro-Steagall *et al.*, 2013). In people, genetic differences in metabolizing enzymes have been identified that account for not only variability in pharmacokinetics, but also as a predisposition for adverse drug effects (Martinez *et al.*, 2004; Kirchheiner & Brockmoller, 2005, Ali *et al.*, 2009). Specifically, the hepatic cytochrome P450 enzyme 2C9 has been linked
to these observations (Martinez et al., 2004; Kirchheiner & Brockmoller, 2005; Ali et al., 2009; Carbonell et al., 2010). Although the genetic variability in canine CYP450 enzymes is less well established, there is evidence for polymorphism in certain breeds, notably Beagles (Paulson et al., 1999; Court, 2013). Currently it is unknown if carprofen is a substrate for these enzymes in dogs.

The etiology of the adverse gastrointestinal effects secondary to NSAID administration has been widely studied and is predominantly due to inhibition of COX enzymes leading to decreased production of protective prostaglandins (Wolfe et al., 1999; Wallace, 2001). However, there is also a component of direct topical injury to the gastrointestinal mucosa, such as that caused by aspirin, which is secondary to this drug’s acidity as well as changes to the protective mucus layer which allows gastric acid to cause further injury (Wolfe et al. 1999). Additionally, the metabolites of certain NSAIDs, for example the acyl glucuronide metabolites formed from carboxylic acid-containing NSAIDs have been associated with toxicity to both the gastrointestinal tract as well as the liver (Seitz & Boelsterli, 1998). One theory for the mechanism of toxicity caused by the acyl glucuronide drug conjugates is disruption of membrane proteins by electrophilic interactions, and protein adduct formation, ultimately leading to cell death (Pumford et al., 1993; Kretz-Rommel & Boelsterli, 1994; Atchison et al., 2000). Adverse gastrointestinal effects have been noted with the use of several NSAIDs in dogs (Lascelles et al., 2005; Wooten et al., 2008; Case et al., 2010; Monteiro-Steagall et al., 2013). In dogs and other species, the non-selective COX inhibitors, such as aspirin or indomethacin, have induced significant gastrointestinal damage (Meddings et al., 1995; Shaw et al., 1997; Reimer et al., 1999; Ward et al., 2003). Thus more COX-2 selective NSAIDs were developed in an attempt to minimize the adverse effects of older NSAIDs on the
gastrointestinal tract. The newer COX-2 selective drugs may have an improved safety profile in terms of gastrointestinal toxicity (Silverstein et al., 2000; Singh et al., 2006), at least in humans, there is still inhibition of prostaglandin synthesis that leads to gastrointestinal injury, including ulceration and perforation, associated with these compounds (Hawkey et al., 2001; Lascelles et al., 2005; Enberg et al., 2006; Case et al., 2010; Machata et al., 2012; Monteiro-Steagall et al., 2013).

Renal damage associated with NSAID administration can occur via local PG inhibition in this tissue, and is more likely to occur in dehydrated or hypovolemic animals (Surdyk et al., 2011). In dogs, both COX-1 and COX-2 are important in the maintenance of normal kidney function, and in fact COX-2 is expressed to a greater degree than in other species, which could predispose them to adverse effects secondary to COX-2 inhibition (Khan et al., 1998). Severe NSAID-induced kidney damage can include acute kidney failure, renal papillary necrosis, and interstitial nephritis (Cheng & Harris, 2005; Harirforoosh et al., 2006; Raekallio et al., 2006; Lomas & Grauer, 2015). Studies in rats have linked renal injury directly to the exposure and distribution of certain NSAIDs (celecoxib and rofecoxib) in renal tissues, suggesting that pharmacokinetics could account for this ADE (Harirforoosh et al., 2006).

Nonsteroidal anti-inflammatory drug-induced liver injury can be caused by both dose-dependent and dose-independent mechanisms. The latter is classified as an idiosyncratic drug reaction, meaning that the development of liver injury or failure is not predictable or dose-related (Trepanier, 2013). These reactions are often not identified in pre-clinical drug studies in healthy dogs, and occur infrequently in the canine population. However, they are of major concern because they have been associated with severe morbidity and mortality (MacPhail et al., 1998). Although carprofen is frequently cited in cases of NSAID-induced hepatopathy
(MacPhail et al., 1998; Mansa et al., 2007; Reymond et al., 2012) other NSAIDs have also been associated with reported liver toxicity, including deracoxib and robenacoxib (McMillan et al., 2011; Reymond et al., 2012).

Because of the various adverse effects associated with NSAID use, general prescribing recommendations in people have been to use the lowest effective dose of NSAID for the shortest time needed to control symptoms (Schnitzer, 2006; Antman et al., 2007). These prescribing recommendations have been applied in veterinary medicine, however they appear to be based on empirical evidence rather than controlled studies that demonstrate improved efficacy and/or safety. Interestingly, recent studies in people have suggested that continuous NSAID administration is more efficacious, and equally well-tolerated as intermittent treatment, and these types of investigations could be similarly applied to veterinary patients (Luyten et al., 2007; Sands et al., 2013).

**Pharmacology and pharmacokinetics of carprofen in dogs**

Carprofen is a carboxylic acid and belongs to the arylpropionic acid class of NSAIDs (Rimadyl® package insert, 2013). Other drugs in this general class include ibuprofen, ketoprofen, and vedaprofen. Many compounds in this class, including carprofen, exist as a racemic mixture of two enantiomers designated as R(-) and S(+) (see Figure 2.2). There are species-specific differences in the pharmacokinetics and pharmacodynamics for each enantiomer of carprofen. For example, in dogs, the R(-) enantiomer exhibits greater plasma area-under-the - curve (AUC) values than the S(+) enantiomer and a slower clearance (McKellar et al., 1994; Priymenko et al., 1998). However, the S(+) enantiomer exhibited significantly greater anti-inflammatory activity, based on an *in vitro* assay, in dogs (Ricketts et al., 1998) and other species when compared to the R(-) enantiomer (Lees et al., 2004). An
enantiospecific assay is typically selected when studying the pharmacokinetics of carprofen, although a number of studies have reported the pharmacokinetics of total (R + S) carprofen (McKellar et al., 1990; Lascelles et al., 1998; Clark et al., 2003).

Figure 2.2: Carprofen racemic and enantiomer structures. From Lees et al., 2012.

Carprofen is approved in the United States and many other countries for the control of pain and inflammation secondary to osteoarthritis and post-operative pain after soft tissue or orthopedic surgery in dogs (Rimadyl® package insert, 2013), and is classified as a COX-2 preferential NSAID (Ricketts et al., 1998; Wilson et al., 2004). The innovator drug Rimadyl® was the first form of carprofen approved by the US Food and Drug Administration (FDA) in 1996 for use in dogs (FDA, 1996). Now there are many generic formulations commercially available, making it one of the most widely used NSAIDs in the United States and elsewhere (Raekallio et al., 2006). Dosage recommendations in the United States are 4.4 mg/kg once daily or 2.2 mg/kg twice daily, typically orally (Rimadyl® package insert, 2013).
Pharmacokinetics and metabolism of carprofen

The pharmacokinetics of carprofen in dogs have been reported in several previous studies (Schmitt et al., 1990; McKellar et al., 1990; Lascelles et al., 1998; McKellar et al., 1994; Priymenko et al., 1998; Lipscomb et al., 2002, Clark et al., 2003). In these studies, carprofen was administered at doses ranging from 0.7 to 4 mg/kg, and by differing routes including intravenous, oral, subcutaneous, and rectal. The majority of these studies used young healthy Beagle dogs as test subjects, although in at least two studies other breeds were used (Lascelles et al., 1998; Lipscomb et al., 2002). The reported range in pharmacokinetic parameter estimates is variable, although some estimates, such as volume of distribution, are consistent among studies (Table 2.1). When administered via extravascular routes such as subcutaneously or orally, bioavailability is high at greater than 90% (Schmidt & Guentert, 1990). Table 2.1 provides a summary of pharmacokinetic parameter estimates for carprofen in dogs that have been obtained from previous studies.
Table 2.1: Summary of carprofen pharmacokinetics in dogs.

<table>
<thead>
<tr>
<th>Study Author, Year</th>
<th>Breed</th>
<th>n</th>
<th>weight in kg (- avg)</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Volume (L/kg), mean (sd)</th>
<th>Cl (mL/hr/kg), mean (sd)</th>
<th>Enantio-selective</th>
<th>Ke (1/hr), mean (sd)</th>
<th>T1/2 (hr), mean (sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schmidt, 1996</td>
<td>Beagles</td>
<td>5</td>
<td>13.4</td>
<td>100 mg total</td>
<td>IV</td>
<td>0.18 (0.06)</td>
<td>17.1 (2)</td>
<td>No</td>
<td>0.66 (0.02)</td>
<td>11.7 (1.0)</td>
</tr>
<tr>
<td>McKellar, 1994</td>
<td>Collie x</td>
<td>6</td>
<td>Not listed</td>
<td>9.7</td>
<td>IV</td>
<td>0.14 (0.02)</td>
<td>16.81 (7.2)</td>
<td>No</td>
<td>0.587 (0.006)</td>
<td>8 (1.2)</td>
</tr>
<tr>
<td>McKellar, 1994</td>
<td>Beagles</td>
<td>2</td>
<td>20</td>
<td>4 total (2 mg/kg per enantiomer)</td>
<td>Oral</td>
<td>0.135 (0)</td>
<td>16.47 (0)</td>
<td>Y - R enantiomer</td>
<td>0.13 (0.05)</td>
<td>5.5 (1.9)</td>
</tr>
<tr>
<td>McKellar, 1994</td>
<td>Beagles</td>
<td>2</td>
<td>10-15 kg</td>
<td>4 total (2 mg/kg per enantiomer)</td>
<td>Oral</td>
<td>0.257 (0)</td>
<td>39.6 (0.61)</td>
<td>Y - S enantiomer</td>
<td>0.14 (0.016)</td>
<td>4.7 (0.5)</td>
</tr>
<tr>
<td>Priymenko, 1998</td>
<td>Beagles</td>
<td>9</td>
<td>10-15 kg</td>
<td>4 total (2 mg/kg per enantiomer)</td>
<td>IV</td>
<td>0.12 (0.03)</td>
<td>17.1 (4)</td>
<td>Y - R enantiomer</td>
<td>0.16 (0.02)</td>
<td></td>
</tr>
<tr>
<td>Priymenko, 1998</td>
<td>Beagles</td>
<td>9</td>
<td>2.2 to 3 mg total</td>
<td>4 total (2 mg/kg per enantiomer)</td>
<td>IV</td>
<td>0.19 (0.05)</td>
<td>28.2 (9.7)</td>
<td>Y - S enantiomer</td>
<td>0.14 (0.016)</td>
<td></td>
</tr>
<tr>
<td>Clark 2003</td>
<td>Beagles</td>
<td>18</td>
<td></td>
<td></td>
<td>Oral</td>
<td>0.19 (0.06)</td>
<td>27.2 (9.1)</td>
<td>N</td>
<td>0.145 (0.04)</td>
<td></td>
</tr>
</tbody>
</table>

The primary pathway of carprofen metabolism in dogs is phase II glucuronidation leading to a conjugated acyl glucuronide compound (Figure 2.3). As previously discussed, this metabolite may be significant in the development of enteropathy in dogs, as work in other species have identified acyl glucuronide conjugates of NSAIDs to be more ulcerogenic than the parent drug (Seitz & Boelsterli, 1998). Both enantiomers are glucuronidated, although in vitro studies using microsomal enzymes show that the R(-) enantiomer is glucuronidated more rapidly than the S(+) enantiomer. Approximately 70% of an IV dose is eliminated in the feces (Rubio et al., 1980). Additionally there is enterohepatic recirculation of the S(+) enantiomer, but not the R(-) enantiomer (Priymenko et al., 1998). Differences in metabolism among breeds have not been reported for carprofen, although as previously discussed, other studies have found that Beagle dogs in general appear to have differing rates of metabolism, attributed to differences in cytochrome P-450 enzymes. These differences have been applicable for other NSAIDs including the COX-2 selective enzyme inhibitor celecoxib as evidenced by “slow”
versus “fast” metabolizers (Paulson et al., 1999; Fleisher et al., 2008; Jeunesse et al., 2013). Very few pharmacokinetic studies involving NSAIDs have assessed these metabolic differences in Beagles, which could impact the safety and/or effectiveness of these drugs in a non-Beagle population (Paulson et al., 1998; Jeunesse et al., 2013).

![Figure 2.3: The acyl glucuronide of carprofen. From Dumasia, et al. (Dumasia et al., 2003)](image)

**Safety of carprofen**

Carprofen is generally well-tolerated by dogs. Long-term safety and tolerability studies were performed in Beagle dogs where doses of 7 mg/kg/day for 1 year were administered, and no abnormalities were found on gross necropsy or histopathology (Rimadyl® Package insert, 2013; Rimadyl FOI). Safety studies performed in healthy research dogs, again Beagles, prior to approval in the United States demonstrated carprofen to be relatively free of adverse effects when tested at up to 10x the recommended dose, and were limited to mild elevations in L-alanine aminotransferase (ALT) and gastrointestinal bleeding, as evidenced by black or bloody stools (Rimadyl FOI). Hypoalbuminemia was identified in 2/8 dogs following administration of 10x the labeled dose for 14 days (Rimadyl® package insert, 2013). When doses up to 36x
the labeled dose were administered daily for 5 days, elevations in alanine aminotransferase (ALT) were detected, as well as decreased hematocrit and hemoglobin levels, but no mortality occurred (Rimadyl FOI). Together, these studies all suggest good tolerability of carprofen at recommended doses, however it is important to note that the majority of these studies were performed in healthy purpose-bred research Beagles.

Recently there have been reviews, prospective, and retrospective studies performed on the safety of carprofen when administered to clinical patients (Moreau et al., 2003; Raekallio et al., 2006; Autefage & Gosselin, 2007; Montiero-Steagall et al., 2013). These studies have reported overall very low numbers of adverse events associated with carprofen administration, with the percentage of dogs experiencing an adverse event being less than 5%, once again, confirming the seemingly good tolerability of this drug (Autefage & Gosselin, 2007, Mansa et al., 2007).

Adverse events associated with carprofen administration

Despite the safety information discussed above, when compared with other NSAIDs used in veterinary medicine, carprofen is the NSAID for which the most adverse drug events (ADE) have been reported on the FDA website (FDA, 2013). However, there are important considerations for this data; first, the ADEs are voluntarily reported and exact medical conditions are unknown, and second, carprofen is also the most frequently prescribed NSAID and the incidence of adverse events per prescription is not known. Nonetheless, common reported ADEs include those already described as associated with NSAID administration; most commonly gastrointestinal irritation and disturbances, such as vomiting and anorexia (Fox & Campbell, 1999; Autefage & Gosselin, 2007). In addition to these, other reported ADEs
include skin reactions, hematologic abnormalities, and hepatotoxicity (MacPhail et al., 1998; Mellor et al., 2005; Banovic et al., 2014).

One of the most serious of the reported ADEs in dogs receiving carprofen is acute hepatocellular toxicosis (McPhail et al., 1998). Acute hepatopathy following carprofen administration has been classified as an idiosyncratic drug reaction (MacPhail et al., 1998; Montiero-Steagall et al., 2013). A commonly cited study reported that Labrador retrievers are over-represented in development of carprofen-induced hepatotoxicity. In these dogs, the following clinical and clinical pathological findings were reported: anorexia, vomiting, icterus, and serum biochemical abnormalities, in particular ALT, ALP, AST, GGT, and bilirubin (McPhail et al., 1998). Significantly increased bilirubin in addition to significant (greater than 3x the upper end of the reference range) increases in the other hepatocellular leakage enzymes is one of the hallmark findings in drug-induced liver injury (DILI) (Kaplowitz, 2005). Subsequent studies performed by the manufacturer and others have investigated the possibility of hepatotoxicity in Labradors, but have been unable to replicate the findings in the report by McPhail and others (McPhail et al., 1998; Hickford et al., 2001). Overall the reported incidence of carprofen-induced hepatotoxicity is low but variable—<0.05% to 1.6% (Mansa et al., 2007; Reymond et al., 2012), but this ADE can be devastating, leading to liver failure and death (Fox & Campbell, 1999). The severe, acute hepatocellular injury associated with carprofen administration in dogs is still poorly understood; in humans, a similarly described reaction is seen in some individuals following diclofenac administration and is hypothesized to be secondary to protein adducts formed with diclofenac metabolites (Aithal et al., 2004). This acute, severe hepatotoxic reaction should not be associated with mild changes that have been observed in dogs receiving NSAIDs, including minor increases (i.e. less than 3x the normal
upper end of the reference range) in serum hepatocellular enzyme activities (ALT, ALP, or GGT) (Autefage & Gossellin, 2007; Steagall et al., 2007; Reymond et al., 2012).

**Drug-drug interactions (DDIs) with carprofen**

Known drug-drug interactions exist for carprofen, some of which may predispose an animal to develop an ADE. The concurrent administration of corticosteroids or other NSAIDs with carprofen is a well-recognized interaction (Rimadyl® package insert, 2013). Concurrent administration of NSAIDs and corticosteroids increases the risk of gastrointestinal injury in people (Lanza et al., 1998) and is likely a risk factor in dogs as well (Lascelles et al., 2005). Other possible DDIs with carprofen include angiotensin converting enzyme inhibitors and furosemide. These drugs have the potential to cause kidney injury secondary to PG inhibition in the kidney or through relative hypovolemia, and therefore decreased renal blood flow and possibly a reduction in glomerular filtration (Surdyk et al., 2012). However, studies in healthy dogs with different NSAIDs, suggests these interactions may not be significant (Fusellier et al., 2005). Lastly, there are suggested potential interactions including concurrent administration with other highly protein bound (i.e., >90%) drugs, leading to a drug displacement interaction resulting in higher concentrations of the less avidly bound drug (Benet & Hoener, 2002). Such drugs include anticonvulsants (phenobarbital), cardiac drugs (digitalis), some antimicrobials (cefovecin, doxycycline), and behavior modification drugs (Rimadyl® package insert, 2013). Although these potential interactions are discussed both in the literature and on the innovator product label, they have not actually been investigated in dogs.

Despite numerous veterinary studies involving carprofen, few to none have assessed whether there could be pharmacokinetic differences or effects that might provide some insight
into the development of adverse effects following carprofen administration in dogs. One type of clinically useful pharmacokinetic analysis involves the use of population pharmacokinetics (PPK). Population pharmacokinetic studies are recommended, and widely accepted, by regulatory authorities because of the value of these models in drug safety and efficacy (Sun et al., 1999; Williams & Ette, 2000). This approach uses sparse sampling methods from a large number of animals, where only a few samples are collected per patient, combined with non-linear mixed effects modeling (NLME) to assess possible effects for patient variability (Sheiner et al., 1977; Sun et al., 1999). The population approach is advantageous for many reasons, but predominantly because it allows one to collect important data in the target treatment population. The sparse sampling approach allows one to study populations where robust sampling design is not possible (i.e. very small animals or animals with anemia). Because animals are more heterogeneous in terms of size, breed, age, gender, and health status differences compared to a small group of healthy experimental animals, this approach could result in changes to dosing regimens (Cox et al., 2010). In addition, inter-individual and intra-individual variability in the PK parameters can be estimated and potentially explained through these patient-specific effects (Aarons, 1991; Ette & Williams, 2004). Recent population pharmacokinetic studies on the NSAIDs mavacoxib and robenacoxib in dogs proved useful in identifying patient factors to explain pharmacokinetic variability (Silber et al., 2010; Cox et al., 2010; Fink et al., 2013). For mavacoxib, this analysis also resulted in a change in dose (from 4 mg/kg to 2 mg/kg), although the dose interval did not change (first dose interval of 15 days, then every 30 days thereafter for a maximum of 6.5 months) for the intended treatment population- a decrease the authors believe will increase the therapeutic index for this drug (Cox et al., 2010). Carprofen is by far more commonly used than these other two NSAIDs, yet no
true population pharmacokinetic analyses for this drug have been performed in veterinary medicine. It is possible that such an analysis would identify patient factors that could influence the pharmacokinetics, and therefore potentially the pharmacodynamics, of this drug in dogs.

**Plasma and tissue distribution of NSAIDs**

Better understanding the tissue distribution and local action of NSAIDs may help prevent adverse effects associated with their administration (Brune & Furst, 2007). However, the total drug concentration ($C_{\text{total}}$) in plasma is most frequently measured in pharmacokinetic studies. By including both protein-bound and unbound drug concentrations in the assay, it does not represent the active fraction. The protein-unbound concentration of a drug is critical to determine, as it is only the free, unbound portion of the drug that is therapeutically active. In other words, while a drug is bound to a plasma protein, it is not available to bind to target receptors to exert an effect. Some drugs exert their effects in sites of the body where plasma protein is low compared to plasma (for example, the interstitial space where many antimicrobials exert their effects). Theoretically, the unbound concentration in plasma should be in equilibrium with the unbound concentration in tissues (the site of action). Thus, understanding the protein-unbound component is important for determining pharmacodynamic activity.

Since pharmacodynamic effects of drugs are dependent on protein-unbound drug concentrations at sites of action, it is important to know effective concentrations in affected tissues. However, few studies actually address this need. For NSAIDs, sites of action include both central (spinal cord and brain) and peripheral (e.g., synovial) tissues. Investigations into the concentrations of NSAIDs directly from inflamed tissue sites can provide important information about both the pharmacokinetic and pharmacodynamic behavior of these
compounds. For carprofen only one such study exists, and due to low numbers of dogs and sampling issues, the results are difficult to interpret (McKellar et al., 1994). Data obtained from such experiments could be used to optimize dosage regimens; disease and insults that lead to inflammation (i.e. osteoarthritis or surgery) have the potential to affect the pharmacokinetics and pharmacodynamics (PK-PD) of NSAIDs through alterations in blood flow, organ function, or other mechanisms such as protein binding (Martinez & Modric, 2010).

Protein binding effects on pharmacokinetics and drug distribution

Protein binding is important in the calculation of primary pharmacokinetic parameters such as volume of distribution and clearance, as shown by the following equations (Benet & Hoener, 2002):

(Equation 1) \[ V = \frac{F(u)}{F(ut)}V_t + V_p; \]

where \( F(u) \) is the fraction of drug unbound in the plasma, \( F(ut) \) is the fraction unbound in tissue, \( V_t \) is volume of tissue, and \( V_p \) is the volume of plasma.

(Equation 2) \[ Cl = \frac{Q_{organ} \times F(u) \times Cl_{int}}{(Q_{organ} + F(u) \times Cl_{int})}; \]

where \( Q_{organ} \) is organ blood flow and \( Cl_{int} \) is the intrinsic organ clearance for the unbound drug.

Equation 2 demonstrates that drug clearance will increase as the fraction unbound increases.

Drug half-life can also be linked to protein binding as shown by the following equation:

(Equation 3) \[ T_{1/2} = \frac{0.632 \times V_d}{Cl}, \]

which incorporates both clearance and volume of distribution.

The reversible association of a drug with plasma proteins is an important aspect of drug delivery and distribution to tissues since only the unbound drug is available to cross biological membranes. Drug protein binding also has a potential role for drug-drug interactions if two
highly protein-bound (i.e., >90%) drugs compete for the same binding site on a protein. The association and dissociation of drugs to proteins is described using the law of mass action (Equation 4), and binding affinities (K_a or K_diss) and maximum binding capacity (B_max) can be calculated from in vitro studies (Equation 5). These data can be used in modeling to predict whether or not displacement interactions are likely to occur in vivo. For example Drug B may have a greater binding affinity than Drug A and is more likely to displace the previously bound Drug A from protein, therefore increasing the unbound concentration of Drug A.

\[
\text{C}_{\text{free}} + \text{C}_{\text{protein}} \rightarrow^{(\text{ka})} \text{C}_{\text{drug-protein complex}} \rightarrow^{(\text{k_diss})} \text{C}_{\text{free}} + \text{C}_{\text{protein}}
\]

\[
\text{C}_{\text{bound}} = \frac{(B_{\text{max}} \times C_{\text{free}})}{(K_{\text{diss}} + C_{\text{free}})}
\]

The primary proteins involved in protein-drug binding are albumin, alpha-1 acid glycoprotein (AAGP), and to a lesser extent, lipoprotein (Wood, 1986). Albumin is the most abundant serum protein in the body, and it is often found at higher concentrations than the therapeutic target concentration for most drugs. Albumin has two major drug binding sites, classically described as Site I (the warfarin site) and Site II (the benzodiazepine site), which gives it a greater capacity for drug binding when compared with AAGP (Sudlow et al., 1975; Christensen et al., 2006). Generally, AAGP is responsible for the binding of basic drugs and albumin is responsible for the binding of acidic drugs (Wood, 1986). Pathology can alter the normal concentrations of these important proteins, which could potentially lead to alterations in the unbound portion of drug available. For albumin, the most common result of pathological states is hypoalbuminemia and perhaps a decreased capacity to bind drug (increased free drug concentration), whereas for AAGP, increased amounts of this protein (and therefore a decreased free drug concentration) are often observed, particularly during inflammation (Ikenoue et al, 2000). NSAIDs are weak acids and bind to albumin. They classically occupy
Site II, although if a protein binding displacement interaction occurs, NSAIDs can bind to Site I (Noctor et al., 1991; Rahman et al., 1993). These different binding sites give albumin a large capacity to bind NSAIDs, particularly if a displacement interaction at one binding site should occur.

The debate over the significance of protein binding in pharmacology has been extensively reviewed in the literature (Benet & Hoener, 2002; Toutain and Bosquet-Melou, 2002; Schmidt et al., 2010; Zeitlinger et al., 2011). However studies are lacking that definitely answer questions related to protein-binding interactions. These questions include whether changes in protein binding are the cause of a significant drug-drug interaction, or whether changes in protein binding alter the pharmacokinetics of a drug significantly enough to cause a pharmacodynamic change. In veterinary medicine, the wide diversity in species and disease states highlights the importance of understanding differences in drug protein binding directly in species of interest and whether these differences would affect the pharmacokinetics or pharmacodynamics of drugs.

Drugs that are extensively bound to plasma proteins will, in theory, have more changes in free drug concentration if the fraction of bound drug is decreased. Examples of highly protein-bound (i.e. >90%) drugs utilized in veterinary medicine include digitalis, many anesthetics and analgesics (propofol and NSAIDs), antineoplastics/immunosuppressants (methotrexate), anti-coagulants (warfarin), and certain antimicrobials (cefovecin, doxycycline). The increases in free drug concentrations may result in different outcomes. For example, a greater drug effect could occur when more unbound drug is available to bind receptors or enzymes. On the contrary, a lesser drug effect could be seen due to more rapid elimination of the unbound drug. However, studies assessing the effects of protein binding and
drug displacement interactions in veterinary medicine are sparse, despite warnings found on the FDA-approved drug labels for many commonly used medications (Rimadyl® Package Insert; Convenia® Package Insert). These warnings are based on *in vitro* studies and speculation, rather than *in vivo* interaction studies.

*Effects of inflammation on NSAID distribution*

During inflammation, NSAID concentrations rise and accumulate in inflamed tissues preferential to non-inflamed tissues. Not all NSAIDs distribute in this manner however, and accumulation in inflamed tissues is believed to be secondary to drug physicochemical properties, such as high plasma protein binding and acidity (pKa 3-5) (Brune & Furst, 2007; Brune & Patrignani, 2015). This work is based on research in cell culture and rat models, where both acidic and non-acidic NSAIDs have been quantified in plasma, inflamed, and non-inflamed tissues (Brune & Furst; 2007). However, it should be recognized that the pH of inflamed tissues does not vary widely, and the influence on tissue pH and ion trapping of acidic drugs such as NSAIDs has been questioned (Cummings & Nordby, 1966; Farr *et al.*, 1985; Punnia-Moorthy, 1987). The distribution of NSAIDs in dogs during pathological states such as inflammation is likely to be different than in healthy dogs, in whom most pre-clinical trials are performed (Martinez & Modric, 2010). Also, the distribution and kinetics of these drugs could change depending on degree of inflammation, whether systemic versus localized, other co-morbidities, concurrent drug administration, alterations in protein binding and pH, and local tissue blood flow. There is a need to better evaluate NSAID distribution and action at relevant sites in the body, in order to optimize treatment and minimize adverse effects. Concern about adverse effects are one of the primary reasons that clinicians forego treatment with NSAIDs to dogs, or discontinue treatment for a chronic condition (e.g., osteoarthritis). Such studies could
allow for the administration of lower doses while maintaining effective therapeutic concentrations at tissue sites of action. In theory, lower doses and lower plasma concentrations could minimize many of the ADEs that are a result of broad COX inhibition, such as gastrointestinal PG inhibition and secondary gastrointestinal ulceration. None of the previously discussed studies have adequately evaluated carprofen concentrations in affected (i.e., inflamed) tissues, and very few have assessed carprofen pharmacokinetics following commonly prescribed uses, such as chronic administration for the treatment of pain associated with osteoarthritis (McKellar et al., 1994; Lipscomb et al., 2002).

Techniques for studying tissue drug concentrations and inflammation

There have been several methods to study the tissue concentrations and distribution of drugs, including blood/plasma sampling, tissue homogenates, tissue cages, microdialysis, ultrafiltration, bronchoalveolar lavage, and skin blister fluid. Each of these techniques has unique advantages and disadvantages which have been recently reviewed (Deitchman & Derendorf, 2014).

Whole blood, serum, or plasma sampling and analysis are convenient and common methods used in PK-PD studies. These techniques are utilized for several reasons related to ease of sampling technique, drug analysis, and understanding of results. The most important disadvantage to this technique is that concentrations in plasma do not reflect those at the site of action (Ryan et al., 1986; Toutain et al., 2001; Lees et al., 2004; Deitchman & Derendorf, 2014).

Tissue homogenates can be used to determine total drug concentrations at tissue sites and are convenient to perform in an animal that is undergoing surgery or having a specific tissue removed. The technique is not practical for repeated samples or for certain tissue types,
such as internal organs. Importantly, results from homogenized tissues assumes that there is equal drug distribution to all compartments, which may not be true. Tissue homogenized drug concentrations can potentially overestimate intracellular drug concentrations while underestimating extracellular concentrations. Ultimately, it is impossible to predict unbound, active drug concentrations at tissue sites from homogenized tissue samples (Mouton et al., 2008).

Tissue cages offer an alternative to collect drugs in inflamed or infected sites. However, the tissue cage is an unnatural environment in which to measure drug concentrations. Encapsulation and vascularization secondary to a foreign object reaction may occur, which can change drug penetration into the cage through the creation of this artificial compartment (Pelligand et al., 2011). Also, the placement of the tissue cages requires surgery and prolonged healing times, and removal of the cages requires a second surgical procedure or euthanasia in order to avoid a second surgical procedure. Lastly and very importantly, the drug collected from tissue cages is bound to proteins and other cellular constituents, which does not represent just the pharmacologically-active form of the drugs.

Despite the disadvantages, the tissue cage is a commonly used method in veterinary medicine. Several papers have been published using this technique in dogs and other veterinary species (McKellar et al., 1994; Lees et al., 2004; Pelligand et al., 2011). The advantages of the tissue cages include: insertion of multiple cages in one subject, allowing animals to serve as their own controls; inflammation or infection can be introduced into the chambers through a variety of pro-inflammatory substances, such as carrageenan or lipopolysaccharide (LPS); repeated samples can be obtained from the cages; and lastly, cages
can be surgically removed at the end of the study thus there is no permanent effect from the model.

Microdialysis (MD) is one of the most commonly used methods to collect local, protein-unbound drug concentrations (Muller et al., 2004). Although there are no reports in other veterinary species, this technique has been used to study NSAID distribution and pharmacodynamics in tissues in humans (Gordon et al., 2002). Advantages of MD are many, and include the collection of unbound, pharmacologically active drug, continuous sampling, and ability to sample from a variety of tissue sites. Placement of MD probes is minimally invasive and does not cause permanent damage to the patient. Disadvantages include complicated calculations to determine recovery, expensive pumps and equipment, requirement that subjects be confined or immobile, and low volume collection of samples. The last of these is a limitation in that there may not be enough sample for simultaneous determination of drug concentrations and pharmacodynamic endpoints such as inflammatory biomarker quantification.

*In vivo* ultrafiltration is a technique very similar to MD in concept, but has important differences that can be considered advantages. This method involves the placement of an ultrafiltration probe into the tissue of interest. Placement is minimally invasive and is performed using local anesthetic and an introducer needle. The probe consists of 3 loops made of a semi-permeable dialysis membrane made of polyacrylnitrile, with a molecular weight cut-off of 30,000 Daltons. Figures 2.4 - 2.6 show a cartoon representation of the probe and subcutaneous placement in a dog, in addition to scanning electron microscopy images of a new and used probe. The specific molecular weight cut-off allows for the exclusion of large proteins and peptides, such as blood and albumin (MW approximately 60,000 Daltons). Tissue
fluid is collected through the ethylene propylene tubing using a vacuum collection system, which allows for continuous fluid sampling at a rate of approximately 100 µL/hr. Multiple samples over time can be obtained with minimal disturbance to the animal, and the probes can be left in place for several days with no adverse consequences.

Other advantages of UF over MD include the absence of added perfusate, which simplifies calculations including the initial analyte recovery. Also, depending on the analyte of interest, less sensitive detection methods can be used since the analyte is not diluted by the perfusate. Lastly, this technique does not require the purchase and use of costly driver pumps or immobilization of the animals during sample collection. The probes are made of non-reactive polymers, thereby causing minimal inflammatory responses and can be left in an animal for several days without any adverse effects (Linhares & Kissinger, 1993).

Figures 2.4: Graphic of an in vivo ultrafiltration probe, vacutainer collection assembly, and placement in a dog. From http://www.basinc.com/mans/uf.pdf
Figure 2.5: Scanning electron microscopy image of cut end of one loop of an unused UF probe, showing hollow fiber and smooth outer surface.

Figure 2.6: Scanning electron microscope image of outer surface of a used UF probe removed from an animal, demonstrating fibrin deposition on outer surface, mixed with inflammatory cells and red blood cells.
Inflammatory models

Different inflammatory models have been used to study distribution and effects of NSAIDs at tissue sites in a variety of veterinary species. These models are very important in human pre-clinical drug discovery and development, and can streamline the drug approval process by more efficiently establishing rational dosing regimens (Lees, et al., 2004; Brune & Patrignani, 2015).

Models of inflammation in dogs (and other animals) have involved the injection of an inflammatory compound such as carrageenan or lipopolysaccharide (LPS) into tissues or tissue cages (Higgins et al., 1984; Lees et al., 2004). Carrageenan is an algae protein and induces mild to moderate, short duration, reversible inflammation. In this model, stimulation of PGE$_2$ is responsible for the edema and hyperalgesia that results following carrageenan injection, thus making it an acceptable biomarker for NSAID activity (Simmons et al., 2004; Pelligand et al., 2011). Lipopolysaccharide is a pro-inflammatory toxin found on the membrane of gram-negative bacteria (Thorn, 2001). Lipopolysaccharide-induced inflammation is characterized by rapid influx of inflammatory cells, including macrophages and neutrophils, which peaks within a few hours of administration and resolves over several days (Palmer & Bertone, 1994; Thorn, 2001).

Experimental models of arthritis in dogs and other species have involved chemical or surgical methods. In chemically-induced arthritis models, the injection of a highly reactive chemical substance such as Freund’s adjuvant or sodium urate crystals into the joint space results in synovitis (Faires & McCarty, 1962; Toutain et al., 1994; Cross et al., 1997; Lees et al., 2004; Vincent et al., 2012). Surgical methods involve destabilization of the meniscus or cranial cruciate ligament transection (Budsberg et al., 2006; Vincent et al., 2012, Moreau et
While these techniques have provided valuable data regarding the inflammatory process, they have significant drawbacks. Injection of pro-inflammatory substances such as Freund’s adjuvant into joints initiates severe joint deterioration and results in permanent damage to the animal (osteoarthritis) (Toutain et al., 2001). Likewise, surgical models are not only very costly due to anesthesia and surgical healing requirements, they also permanently damage the joints in these animals. From an animal welfare perspective and refinement of the use of animals in research, there are several ideal features of in vivo models. These features include a model that is reversible or doesn’t result in permanent damage to the animal, reproducible, affordable, is minimally invasive, results in inflammation that resembles clinical disease, and allows for the collection of pharmacologically active drug concentrations directly at sites of action (Vincent et al., 2012). A summary table containing the advantages and disadvantages of inflammatory models and methods of sample collection in animals is provided below (Table 2.2).
Table 2.2: Summary table of inflammatory models and methods of sample collection.

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Tissue cage (carrageenan)</td>
<td>Yes</td>
<td>No</td>
<td>Yes, but includes protein-bound drug</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Kaolin paw inflammation</td>
<td>No</td>
<td>Possibly</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Chemically-induced synovitis</td>
<td>No</td>
<td>Yes, depending on the chemical</td>
<td>Yes, but includes protein-bound drug</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>LPS paw inflammation</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Surgically induced arthritis</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes, but includes protein-bound drug</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>In vivo UF (carrageenan)</td>
<td>No</td>
<td>No</td>
<td>Yes, protein-unbound drug (active portion)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Combining in vivo ultrafiltration methodology with a previously established inflammatory model (carrageenan) may offer a novel and refined means to study both drug pharmacokinetics and pharmacodynamics directly at effect sites in the body. This technique would allow for the repeated collection of protein-unbound drug directly from inflamed sites, allowing for the generation of pharmacologically active drug concentration-effect data, a feat that cannot be accomplished with other methods such as tissue cages. Ultimately these
techniques may lead to new discoveries and information regarding in tissue drug concentrations, as well as effects, which could result in a modification of currently utilized dosing regimens.

_Could differences in carprofen pharmacokinetics in dogs explain the variability in response and/or the development of adverse drug events_?

Despite many known mechanisms of some NSAID-induced adverse events discussed above, the underlying cause as to why some dogs develop adverse events and others do not is largely unknown. Very few veterinary studies have adequately investigated patient disease or other effects on carprofen pharmacokinetics. The work in this dissertation attempts to address some of these issues through separate studies investigating potential factors that could result in changes in carprofen pharmacokinetics in dogs. The combination of well-established models, such as carrageenan-induced inflammation, with more unique sampling and analysis methodologies offer new strategies to investigate carprofen pharmacokinetics. Specifically, the effects of protein binding drug-drug interactions on carprofen pharmacokinetics, studies of carprofen pharmacokinetics in a representative clinical dog population, and investigation into the tissue distribution and pharmacodynamic activity of carprofen in inflamed tissue sites are investigated and discussed.
REFERENCES


3. THE INFLUENCE OF CEFOVECIN ON THE ENANTIOSELECTIVE PHARMACOKINETICS OF CARPROFEN IN DOGS

Kristen M. Messenger and Mark G. Papich

ABSTRACT

Objective- To determine whether concurrent cefovecin administration alters the plasma pharmacokinetics of carprofen in dogs.

Animals- 6 healthy adult purpose-bred Hound dogs.

Procedures- Carprofen was administered intravenously (4 mg/kg) alone and in combination with subcutaneously administered cefovecin (8 mg/kg) in a two-period crossover study. Blood samples were obtained at pre-determined intervals. Plasma concentrations of carprofen enantiomers were analyzed by HPLC and pharmacokinetics were determined using compartmental methods.

Results- Cefovecin administration did not significantly alter the pharmacokinetics of S(+) carprofen, but did affect the R(-) enantiomer. Area under the curve (AUC) and clearance were significantly less following concurrent carprofen and cefovecin administration when compared with carprofen alone. The R(-) enantiomer median estimates for AUC were 182.9 and 150.3 μg/mL*hr; Cl were 11.0 and 13.3 mL/hr/kg for carprofen alone and in combination with cefovecin, respectively. Volume of distribution at steady-state was unchanged and was approximately 0.1 L/kg for both enantiomers.
Conclusions and Clinical Relevance- Although some of the pharmacokinetic parameters of the R(-) enantiomer were significantly altered by concurrent cefovecin administration, the more pharmacologically active S(+) enantiomer was not. The results of this study indicate that a clinically significant drug interaction is unlikely to occur following concurrent administration of carprofen and cefovecin in dogs.

INTRODUCTION

Drug-drug interactions (DDI) are a source of morbidity, mortality, and increased healthcare costs in both human and veterinary medicine (McDonnell et al., 2002). There are many complicated mechanisms for a DDI, such as competition at receptors, enzyme induction/inhibition of metabolism, and protein binding interactions (Hochman et al., 2015; Varma et al., 2015). A protein binding interaction can occur in theory because one highly protein bound drug displaces a second highly bound drug from its binding sites on plasma proteins (Benet & Hoener, 2002). Because protein-unbound concentrations are the pharmacologically active components of drugs, this interaction could cause an increase in the unbound concentration of the displaced drug. Increased amounts of unbound drug could then be available to exert a greater pharmacologic effect, or perhaps produce adverse effects. The theory behind these interactions has been previously reviewed (Benet & Hoener, 2002; Toutain & Bousquet-Melou, 2002; Roberts et al., 2013).

Carprofen and cefovecin (Convenia®) are two highly protein bound drugs that are FDA-approved for use in dogs in the United States. Carprofen is a non-steroidal anti-inflammatory drug (NSAID) that is administered once or twice daily for the treatment of pain associated with soft tissue injury or osteoarthritis. Carprofen is among the most commonly prescribed NSAID for dogs in the world, although its use has also been associated with adverse
events, including death (MacPhail et al., 1998; Hampshire et al., 2004). Carprofen is a chiral compound, administered as a racemic formulation, with the S(+) enantiomer responsible for the majority of pharmacologic activity (Lees et al., 2004). Additionally, carprofen exhibits enantioselective pharmacokinetics (Delatour et al., 1993; Mckellar et al., 1994). Carprofen has been reported as being > 99% protein bound (Schmidt & Guentert, 1990; Rimadyl® package insert, Zoetis, New York, NY), but protein binding for each enantiomer have not been reported in dogs. In humans, carprofen and other chiral NSAIDs exhibit enantioselective protein binding (Evans, 1991). If there are differences in protein binding of each enantiomer, pharmacokinetic differences are possible if a protein binding interaction occurs.

Cefovecin is classified as a third-generation cephalosporin and is unique amongst other cephalosporins because it has a plasma half-life of approximately 5 days in dogs (Stegemann et al., 2006), which allows for a duration of effect of either 7 or 14 days, depending on the indication, according to the U.S. FDA approved dose (Convenia® Package Insert, Zoetis; New York, NY). Cefovecin is also unique among cephalosporins in that the protein binding is very high and ranges from 96.0 to 98.5 % in canine plasma at concentrations of 100 µg/mL and 10 µg/mL, respectively (Stegemann et al., 2006). The FDA-approved labels for carprofen and cefovecin list a precautionary statement that their concurrent administration could result in a clinically significant drug-drug interaction caused by protein binding displacement and careful patient monitoring is warranted (Rimadyl® package insert, Zoetis; New York, NY; Convenia® Package Insert, Zoetis; New York, NY). However, there is no data documenting these interactions from studies performed in dogs. Such precautions could result in veterinarians’ reluctance to use these medications together. Because cefovecin is present in dogs for weeks after administration, veterinarians may avoid treating a dog with carprofen for a painful
condition if the label recommendation is followed. The precaution on the FDA-approved label is not based on empirical evidence, therefore it is important to determine if a relevant interaction occurs in vivo when these two drugs are concurrently administered.

The objectives of this study were to (1) determine the enantiospecific protein binding of carprofen in vitro, (2) determine if an enantiospecific drug-drug interaction occurs in vitro between carprofen and cefovecin, and (3) determine if cefovecin alters the enantiospecific pharmacokinetics of carprofen in vivo in dogs. We hypothesized that, based on theoretical evidence (Benet & Hoener, 2002; Toutain & Bousquet-Melou, 2002), a significant drug-drug displacement interaction would occur in vitro, and in vivo this interaction would result in changes in the pharmacokinetics of carprofen enantiomers in dogs.

MATERIALS AND METHODS

In vivo carprofen-cefovecin drug interaction study

In vivo drug-drug interactions between carprofen and cefovecin were studied in 6 healthy adult dogs (4 intact females and 2 intact males, all purpose-bred hounds), with a mean (±SD) weight of 23.0 ± 4.7 kg and age of 2.43 ± 0.56 years. All dogs were determined to be healthy on the basis of history, physical exam, complete blood count and serum biochemistry panels. The only medication these dogs received prior to the study was monthly heartworm prophylaxis. The dogs were housed individually and were fed their normal diet twice daily and had ad libitum access to water. The study was a 2-period sequential design.

One day prior to each drug administration, dogs were sedated (Detomidine Gel, Orion, Finland, 1 mg/m², oral-transmucosal) and a sampling catheter (BD Intracath™, Becton Dickinson, New Jersey, USA) was aseptically placed in one jugular vein. On the morning of each drug administration, dogs were fasted and a 20 or 22-gauge IV catheter (BD Angiocath™,
Becton Dickinson, New Jersey, USA) was aseptically placed in one cephalic vein for carprofen administration.

**Drug administration**

**Phase 1**

Racemic carprofen (Rimadyl®, Zoetis, New Jersey, USA), 4 mg/kg, was administered intravenous via the pre-placed catheter in the cephalic vein. Blood samples were obtained via a preplaced jugular vein catheter prior to drug administration and at the following time points (in hours) after carprofen administration: 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, and 24.

Blood samples were collected into lithium heparin tubes (BD Vacutainer®, Becton Dickinson and Company, New Jersey, USA) and immediately placed on ice. Samples were processed within one hour of collection. Each sample was centrifuged 1509 x g for 10 minutes. Plasma was harvested and stored at -80°C until analysis by HPLC as described previously.

**Phase 2:**

After a minimum of 84 hours washout, the same procedure as described above was performed, except that each dog received a single subcutaneous injection of cefovecin (Convenia®, Zoetis, New Jersey, USA), 8 mg/kg, between the shoulder blades 2 hours prior to IV carprofen administration. The 2 hour time period was chosen because it was consistent with the peak (C\text{MAX}) concentrations in plasma after cefovecin administration (Stegemann et al., 2006). Blood samples were obtained from the jugular catheter at the same time points as described above following intravenous carprofen administration. Further blood samples were obtained in 4 dogs, via direct venipuncture once the jugular catheters had been removed, at
approximately the following time points (in hours) for cefovecin analysis: 2.5, 3, 4, 6, 8, 10, 12, 14, 24, 48, 72, 96, 120, 144, 216, 336, 432, and 504.

All dogs were monitored at least twice daily for the development of adverse effects, which included vomiting, diarrhea, lethargy, or inappetence.

**HPLC Drug Analysis:**

Canine plasma samples were analyzed by high-pressure liquid chromatography (HPLC) with a method developed in the Clinical Pharmacology Laboratory at NCSU. Racemic carprofen reference standard was obtained from US Pharmacopeia (Rockville, MD, USA). Carprofen reference standard was weighed and dissolved in 100% HPLC-grade methanol to a concentration of 1 mg/mL. From this stock solution, further dilutions were made in HPLC-grade distilled water to make up fortifying solutions for plasma in order to prepare quality control samples, calibration curve samples, and for development of these methods. The stock solution was kept at 4 °C in a tightly sealed dark vial when not in use, but was made fresh on each day’s analysis. The fortifying solutions made from the stock solution were added to blank (control) plasma, to make up seven calibration standards, including zero (range 0 to 100 µg/mL).

The mobile phase consisted of 55% 0.02 M potassium phosphate in water buffered to a pH of 3.0 using phosphoric acid, and 45% acetonitrile at a flow rate of 1 mL/min. Fresh mobile phase was prepared, filtered (0.45 µm), and degassed for each day's run. The HPLC system consisted of a quaternary solvent delivery system (Agilent Technologies, Wilmington, DE, USA) at a flow rate of 1 mL/min, an autosampler (1100 Series Autosampler, Agilent Technologies), and fluorescence detector set at an excitation wavelength of 310 nm and emission wavelength of 375 nm (Agilent 1100 series fluorescence detector, Agilent
Technologies, Wilmington, DE, USA). The chromatograms were integrated with a computer program (1100 Series Chemstation software, Agilent Technologies). The column was a reverse-phase, 4.6 mm × 15 cm C18 column (Zorbax Eclipse XDB-C18 column) and Eclipse guard column (Agilent Technologies, Wilmington, DE, USA) kept at a constant temperature of 40 °C. Retention time for the peaks of interest were approximately between 8.5-9.5 minutes.

All plasma samples, calibration samples, and blank (control) plasma samples were prepared identically using solid-phase extraction. Solid-phase extraction cartridges (Waters Oasis HLB cartridges, Waters Associates, Milford, MA, USA) were conditioned with 1 mL methanol followed by 1 mL distilled water. A plasma sample of 250 μL was added to a conditioned cartridge (125 μL sample + 125 μL pooled blank canine plasma) which was acidified using 250 μL of 4% phosphoric acid, followed by a wash step of 1 mL distilled water: methanol (95:5). Carprofen was eluted with 1 mL 100% methanol and collected in clean glass tubes. The tubes were evaporated at 40 °C for 15–20 min in an evaporator. Each tube was then derivitized with L-leucinamide according to previous methods in order to separate the R and S enantiomers (Spahn et al., 1988; Priymenko et al., 1998) and vortexed. Briefly, 100 μL of 50 mM TEA in acetonitrile was added to each sample, followed 30 seconds later by 50 μL of 60 mM ethylchloroformate in acetonitrile, followed 30 seconds later by 50 μL of a 1:1 mixture of 1 M TEA in methanol and 1 M L-leucinamide in methanol, followed 2 minutes later by 50 μL of water. From each tube, 5 μL was then injected into the HPLC system.

A fresh set of calibration, blank, and quality control samples were prepared for each day's run. The quality control samples were prepared using blank sample matrix fortified with a range of concentrations of carprofen. Quality control samples were back-calculated and were within 15% of the nominal value, which was within our acceptance criteria. All calibration
curves were linear with an \( r^2 \) value of 0.99 or higher. Limit of quantification (LOQ) for this study was 0.1 \( \mu \text{g/mL} \), which was determined from the lowest point on a linear calibration curve that met our acceptance criteria and using guidelines published by the United States Pharmacopeia (United States Pharmacopeia, 2011). Average accuracy (%) for the R(-) and S(+) enantiomers in plasma were 95.8 ± 3.2 and 95.1 ± 4.6, respectively. Average precision (RSD, %) for the R (-) and S(+) enantiomers were 7.7 ± 2.7 and 8.1 ± 2.9, respectively.

**Cefovecin analysis**

Plasma concentrations of cefovecin were determined by HPLC analysis. Cefovecin reference standard (Zoetis, Kalamazoo, MI) was used to prepare all calibration and quality control samples, which were made fresh on each day’s analysis using pooled blank canine plasma and prepared with fresh stock solution diluted in water. Cefovecin was extracted from plasma using a liquid extraction method. One mL of acetonitrile was added to 400 \( \mu \text{L} \) of plasma, which was vortexed and centrifuged at 1509 x \( g \) for 10 minutes. One mL of supernatant was transferred to a clean test tube, which was then evaporated under a stream of air at 40 °C for 15 min. The dried samples were reconstituted with 200 \( \mu \text{L} \) of mobile phase, and vortexed. Thirty \( \mu \text{L} \) of sample was injected onto a Zorbax Eclipse XDB-C18 column (4.6 mm X 15 cm) at a column temperature of 40 °C. The mobile phase was 83% water and 17% acetonitrile with 2% trifluoroacetic acid, at a flow rate of 1 mL/min. Cefovecin was detected using ultraviolet detection at a wavelength of 295 nm. Acceptance criteria for linearity and LOQ (1 \( \mu \text{g/mL} \)) for the method were the same as described previously. Average accuracy and precision for the cefovecin method was 97.6 ± 6.5% and 3.1 ± 2.6, respectively.
Protein Binding

Plasma protein binding of R (-) and S (+) carprofen enantiomers was determined using ultracentrifugation as previously described (Bidgood & Papich, 2002) but modified for carprofen. Prior to protein binding determination, carprofen adsorption to the ultrafiltration membrane was tested and determined to be insignificant. Pooled blank canine plasma was fortified with carprofen at 3 different concentrations: 10, 25, and 50 µg/mL. Five replicates at each concentration were prepared and incubated in a water bath at 37 °C for 30 minutes. One mL of each replicate was placed into the micropartition device (Centrifree® Ultrafiltration Device, EMD Millipore, Massachusetts, USA). Devices were centrifuged at 1500 x g for 30 min in a temperature-controlled centrifuge, according to manufacturer’s instructions. Two hundred µL of ultrafiltrate was removed from the collection reservoir and dried under a stream of air for 30 minutes at 40°C. The samples were then reconstituted, derivitized and analyzed as described above. The assay range for carprofen in ultrafiltrate was 1 to 1000 ng/mL. Calibration curves were linear with a correlation coefficient (R²) value of 0.99 or greater. The LOQ was 1 ng/mL. Average accuracy (%) for the R(-) and S(+) enantiomers were 102.1 ± 3.8 and 101.3 ± 4.2, respectively. Average precision (RSD, %) for the R(-) and S(+) enantiomers were 4.0 ± 1.7 and 4.1 ± 2.5, respectively. A second set of fortified samples was prepared in replicates of 5 at the same concentrations, but omitting the ultracentrifugation step.

Plasma samples for total (bound + unbound) carprofen concentrations (5 replicates) were extracted using solid phase extraction and concentrations of each enantiomer were determined using HPLC after derivitization as described previously.

Percentage of the bound fraction of each enantiomer was calculated by the following formula:
% Protein Binding

\[
\text{\% Protein Binding} = \left( \frac{\text{total concentration} - \text{unbound concentration}}{\text{total concentration}} \right) \times 100 \quad (\text{Equation 1})
\]

*In vitro cefovecin-carprofen drug interaction study*

Plasma carprofen samples were incubated with, and without cefovecin to measure the *in vitro* effect on protein binding. Blank plasma was pooled from healthy dogs. Replicates of 5 plasma samples were fortified with carprofen at concentrations that represent a range of concentrations attained after oral administration of approved doses in dogs (50 and 100 µg/mL of total carprofen, which corresponds to 25 and 50 µg/mL of each enantiomer, respectively). Samples were incubated in a water bath at 37° C for 30 minutes, then 1 mL of each sample was used for determination of unbound carprofen via ultracentrifugation, and 125 µL of each replicate was used for determination total (bound + unbound) carprofen.

A second set of plasma was fortified with 80 µg/mL of cefovecin, representing a concentration attained after administration of an approved dose (8.0 mg/kg subcutaneously) in dogs, and incubated at 37° C for 30 minutes. Following this incubation, carprofen was added at the above concentrations (25 and 50 µg/mL of each enantiomer), in replicates of 5. Samples were incubated for an additional 30 min and processed as described above.

*Pharmacokinetic analysis*

*Carprofen*

Pharmacokinetic modeling was performed by least squares regression analysis using a commercial software program (Phoenix WinNonLin ver.6.3, Certara, St. Louis, MO) to estimate the plasma pharmacokinetic parameters for each enantiomer of carprofen. Data were
weighted by the reciprocal of the predicted concentration squared, and analyzed by compartmental methods. The model that best fit the data was determined by visual inspection of the concentration vs. time curves, plots of the residuals, and minimum Aikake information criterion (Yamaoka et al., 1978). A 2-compartment model for IV bolus input was used and is described by the following equation:

$$C_p^t = Ae^{-\alpha t} + Be^{-\beta t} \quad \text{(Equation 2)}$$

where A and B are the coefficients (µg/mL) and α (distribution rate constant) and β (terminal elimination rate constant) are exponents (per hour) and $C_p^t$ is the plasma concentration (µg/mL) of carprofen at time t. Secondary parameter estimates were obtained using standard compartmental equations (Gibaldi & Perrier, 1982).

**Cefovecin**

Four of the dogs from the experiment were selected for determination of cefovecin pharmacokinetics. The parameters were determined using non-compartmental methods (Phoenix WinNonLin ver.6.3, Certara, St. Louis, MO). Least squares regression analysis was used to obtain the elimination rate constant (λz). The log linear trapezoidal method was used to estimate the area under the curve, and data were uniformly weighted. Standard noncompartmental equations were used to obtain estimates for mean residence time, clearance, and volume of distribution at steady state (Gibaldi & Perrier, 1982).

**Statistical analysis:**

Data were analyzed for normality using a Kolmogorov-Smirnov test. Differences in pharmacokinetic parameters of carprofen with and without cefovecin were analyzed using a paired t test or Wilcoxon signed rank test, as appropriate, and significance was established at a $p \leq 0.05$. Differences in protein binding were analyzed using an unpaired t test.
RESULTS

In vitro protein binding of carprofen enantiomers and cefovecin interaction

Both enantiomers were very highly protein bound (>99.9%) at all concentrations tested. The R enantiomer was 99.97 and 99.92 % and the S enantiomer was 99.94 and 99.80 % protein bound at 25 and 50 µg/mL, respectively. The R enantiomer was found to have significantly greater protein binding than the S enantiomer at both 25 and 50 µg/mL concentrations (p< 0.001). At 10 µg/mL, concentrations of carprofen collected in the ultrafiltrate were below the LOQ for the assay and therefore protein binding at this concentration could not be calculated.

A drug-drug interaction was observed following in vitro protein binding experiments. At both 25 and 50 µg/mL, the unbound concentrations of both the R and S enantiomers were significantly increased when cefovecin was added to the plasma (p ≤ 0.001). Figures 3.1 and 3.2 depict the displacement interactions at 25 and 50 µg/mL of carprofen alone and with 80 µg/mL cefovecin added.

In vivo carprofen-cefovecin pharmacokinetic interactions

There were not any clinical adverse effects observed in any of the treated dogs. Plasma concentration versus time curves and pharmacokinetic parameter estimates for total (bound and unbound) carprofen enantiomers following administration alone, and with cefovecin, are shown in Figure 3.3 and Table 3.1. The following parameter estimates for the total drug concentrations were significantly different for the R(-) enantiomer when cefovecin was administered concurrently: AUC (p= 0.02), Cl (p= 0.01), mean residence time (p=0.04), β (p=0.02) , and β half-life (p=0.04). There were no significant differences in parameter estimates for the carprofen S(+) enantiomer when cefovecin was concurrently administered.
with carprofen. The plasma concentration versus time curve for cefovecin and parameter estimates for 4 dogs are shown in Figure 3.4 and Table 3.2.

DISCUSSION

The results from the *in vitro* studies show that drug-drug protein binding displacement interactions can occur in a “closed system,” i.e. one in which displaced drug cannot be metabolized or eliminated by the body. The addition of cefovecin in an *in vitro* system increased the free concentration of carprofen significantly (Figures 3.1 and 3.2). The *in vivo* experiments showed that changes in plasma pharmacokinetics for total (bound + unbound) carprofen occurred only for the R(-) enantiomer. Pharmacokinetics of the S(+) enantiomer were not changed, which is clinically relevant because the R(-) enantiomer is largely pharmacologically-inactive in dogs (Ricketts *et al.*, 1998; Lees *et al.*, 2004). Therefore, we can conclude that administration of cefovecin to healthy research dogs did not affect the clinically-relevant S(+) plasma concentrations of carprofen. Additionally, plasma concentrations of cefovecin did not appear to change with the concurrent administration of carprofen, as the pharmacokinetics from this study (Figure 3.3, Table 3.2) were similar to the pharmacokinetics previously published (Stegemann *et al.*, 2006). However, a direct comparison was not performed because we did not administer cefovecin alone in this study thus a statistical comparison was not possible.

If two highly protein-bound drugs are concurrently administered, they may compete for binding sites on plasma proteins. If this occurs, a drug displacement interaction may result, causing an increase in free drug plasma concentrations of the displaced drug. *In vivo*, changes in total (bound + unbound) drug pharmacokinetics are possible because of drug-drug protein binding interaction, but the free drug concentration is not expected to change due to increased
drug elimination (Toutain & Bousquet-Melou, 2002). In this study, only some parameters for the R(-) enantiomer were different following cefovecin administration; the most important of which being clearance. An increase in total clearance for the R(-) enantiomer of carprofen occurred when cefovecin was administered, suggesting that cefovecin displaced carprofen from its binding sites on albumin in vivo, which was similarly observed in the in vitro experiment. A significant decrease in terminal half-life was also detected, which would be predicted because half-life is a dependent parameter that is influenced by both volume of distribution and clearance. Also, the mean residence time, MRT, was significantly shorter when cefovecin was concurrently administered which could be predicted based on the decreased AUC (Table 2.1).

Differences in volume of distribution might also be predicted when a protein binding displacement interaction occurs, since an increase in the unbound drug concentration would result in both increased distribution and clearance (Benet & Hoener, 2002). The volume of the central compartment (V1) and volume of distribution at steady state (Vdss) were not significantly different in this study. The lack of a significant difference in these volumes of distribution may be due to a Type II statistical error, as a post hoc power analysis revealed that power was low.

An unexpected finding was that the pharmacokinetics of S(+) enantiomer did not differ significantly in vivo, as we observed for parameters for the R (-) enantiomer. There are several potential explanations for this observation, the first being the relatively lesser effect on protein displacement for the S(+) enantiomer compared to the R(-) enantiomer based on our in vitro results (Figure 3.1). The small amount of displacement that occurred for the S(+) enantiomer, although statistically significant, was likely not of a large enough magnitude to affect the
pharmacokinetics of the drug *in vivo*. The amount of drug displacement observed by the interaction (Figure 3.1) amounts to only a very small percent of the total concentration (less than 0.1%). These concentrations are probably much lower than the concentrations that can saturate protein binding sites to produce a drug displacement interaction *in vivo*, which has been reported in studies with other NSAIDs (Borgå & Borgå, 1997). Another possible explanation for our findings could be that a displacement occurred at only one binding site on albumin (i.e., Site II), such that cefovecin displaced carprofen from Site II only to have it move to Site I (the warfarin site). Both carprofen and cefovecin contain carboxylate groups, which is a characteristic of drugs that bind to Site II, however studies with NSAIDs and albumin proteins from multiple other species have demonstrated multiple binding sites for NSAIDs so a displacement from one site, with binding at different site is possible (Rahman *et al.*, 1993; Rahim & Aubry, 1995; Yamasaki *et al.*, 2000).

However, there are also pharmacokinetic differences between the enantiomers that may explain our observations. Carprofen is metabolized by the liver and undergoes glucuronidation in dogs and other species (Rubio *et al.*, 1980). The S(+) enantiomer of carprofen is subject to greater enterohepatic recirculation than the R(-) enantiomer (Priymenko *et al.*, 1998). Therefore, if hepatic clearance for the S(+) enantiomer was increased because of protein binding displacement by cefovecin, the glucuronide conjugate for S(+) carprofen may be hydrolyzed back to the parent compound, and therefore recirculated. Ultimately this might result in either no change, or perhaps an increase, in total S(+) drug concentrations. The latter result was in fact observed in this study, with slight increases in total S(+) concentrations observed from 2-8 hours after drug administration (Figure 3.3). The increase in total S(+) concentrations during this time did not affect the pharmacokinetics however.
The in vitro protein binding differences between carprofen enantiomers has not been previously reported for dogs. Although both enantiomers were highly protein bound (>99%), there was a statistically significant difference in protein binding between the two carprofen enantiomers, with the R(-) enantiomer being more highly protein-bound in dogs. Differences in protein binding for carprofen enantiomers have been reported in people as well, although the S(+) enantiomer was found to have a greater affinity/protein binding for albumin than the R(-) enantiomer (Iwakawa et al., 1990). Several of the chiral NSAIDs exhibit some degree of enantioselective protein binding, and studies with many of these compounds have demonstrated significant clinical implications from these differences, including differences in pharmacokinetics and pharmacodynamics (Evans, 1992). The overall magnitude of the difference in protein binding is low compared to the extent of binding for this drug (>99%), therefore the degree of clinical relevance is undetermined but is likely low.

The pharmacokinetic parameter estimates obtained for carprofen in this study were slightly different than those previously reported. Priymenko et al. (1998) reported higher estimates for clearance compared with our study; specifically clearance for the S(+) enantiomer was 28 mL/h/kg whereas this study estimated a value of 12 mL/h/kg (Priymenko et al., 1998). The values obtained for the R(-) enantiomer were more similar (17 mL/h/kg versus 11 mL/h/kg in the current study). The reason for these differences is undetermined. Each study used a different breed of dog (Beagle dog versus Hound dog), and differences in metabolism are known to occur among dog breeds, which is a possible explanation for the observed differences and this warrants further investigation (Paulson et al., 1999; Fleischer, et al., 2008). The remainder of the parameter estimates for each enantiomer in our study were very similar to those reported by others (McKellar et al., 1994; Priymenko et al., 1998).
The plasma pharmacokinetic parameter estimates for subcutaneous cefovecin in these dogs studied were remarkably similar to a previously published report by Stegemann and others, suggesting that administration of carprofen did not change the pharmacokinetics of cefovecin appreciably (Stegemann et al., 2006). A direct statistical comparison was not possible because we did not perform a study with cefovecin administered alone. The Cmax in our study was only slightly less; 102 µg/mL compared to 116 µg/mL, and the Tmax slightly longer at 4 hours compared to 2.7 hours. These minor differences are likely a result of differences in study design (i.e. time points selected for blood sampling). The AUC and elimination half-life estimated in this study were nearly identical to those estimated previously (Stegemann et al., 2006). We believe that there would be little, if any, effect on the clinical activity of cefovecin following a single administration of carprofen because of the relatively short half-life of carprofen compared to cefovecin. We cannot speculate on the potential effect of multiple administration of carprofen (e.g., every 12 hours) on these results however.

The clinical importance of protein binding drug interactions have been discussed in other highly cited reviews (Toutain & Bousquet-Melou, 2002; Benet & Hoener, 2002). The review by Benet & Hoener (2002) listed certain criteria that must be met for clinically significant protein binding displacement interactions to occur. Carprofen does not meet any of these criteria. Thus, these authors argue that there is little clinical importance for protein binding interactions for the drugs administered in this study. Examples include drugs administered intravenously that have a high hepatic extraction ratio and a narrow therapeutic index. Carprofen has a low hepatic extraction ratio (Schmitt & Guentert, 1990; Evans, 1992) and clearance for drugs with low extraction ratios depends on the fraction of unbound drug in the plasma (fu) according to the equation:
\[ Cl = \frac{[Q_{\text{organ}} \cdot fu \cdot Cl_{\text{int}}]}{[Q_{\text{organ}} + fu \cdot Cl_{\text{int}}]} \] (Equation 3),

which is simplified for low ER drugs into the equation:

\[ Cl \approx fu \cdot Cl_{\text{int}} \] (Equation 4),

where \( Cl \) is organ clearance, \( Q_{\text{organ}} \) is blood flow to the clearing (eliminating) organ, and \( C_{\text{int}} \) is the intrinsic organ clearance of the unbound drug (Benet & Hoener, 2002), thus an increase in the \( fu \) would result in an increase in total drug clearance, which was demonstrated in this experiment for the \( R(-) \) enantiomer (Table 3.1). Speculation for why \( Cl \) was not changed for the \( S(+) \) isomer was discussed previously.

Both \textit{in vitro} and \textit{in vivo} studies were conducted for this investigation. Toutain and Bousquet-Melou (2002) discussed how the relationships between the steady state free and total drug concentrations are fundamentally different in \textit{in vitro} versus \textit{in vivo} systems. \textit{In vitro}, the interaction between drug and binding protein occurs in a closed system and there is an increase in unbound drug concentrations because of displacement of one drug from its binding site(s) with no mechanism for drug elimination. In contrast, \textit{in vivo} drug concentrations are controlled by the elimination process. When an interaction occurs, the amount of drug displaced from the binding protein will be redistributed and eliminated by the body, causing a change in the total drug concentration but ultimately resulting in no change in the free drug concentration and consequently no change in drug effect (Toutain & Bousquet-Melou, 2002). Based on \textit{in vitro} results and the arguments proposed by Toutain and Bousquet-Melou, pharmacokinetic changes may have been anticipated but statistically significant changes were observed only in the \( R(-) \) enantiomer. The greater anti-inflammatory activity of the \( S(+) \) enantiomer as compared with the \( R(-) \) enantiomer suggests that the pharmacodynamics of carprofen in this interaction are likely to be unaffected in dogs.
In conclusion, this study demonstrated the enantioselective protein binding of carprofen in canine plasma, with the R(-) enantiomer being more highly protein bound than the S(+) enantiomer. Both enantiomers underwent a small, but statistically significant displacement interaction *in vitro* when combined with the highly protein bound antimicrobial cefovecin. However, *in vivo*, the concurrent administration of carprofen and cefovecin altered the pharmacokinetics only for the R(-) enantiomer of carprofen and not the S(+) enantiomer. The R(-) enantiomer was cleared more rapidly when cefovecin was concurrently administered. This drug-drug interaction is likely to have little clinical relevance due to the low pharmacodynamic activity of the R(-) enantiomer of carprofen. Therefore, we see little basis for concern as stated on the current approved U.S. label that states “Concurrent use of these (including carprofen) or other drugs that have a high degree of protein-binding may compete with cefovecin binding and cause adverse reactions”.
REFERENCES


Previcox® Package Insert (2013) Merial Ltd., Duluth, GA.


Table 3.1: Pharmacokinetic parameter estimates for R(-) and S(+) carprofen without and with concurrent cefovecin administration. † denotes significantly different (p≤0.05). 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; Vdss: volume of distribution at steady state; Cl, total plasma clearance.

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Table 3.2: Pharmacokinetic parameter estimates for cefovecin in 4 dogs using noncompartmental methods. AUC: Area under the curve; AUC % Ext: AUC percent extrapolated; AUMC: area under the first moment curve; Cmax: maximum concentration; half-life $\lambda_z$: elimination half-life; $\lambda_z$: elimination rate constant; MRT: mean residence time; Tmax: time to maximum concentration.

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<td>Tmax</td>
<td>h</td>
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Figure 3.1: In vitro protein binding displacement interaction for 25 µg/mL carprofen alone (R25 and S25 represent the R(-) and S(+) carprofen enantiomers, respectively) and in combination with 80 µg/mL cefovecin (R25Cef and S25Cef represent the R(-) and S(+) carprofen enantiomers with cefovecin present). Experiments were performed on 5 replicates; one outlier in the 25 µg/mL group was not included in the statistical analysis and is not shown in the figure. The outlier values for R(-) and S(+) free concentrations of carprofen from this replicate were 18.3 µg/mL and 27.0 µg/mL, respectively. * Denotes significantly different from carprofen alone (p < 0.001).
Figure 3.2: In vitro protein binding displacement interaction for 50 µg/mL carprofen alone and in combination with 80 µg/mL cefovecin (n= 5). * Denotes significantly different from carprofen alone (p < 0.001).
Figure 3.3: Mean (SD) total (bound + unbound) plasma concentration versus time profiles for carprofen enantiomers following intravenous administration alone (4 mg/kg, intravenously), and with cefovecin (8 mg/kg, subcutaneously) (n=6 dogs).
Figure 3.4: Mean (SD) plasma concentration versus time profiles for cefovecin (8 mg/kg, subcutaneously) (n=4 dogs).
ABSTRACT

Background and Objective: Carprofen is one of the most commonly administered non-steroidal anti-inflammatory drugs (NSAIDs) in dogs. Despite its widespread use, there is little pharmacokinetic data available for carprofen in the intended population of dogs at recommended doses. The objective of this study was to describe the pharmacokinetics of orally administered carprofen in a large population of clinical patients and assess the influence of covariates that may alter the pharmacokinetics in certain individuals.

Animals: This study analyzed 216 plasma samples from 73 dogs that were prescribed carprofen for medical reasons, including osteoarthritis and analgesia following soft tissue surgery.

Methods: Population pharmacokinetic parameter estimates for R(-) and S(+) carprofen following either once or twice daily dosing were obtained using non-linear mixed effects modeling (NLME). Population estimates for apparent volume of distribution (V/F), apparent clearance (Cl/F), absorption rate constant (Ka), and lag time (Tlag) were obtained using separate models for each enantiomer. The effects of age, weight, breed (small or large), gender, dosing interval, elevated liver or kidney chemistry values, presence of osteoarthritis (OA), and concurrently administered supplements or tramadol on carprofen pharmacokinetics were also investigated.

Results: There were very few outlier animals with either very low or very high concentrations of carprofen in plasma. Overall the population pharmacokinetic estimates for carprofen were...
found to be similar to those previously reported, with the exception of Cl/F, which was estimated to be lower in this clinical population of dogs studied. A significant covariate effect was identified for dogs with OA, which resulted in a 27% lower Cl/F for the S(+) enantiomer in these dogs. There was no apparent advantage, in terms of overall exposure to carprofen, in dogs dosed twice daily at lower doses versus those dosed once daily at a higher dose.

Conclusions: The Cl/F for S(+) carprofen in this clinical population was lower than previously reported in healthy dogs, and the presence of OA was a significant covariate to account for reduced Cl/F and greater carprofen exposure in a clinical population. The clinical significance of these findings in terms of increased risk for adverse effects or clinical response is undetermined.

INTRODUCTION

Carprofen is one of the most commonly prescribed nonsteroidal anti-inflammatory drugs (NSAIDs) for dogs, and is used to treat pain and inflammation associated with osteoarthritis (OA) and soft tissue injury, including surgery. Carprofen is generally considered a mild cyclooxygenase (COX) inhibitor; some studies report greater COX-2 inhibition than COX-1 resulting in classification as a COX-2 preferential NSAID (Mckellar et al., 1994; Ricketts et al., 1998; Lees et al., 2004). Orally administered carprofen is approximately 100% bioavailable and reaches a peak plasma concentration, approximately 1-2 hours after administration in dogs (Schmitt & Guentert, 1990). Carprofen, like many NSAIDs of the 2-aryl propionic class, is administered as a racemic mixture, but exhibits species-specific enantioselective differences in pharmacokinetics and metabolism (Priymenko et al., 1998; Maire-Gauthier et al., 1998; Lees et al., 2004). The R(-) enantiomer has been reported to have a greater overall exposure in canine plasma (Mckellar et al., 1994), but the S(+) enantiomer
possesses significantly greater anti-inflammatory activity and is responsible for the pharmacodynamic effects (Ricketts et al., 1998). Metabolism occurs via the liver, predominately through glucuronidation, and ultimately is excreted in the feces (Rubio et al., 1980). The S (+) enantiomer undergoes enterohepatic recirculation, but the R(-) does not (Priymenko et al., 1998). The pharmacokinetics of carprofen remain largely unchanged after single or multiple doses, and have been variable among dogs (Lipscomb et al., 2002; Clark et al., 2003).

There is wide variation in ages, breeds, and disease states of dogs that receive carprofen for pain associated with soft tissue surgery, OA, and other painful inflammatory conditions. Traditional pharmacokinetic (PK) studies have been performed for carprofen in a small number of healthy research dogs, or a narrow group of clinical patients; but the pharmacokinetics have not been examined in a larger population of clinical patients (Mckellar et al., 1994; Lascelles et al., 1998; Priymenko et al., 1998; Lipscomb et al., 2002; Clark et al., 2003). The pharmacokinetic parameters of NSAIDs reported from studies performed in healthy research animals can be substantially different from the intended treatment population of dogs with OA or other painful conditions (Cox et al. 2011; Silber et al., 2010).

When studied in a small population of healthy research dogs, even at high doses, carprofen was judged to be safe and effective (Hickford et al., 2001; FDA Freedom of Information Summary). But in the general treatment population, carprofen has the largest number of adverse events and number of animals experiencing adverse events compared to any other NSAID listed on the FDA's Adverse Drug Experience Reports (FDA, 2014). Adverse events include liver toxicity (acute hepatocellular necrosis, currently considered an idiosyncratic liver injury), vomiting, diarrhea, lethargy, and death, and Labrador retrievers
have been over-represented in some of these reports (MacPhail et al., 1998; Hampshire et al., 2004). It is still undetermined if Labrador retrievers are particularly sensitive to carprofen. While the incidence of reported adverse effects associated with carprofen is low, with an estimate of approximately 3.7% or less (Holloway et al., 2012), they can produce significant morbidity and result in extensive medical expense. The underlying mechanism(s) of these adverse events is largely unknown and may be multi-factorial. It has also been observed that some dogs treated with carprofen for OA fail to improve. In a multi-center clinical trial, between 20% - 44% of dogs with OA treated with carprofen had a negative response, meaning that they either did not show improvement or became worse in lameness scores assessed by a force plate evaluation, as well as subjective owner and veterinarian evaluations (Vasseur et al., 1995). Other studies have reported that approximately 25% of dogs show either no improvement or only mild improvement in lameness after receiving carprofen, although only one of these studies concurrently assessed the pharmacokinetics of carprofen and this was only in a small number (n=6) of dogs (Lipscomb et al., 2002; Mansa et al., 2007; Holloway et al., 2012).

An aim of this study was to investigate potential reasons for variable response to carprofen (e.g., adverse effects or lack of an adequate response) that could be attributed to differences in pharmacokinetics of carprofen in a clinical population of dogs. The clinical response to NSAIDs do not always correlate to anti-inflammatory activity at effect sites but adverse effects could be associated with the plasma concentrations (Lees et al., 2004; Brune & Furst, 2007). Additionally, in people, genetic differences in metabolizing enzymes have been identified that account for both variability in pharmacokinetics and have been cited as a predisposition for adverse drug effects (Martinez et al., 2004; Kirchheiner & Brockmoller,
Specifically, a deficiency in the hepatic cytochrome P450 enzyme 2C9 has been linked to these observations (Martinez et al., 2004; Kirchheiner & Brockmoller, 2005; Carbonell et al., 2010). We suspect that clinical cases of dogs treated with carprofen will have differences in pharmacokinetics of carprofen caused by factors such as breed, age, liver or kidney dysfunction, or presence of OA which puts them at risk to experience an adverse event, including lack of response, following carprofen administration.

The objective of this study was to perform a population pharmacokinetic analysis of carprofen in dogs receiving carprofen for OA or another painful condition, and to evaluate the effects of age, weight, breed, gender, dose frequency, changes in serum creatinine or blood urea nitrogen (BUN), elevated serum hepatic enzyme activities, the presence of OA, and currently administered supplements or drugs on carprofen pharmacokinetics. We hypothesized that there would be differences in population parameter estimates for carprofen in this population of dogs, and the variability in parameter estimates would be minimized through covariate analysis.

MATERIALS AND METHODS

Animals

This study was approved by the North Carolina State University Institutional Animal Care and Use Committee and owner consent was obtained prior to beginning the study. Inclusion criteria were dogs over 3 months of age, weighing between 5 and 100 kg, and receiving approximately 4.4 mg/kg/day of carprofen orally (2.2 mg/kg twice daily, or 4.4 mg/kg once daily) for medical conditions including osteoarthritis, lameness from soft tissue injury, post-operatively for analgesia. Exclusion criteria were dogs also receiving
corticosteroids (a known drug-drug interaction), those with resistant bacterial infections or aggressive demeanor, and animals weighing less than 5 kg.

**Sample Collection**

A physical examination was performed on all dogs at admission. Carprofen was administered orally to each patient by either the study coordinator or the owner according to the animal’s prescription (a targeted dose of either 2.2 mg/kg every 12 hours or 4.4 mg/kg every 24 hours). The exact amount (in mg) and time of carprofen administration and blood sample collection was recorded for each animal. Blood samples (approximately 2-4 mL) were collected via venipuncture into tubes containing lithium heparin (BD Vacutainer®, Becton Dickinson and Company, New Jersey, USA) and immediately centrifuged at 1509 x g for 10 minutes. Plasma was harvested and aliquoted into cryovials, and stored at -80°C until analysis. Blood was also collected for hematological (CBC) and serum biochemistry analysis, and urine was collected via free catch for urinalysis. All clinical pathology tests were performed by the Clinical Pathology Laboratory at North Carolina State University College of Veterinary Medicine.

In most cases animals were randomly assigned to a sample collection schedule that consisted of samples obtained at 3-4 of the following time points: 0 (prior to carprofen administration), 0.5, 1, 2, 4, 6, 8, 12, 16, and 24 hours after carprofen administration (Table 4.1). Due to limitations in the dog owner’s schedule, some dogs were not randomly assigned, but were placed into a sample schedule that coincided with the owner’s hospital visit time. These sample times were selected to obtain a complete concentration–time profile over a 24 hour dosing interval. Only dogs receiving once daily carprofen were included in sample
schedules out to 24 hours; dogs receiving twice daily doses were in sample schedules with collection up to 12 hours.

Eighty-five dogs were enrolled in the study, however due to missing or incorrect data, lack of compliance, or dosing errors, a total of 73 dogs were included in the final pharmacokinetic analysis, with 53 dogs that received approximately 2.2 mg/kg of carprofen orally once every 12 hours, and 20 dogs that received approximately 4.4 mg/kg of carprofen orally once every 24 hours.

Carprofen Analysis

Canine plasma samples were analyzed by high-pressure liquid chromatography (HPLC) with a method developed and validated in the Clinical Pharmacology Laboratory at NCSU. Racemic carprofen reference standard was obtained from US Pharmacopeia (Rockville, MD, USA). Carprofen reference standard was weighed and dissolved in 100% HPLC-grade methanol to a concentration of 1 mg/mL. From this stock solution, further dilutions were made in HPLC-grade distilled water to make fortifying solutions for plasma in order to prepare quality control and calibration curve samples as well as for method development. The stock solution was kept at 4 °C in a tightly sealed dark vial when not in use, but was made fresh on each day’s analysis. The fortifying solutions made from the stock solution were added to blank (control) plasma, to make up seven calibration standards, including zero (range 0 to 100 μg/mL).

The mobile phase consisted of 55% 0.02 M potassium phosphate in water buffered to a pH of 3.0 using phosphoric acid, and 45% acetonitrile at a flow rate of 1 mL/min. Fresh mobile phase was prepared, filtered (0.45 μm), and degassed for each day's run. The HPLC system consisted of a quaternary solvent delivery system (Agilent Technologies, Wilmington,
DE, USA) at a flow rate of 1 mL/min, an autosampler (1100 Series Autosampler, Agilent Technologies), and fluorescence detector set at an excitation wavelength of 310 nm and emission wavelength of 375 nm (Agilent 1100 series fluorescence detector, Agilent Technologies, Wilmington, DE, USA). The chromatograms were integrated with a computer program (1100 Series Chemstation software, Agilent Technologies). The column was a reverse-phase, 4.6 mm × 15 cm C18 column (Zorbax Eclipse XDB-C18 column) and Eclipse guard column (Agilent Technologies, Wilmington, DE, USA) kept at a constant temperature of 40 °C. Retention time for the peaks of interest were approximately between 8.5-9.5 minutes.

All plasma samples, calibration samples, and blank (control) plasma samples were prepared identically using solid-phase extraction. Solid-phase extraction cartridges (Waters Oasis HLB cartridges, Waters Associates, Milford, MA, USA) were conditioned with 1 mL methanol followed by 1 mL distilled water. A plasma sample of 250 μL was added to a conditioned cartridge (125 μL sample + 125 μL pooled blank canine plasma) which was acidified using 250 μL of 4% phosphoric acid, followed by a wash step of 1 mL distilled water: methanol (95:5). Carprofen was eluted with 1 mL 100% methanol and collected in clean glass tubes. The tubes were evaporated at 40 °C for 15–20 min in an evaporator. Each tube was then derivitized with L-leucinamide according to previous methods (Spahn et al., 1988; Priymenko et al., 1998) and vortexed. Briefly, 100 μL of 50 mM TEA in acetonitrile was added to each sample, followed 30 seconds later by 50 μL of 60 mM ethylchloroformate in acetonitrile, followed 30 seconds later by 50 μL of a 1:1 mixture of 1 M TEA in methanol and 1 M L-leucinamide in methanol, followed 2 minutes later by 50 μL of water. From each tube, 5 μL was then injected into the HPLC system.
A fresh set of calibration, blank, and quality control samples were prepared for each day's run. The quality control samples were prepared using blank sample matrix fortified with a range of concentrations of carprofen. Quality control samples were back-calculated and were within 15% of the nominal value, which was within our acceptance criteria. All calibration curves were linear with an $r^2$ value of 0.99 or higher. Limit of quantification (LOQ) for this study was 0.1 μg/mL, which was determined from the lowest point on a linear calibration curve that met our acceptance criteria and using guidelines published by the United States Pharmacopeia (United States Pharmacopeia, 2014). Average accuracy (%) for the R(-) and S(+) enantiomers in plasma were 95.8 +/- 3.2 and 95.1 +/- 4.6, respectively. Average precision (RSD, %) for the R (-) and S(+) enantiomers were 7.7 +/- 2.7 and 8.1 +/- 2.9, respectively.

**Pharmacokinetic Analysis of Carprofen in a Clinical Population of Dogs**

Non-linear mixed effects modeling of carprofen enantiomer concentrations was performed with a commercially available software program (Phoenix® NLME Ver. 1.3, Certara, St. Louis, MO). Different compartmental models were tested to determine the best fit base model for each enantiomer. The models were parameterized by clearance, and run with first order conditional estimation with the extended least squares algorithm. Model selection was based on goodness of fit plots, statistical significance between models using AIC values obtained in the Phoenix software, and coefficient of variation (CV%) of parameter estimates. Secondary parameter estimates were obtained using standard compartmental equations (Gabrielsson & Weiner, 2006). Inter-individual variability (variance of a parameter among different subjects, or random effects) was expressed using an exponential error model according to the equation:

$$P_i = \theta \ P_{\text{pop}} \times \exp^{(\eta_i P)}, \quad \text{(Equation 1)}$$
where $P_i$ is the parameter of interest for the individual $i$, $\theta P_{\text{pop}}$ is the population estimate for the parameter of interest, and $\eta_iP$ is the $\eta$ for the individual and parameter of interest. The $\eta$ values were assumed to be independent and have a normal distribution with a mean of zero and variance of $\omega^2$. Assessment of the variance-covariance matrix of the random effects determined that a full matrix structure was better than a diagonal matrix.

An additive model was used to describe the residual random variability ($\varepsilon$) of the data for both enantiomers, where $\varepsilon$ is the residual intrasubject variability with a mean of zero and a variance of $\sigma^2$, according to the equation:

$$C_{\text{obs}} = C_{\text{pred}} + \varepsilon,$$  \hspace{1cm} (Equation 2)

where $C_{\text{obs}}$ is the observed concentration for the individual and $C_{\text{pred}}$ is the model predicted concentration plus the error value ($\varepsilon$).

An examination of covariates was performed to determine if there are factors that may explain the variability in the $V/F$, $Cl/F$, and/or $Ka$. Covariates investigated included weight, age, breed (categorized as large versus small breed due to low overall numbers of each breed), body condition score (1-9 scale), dosing interval (2.2 mg/kg every 12 hours versus 4.4 mg/kg every 24 hours), presence of increased serum alanine aminotransferase (ALT), presence of increased serum blood urea nitrogen (BUN) and/or creatinine (CREA), presence of osteoarthritis, or concurrent tramadol or supplement administration. In this study, dogs weighing less than 12.5 kg and having a body condition score $>4$ were categorized as small breed, and dogs weighing 12.5 kg or greater, and with a normal BCS (i.e., BCS $< 6$), were categorized as a large breed. This criteria was established in the event that an obese small breed dog would not be categorized as large breed based on body weight alone. The continuous variables weight and age were centered on the mean of the population. Covariates were tested
on parameters in a stepwise approach with forward inclusion and backward elimination. The effects of the covariate on a parameter was evaluated based on changes in the -2LL (equivalent to the objective function value (OFV) in NONMEM) and results were considered statistically significant if there was a decrease in 3.84 units ($p<0.05$), as well as improvement of fit based on visual inspection of diagnostic plots. A backward elimination step was used to assess the significance of the covariate, and an increase in the -2LL of 6.63 units ($p<0.01$) was considered significant and the covariate would remain in the final model.

The predictive accuracy of the final model was tested using the bootstrap method on 500 datasets. The bootstrapped sample parameter estimates were assessed for minimal or improved change in variation of estimates to be considered valid.

*Pharmacokinetic Analysis of Carprofen in Healthy Research Dogs Compared to Clinical Dogs*

A second population model was created by combining the data obtained in the present study with data obtained from two previous studies (Messenger *et al.*, 2015; Messenger & Papich, 2013). The rationale behind the development of this model was to assess possible differences in the pharmacokinetics of healthy research dogs (Beagles and mongrel Hounds), compared to each other, as well as compared to the general population of clinical dogs used in the model described above. The second population model was developed due to differences noted in Cl/F in the client-owned population of dogs during the preliminary model assessment as compared with Cl values previously reported in the veterinary literature (Schmitt & Guentert, 1990; Lascelles *et al.*, 1998; Lipscomb *et al.*, 2002; Clark *et al.*, 2003). The same methodology was utilized as described above for model building, covariate analysis, and final model evaluation with this dataset.
RESULTS

A total of 216 concentration-time samples from 73 dogs were included in the population pharmacokinetic modeling for each enantiomer of carprofen. Post-carprofen plasma concentrations ranged from 0.6 to 34.2 µg/mL for the R(-) enantiomer, and 0.5 to 29.4 µg/mL for the S(+) enantiomer, and are shown in Figures 4.1 and 4.2. Dogs’ weights ranged from 5.4 to 69.2 kg, with a median value of 26 kg. Ages ranged from 0.42 to 14 years, with a median of 7 years of age (Table 4.2). The majority of dogs were categorized as large breed (Tables 4.3 and 4.4). None of the 73 dogs included in this analysis were reported to experience a significant adverse event during the study period, although an increased alanine aminotransferase (ALT) above the reference range (reference range: 12-54 IU/L) was detected in 21/73 dogs, with the highest being from a yellow Labrador retriever (ALT 187 IU/L). An increased gamma-glutamyl transferase (GGT; reference range: 0 – 6 IU/L) was noted in 1/73 dogs, and increased alkaline phosphatase (ALKP; reference range: 16 – 140 IU/L) in 8/73 dogs. An increased blood urea nitrogen (BUN; reference range: 6 -26 mg/dL) or creatinine (CREA; reference range: 0.7-1.5 mg/dL) was detected in 4/73 dogs, all three of which were 12 - 14 years of age. Table 4.4 contains the categorical covariate summary data for the population of dogs studied.

A one-compartment model with first order absorption and elimination with an absorption lag time (T lag) was chosen to model the plasma concentration data for both enantiomers of carprofen. The lag time accounts for tablet dissolution and stomach emptying. Including osteoarthritis as a categorical covariate improved the model estimate of Cl/F for the S(-) enantiomer of carprofen. The following equation describes the covariate effect of breed on Cl/F:
\[ \frac{\text{Cl}}{\text{F}}_i = \theta \frac{\text{Cl}}{\text{F}} \times \exp^{\text{OA}} \times \exp^{(\eta_i \frac{\text{Cl}}{\text{F}})} \], \quad \text{(Equation 3)}

where \( \frac{\text{Cl}}{\text{F}} \) is the clearance estimation for the individual, \( \theta \frac{\text{Cl}}{\text{F}} \) is the population estimate for clearance, \( \text{OA} \) is value for the effect of OA, and \( \eta_i \frac{\text{Cl}}{\text{F}} \) is the \( \eta \) of the individual for the clearance. The presence of OA resulted in an approximate 27% reduction in \( \frac{\text{Cl}}{\text{F}} \).

No other covariates were found to significantly improve model predictions for any other parameter for either enantiomer of carprofen. Population parameter estimates and effects are reported in Table 4.5. The goodness of fit plots for the population predicted versus observed concentrations and individual predicted versus observed concentrations are shown in Figures 4.2 and 4.3. Visual predictive plots showing the 5th and 95th confidence intervals of predicted quantiles based on 40 simulations are shown in Figure 4.6.

Absorption rate estimates (\( \text{Ka} \)) and lag times were highly variable. The overall residual error values were moderate in all of the models, and are reported in Table 4.5.

The effects of healthy Beagle and mongrel Hound dogs were assessed by combining data sets and creating a new model. In this model, Beagle breed was a significant covariate effect for apparent clearance, with Beagles having an overall clearance of almost double the value compared to the rest of the population (Table 4.6). Osteoarthritis continued to be a significant covariate effect for apparent Cl, resulting in an estimate of 11.8 mL/kg/hr.

DISCUSSION

Carprofen has been available since the 1990s and is the most common oral NSAID prescribed to treat osteoarthritis in dogs in the U.S. Despite this wide-spread use, this is the first study to describe the population pharmacokinetics of orally administered carprofen in a clinical population of mixed breeds and ages of dogs. The previous pharmacokinetic parameter estimates for carprofen were generated from healthy laboratory Beagle dogs or from very small
numbers of clinical patients. In these studies, carprofen was typically administered at lower than label-recommended doses (Mckellar et al., 1990; Mckellar et al., 1994; Priymenko et al., 1998; Lipscomb et al., 2002; Clark et al., 2003). With the exception of Cl/F for the S(+) enantiomer, the pharmacokinetic parameters estimated by the population models in this study were similar to those previous reports (Table 4.2).

The present study identified a lower value obtained for Cl/F for the S(+) enantiomer than has been previously reported following oral administration of carprofen (Lipscomb et al., 2002; Clark et al., 2003 Lipscomb et al, 2002; Clark et al 2003). Clearance, when corrected for bioavailability, following extravascular administration for S(+) carprofen has been consistently reported to range between approximately 17 mL/kg/hr and 40 mL/kg/hr with some outliers (Schmitt & Guentert, 1990; Lascelles et al., 1998; Lipscomb et al., 2002; Clark et al., 2003). We have reported Cl/F in this population of dogs as approximately between 10 to 15 mL/kg/hr for the S(+), and 12 mL/kg/hr for the R(-) enantiomers, respectively. The parameter of clearance from an oral study is actually CL/F, (per fraction absorbed); therefore true clearance or fraction absorbed (F) is not known. A lower CL/F can either be attributed to lower systemic clearance or higher fraction absorbed. Without additional studies, we cannot determine which of these parameters account for the differences observed, but it should be noted that carprofen, like other NSAIDs, is a BCS Class 2 drug (Papich & Martinez, 2015). As a weak acid, it exhibits consistent, almost 100%, oral absorption. It is unlikely that changes in F could have explained our observed differences in CL/F in this population.

It is well known that Beagles have polymorphisms in hepatic cytochrome-P450 enzymes that contribute to rapid metabolism of some NSAIDs, although this has not been documented specifically for carprofen (Paulson et al., 1999; Fleischer et al., 2008; Jeunesse et
In the second population analysis performed in this study using data obtained from healthy Beagle dogs and Mongrel hound dogs, the Beagle dogs had a significantly higher apparent clearance (approximately 27-33 mL/kg/hr) than the population estimate of 15.75 mL/kg/hr for this combined population (Table 4.6). These findings suggest there could be metabolic differences in the metabolism of carprofen enantiomers between Beagles and other dog breeds.

Two other NSAIDs have undergone population pharmacokinetic analyses in dogs: mavacoxib and robenacoxib (Cox et al., 2010; Silber et al., 2010). Similar to our results, these studies found differences in pharmacokinetic parameter estimates between healthy research Beagle dogs and other canine populations; for example osteoarthritic dogs had a significantly lower value for Cl/F with both of these NSAIDs compared to healthy Beagles (Silber et al., 2010; Cox et al., 2010; Cox et al., 2011; Lees et al., 2015). The authors suggested that this difference could be attributed to chronic inflammation associated with osteoarthritis because inflammatory cytokines may inhibit metabolizing enzymes in the liver (Cox et al., 2011; Fink et al., 2013). In our study, dogs with OA had approximately 27% lower clearance of the S(+) carprofen than dogs without OA.

The dogs included in our study were prescribed carprofen for either acute post-operative pain (such as following orthopedic surgery) or chronically as needed for OA. These conditions are associated with inflammation that might interfere with carprofen clearance, but it was not possible to quantitate the level of inflammation present in these dogs. Lascelles et al. (1998) reported an approximately 35% decrease in Cl/F when carprofen was administered subcutaneously post-operatively compared to pre-operatively for ovariohysterectomy surgery. The clearance values in that study had a large range of 9.6 to 61 mL/kg/hr, which was
speculated at that time to have been caused by anesthetic effects on hepatic blood flow and anesthetic drug effects on metabolism rather than inflammation.

Breed, categorized as large or small, was also assessed as a covariate in the present study. Small breed dogs were estimated to have a higher Cl/F than their large breed counterparts. One could hypothesize that smaller dogs would have a higher clearance than larger dogs because of allometric scaling based on body weight. However, drugs with a high hepatic extraction ratio (those drugs that are flow-limited) are typically the drugs that demonstrate allometric differences (Lave et al., 1999; Espie et al., 2009). Carprofen is a low extraction drug, and its metabolism is more dependent on an animal’s metabolic enzyme capacity (Riviere, 2011). In our study, Cl/F estimates in small breed dogs was between 13 and 17 mL/h/kg, which is closer to clearance estimates from previous studies in healthy research (Beagle) dogs (Schmitt & Guentert, 1990; McKellar et al., 1990; McKellar et al., 1994; Priymenko et al., 1998; Clark et al., 2003). While there was some improvement in the model for the S(+) enantiomer when breed was included as a covariate, the improvement was not statistically significant according to our criteria and therefore was not included in the final model.

Our study population consisted of a population of dogs that received both once-daily and twice-daily doses of carprofen, and assumed to be at steady-state based on the multiple dosing regimen required for entry into the study. At the FDA-approved dose, carprofen may be administered at either 2.2 mg/kg twice daily or 4.4 mg/kg once daily. As expected, we observed that when a higher, once-daily dose was administered to dogs, it produced a higher Cmax and AUC for both enantiomers. But these concentrations were only slightly greater than values in the dogs receiving half the dose twice daily when the dosing interval was taken into
account (Figure 4.1). When concentrations were normalized for dose, the concentrations were similar to the ranges reported in previous studies. These findings suggest that there is likely no difference, in terms of overall daily exposure to carprofen, when dosing twice daily versus once daily. Clinicians at the authors’ institution prefer to prescribe the lower dose of carprofen twice daily because of a false assumption that it is associated with fewer adverse effects. Our study does not support the contention that adverse effects are correlated to plasma concentrations, or that fractionating the once-daily dose to a twice-daily dose will decrease the risk of adverse reactions. Most of the dogs, regardless of dose, achieved a plasma concentration presumed to be therapeutically effective. Lees, et al, (2004) recommended that to achieve clinically relevant effects, 80% inhibition of the COX-2 enzyme is needed. For the S(+) enantiomer of carprofen, based on extrapolation from in vitro studies, this concentration would be approximately 2-3 µg/mL (Ricketts et al., 1998; Brideau et al., 2001). The majority of dogs in this study exhibited plasma concentrations of the S(+) enantiomer well within this range for the 12-24-hour dosing intervals studied. Most of the plasma concentrations, regardless of which dose regimen, ranged between 1-15 µg/mL.

There were two dogs with plasma concentrations of carprofen that were much higher than the rest of the population – one was a yellow Labrador retriever, and another a Greyhound. Despite the high plasma drug concentrations, these dogs had no overt evidence of adverse drug effects. However, the Labrador had an elevated ALT (187 IU/L, reference range 12-54 IU/L) that returned to normal range after carprofen was discontinued. The other liver enzyme activity values (bilirubin, GGT, and ALKP) in this dog were within the normal reference range for our laboratory at the time of carprofen administration. Target animal safety studies performed by the drug sponsor found doses up to 44 mg/kg/day, given orally for 14 days,
resulted in no deaths and minimal adverse effects in healthy Beagles (Rimadyl® package insert, Zoetis, New York, NY); additionally other pharmacokinetic studies in healthy Beagles have reported much higher plasma concentrations than those in the present study without adverse effects (Schmitt & Guentert, 1990). Thus, adverse reactions to carprofen are believed to be idiosyncratic (not dose-dependent or linked to high drug concentrations) (Montiero-Steagall et al., 2013).

We did not find any association between elevations in serum biochemical analyses— in particular the activities of ALT, ALKP, GGT, bilirubin, BUN, creatinine—and the pharmacokinetic parameter estimates for carprofen enantiomers in dogs in this study. While approximately 30% of the dogs in this study had either serum kidney or liver enzyme activity above the normal reference range for our clinical pathology laboratory, this did not account for significant variability of Cl/F or V/F in our model. Carprofen is predominately metabolized by the liver, with minimal excretion by the kidneys (Rubio et al., 1980). Therefore it would be unlikely that minor changes in renal values would alter carprofen clearance. Despite the possibility of a covariate effect for elevated liver enzyme activity, a small study found no effect of liver disease on the pharmacokinetics of carprofen in people (Holazo et al., 1985). The cause of elevated serum hepatic and kidney enzyme activities in the dogs in the present study was undetermined.

Our covariate assessments were unable to identify an underlying association to account for the variability in absorption rates. Carprofen was rapidly absorbed in most dogs in the study, however the absorption rate constants (Ka), were difficult to estimate in these analyses, as evidenced in the large coefficient of variation for these estimates (Table 4.5). A wide range of covariates were tested to explain these findings, but none were found to be significant.
predictors of the pharmacokinetic parameter estimate for absorption. Variable absorption in some dogs may have contributed to the variability in this parameter, as well as our sampling time points during the absorption phase. We elected approximately 3 sampling time points during the absorption phase (0.5, 1, and 2 hours post administration), which may not have been adequate to fully characterize this phase and accurately predict the Ka. In addition, the product formulation (i.e. chewable versus caplet) and the effects of feeding were not assessed in this study. Feeding schedules do influence the pharmacokinetics of some orally administered compounds and might have contributed to the variable absorption rate that was observed (Martinez & Papich, 2009). Despite the variability in absorption rate, this parameter estimate does not affect the overall exposure (AUC) or other parameters estimated in the model and is therefore less clinically relevant in this study.

We acknowledge that this study has some limitations. For example, the study design may have been biased by case selection for dogs that tolerate carprofen, in other words, dogs who had a previous history of problems with carprofen or other NSAIDs were unlikely to be enrolled in this study. Many clinicians would withdraw NSAID administration at the first indication of evidence of changes in either renal function or liver enzyme activities, or the development of vomiting and diarrhea. Another limitation was the sampling design, in particular for capturing the absorption phase. In future studies of similar design it would be useful to capture more intensive plasma concentration data per dog.

In conclusion, the most significant findings of this study were that dogs with OA exhibited a slower apparent plasma clearance for the more pharmacologically active S(+) enantiomer of carprofen compared to dogs without OA. Whether or not the greater exposure to carprofen produced in these dogs has increased clinical benefit is undetermined. Plasma
clearance of both enantiomers of carprofen in this study is lower than previous reports in healthy (mostly Beagle) dogs, the cause of which remains unknown. In our model, Beagle dogs exhibited a higher plasma clearance that appeared to be unrelated to body weight or size, suggesting this breed may metabolize carprofen more rapidly than other breeds. Also, there appears to be no benefit, in terms of exposure for either enantiomer, to twice daily dosing over once daily dosing of carprofen. As none of the dogs in this study experienced significant adverse events, which some would speculate to be related to increased drug exposure, our findings support the previously held hypothesis that significant adverse drug reactions to carprofen are likely idiosyncratic. No dose adjustment to clinical dosing regimens based on these results is recommended at this time. There is wide variation in the canine population regarding overall carprofen concentrations and pharmacokinetic parameter estimates.
REFERENCES


Table 4.1: Schedule table for blood sampling following carprofen administration.

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<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>10</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2: Summary statistics of continuous covariates for the population studied (n=73 dogs).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean (SD)</th>
<th>Median (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>6.6 (4.0)</td>
<td>7.0 (0.4-14.0)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>26.2 (12.3)</td>
<td>25.6 (5.4-69.2)</td>
</tr>
<tr>
<td>Carprofen dose (mg/kg)</td>
<td>2.6 (0.8)</td>
<td>2.3 (1.2-4.7)</td>
</tr>
<tr>
<td>Body condition score (1-9)</td>
<td>5.4 (1.0)</td>
<td>5 (3-8)</td>
</tr>
</tbody>
</table>
Table 4.3: Summary of breeds included in the clinical population analysis (n=73 dogs).

<table>
<thead>
<tr>
<th>Breed</th>
<th>N</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed Breed</td>
<td>14</td>
<td>19.18</td>
</tr>
<tr>
<td>Staffordshire Terrier</td>
<td>10</td>
<td>13.70</td>
</tr>
<tr>
<td>German Shepherd</td>
<td>6</td>
<td>8.22</td>
</tr>
<tr>
<td>Labrador Retriever</td>
<td>5</td>
<td>6.85</td>
</tr>
<tr>
<td>Golden Retriever</td>
<td>4</td>
<td>5.48</td>
</tr>
<tr>
<td>Australian Shepherd</td>
<td>3</td>
<td>4.11</td>
</tr>
<tr>
<td>Bassett Hound</td>
<td>3</td>
<td>4.11</td>
</tr>
<tr>
<td>Greyhound</td>
<td>3</td>
<td>4.11</td>
</tr>
<tr>
<td>Boston Terrier</td>
<td>2</td>
<td>2.74</td>
</tr>
<tr>
<td>English Pointer</td>
<td>2</td>
<td>2.74</td>
</tr>
<tr>
<td>Rottweiler</td>
<td>2</td>
<td>2.74</td>
</tr>
<tr>
<td>Jack Russell Terrier</td>
<td>2</td>
<td>2.74</td>
</tr>
<tr>
<td>Beagle</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Bernese Mountain Dog</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Boxer</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Brittany Spaniel</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Catahoula Leopard Dog</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Dachshund</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Doberman</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>English Bulldog</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>French Bulldog</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Great Dane</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Mastiff</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Newfoundland</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Miniature Poodle</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Pug</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Rat Terrier</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Vizsla</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Yorkshire Terrier</td>
<td>1</td>
<td>1.37</td>
</tr>
</tbody>
</table>
Table 4.4: Summary statistics of categorical covariates for the population studied (n=73 dogs).

<table>
<thead>
<tr>
<th>Categorical Data</th>
<th>N</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, neutered or intact</td>
<td>43</td>
<td>58.9</td>
</tr>
<tr>
<td>Female, spayed or intact</td>
<td>30</td>
<td>41.1</td>
</tr>
<tr>
<td>Small Breed</td>
<td>12</td>
<td>16.4</td>
</tr>
<tr>
<td>Large Breed</td>
<td>61</td>
<td>83.6</td>
</tr>
<tr>
<td>OA Present</td>
<td>33</td>
<td>45.2</td>
</tr>
<tr>
<td>Dose received every 12 hours</td>
<td>53</td>
<td>72.6</td>
</tr>
<tr>
<td>Dose received every 24 hours</td>
<td>20</td>
<td>27.4</td>
</tr>
<tr>
<td>Concurrent supplement received</td>
<td>33</td>
<td>45.2</td>
</tr>
<tr>
<td>Concurrent tramadol received</td>
<td>19</td>
<td>26.0</td>
</tr>
<tr>
<td>Serum Creatinine or BUN outside of reference range</td>
<td>4</td>
<td>5.8</td>
</tr>
<tr>
<td>Serum ALT outside of reference range</td>
<td>21</td>
<td>28.8</td>
</tr>
<tr>
<td>Serum GGT outside of reference range</td>
<td>1</td>
<td>1.4</td>
</tr>
<tr>
<td>Serum ALKP outside of reference range</td>
<td>8</td>
<td>11.0</td>
</tr>
</tbody>
</table>
Table 4.5: Population pharmacokinetic parameter estimates for carprofen enantiomers in canine plasma (mean, CV%) from 73 dogs. Legend: Ka, absorption rate constant; V/F, apparent volume of distribution, Cl/F, total body clearance; Ke, elimination rate constant; Tmax, time to maximum concentration; AUC, area under the concentration versus time curve; Cmax, maximum concentration; Ka T1/2, absorption half-life; Ke T1/2, elimination half-life; N/A, not applicable; N/E, not estimated. *When OA is present, the value -0.32 is entered into Equation 3. # This value corresponds to a 27% decrease in Cl/F when a dog has OA.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>R(-) enantiomer</th>
<th>Estimate</th>
<th>CV%</th>
<th>S(+) enantiomer</th>
<th>Estimate</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ka</td>
<td>1/hr</td>
<td>1.09</td>
<td>201.00</td>
<td>0.95</td>
<td>0.95</td>
<td>146.91</td>
<td>146.91</td>
</tr>
<tr>
<td>V/F</td>
<td>mL/kg</td>
<td>131.21</td>
<td>43.43</td>
<td>130.92</td>
<td>25.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl/F</td>
<td>mL/kg/hr</td>
<td>11.96</td>
<td>46.61</td>
<td>15.10</td>
<td>45.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tlag</td>
<td>hr</td>
<td>0.40</td>
<td>N/E</td>
<td>0.37</td>
<td>58.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effect of OA on Cl/F*</td>
<td>N/A</td>
<td>N/A</td>
<td>-0.32 #</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual error</td>
<td>µg/mL</td>
<td>2.14</td>
<td>N/A</td>
<td>1.78</td>
<td>1.78</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ke</td>
<td>1/hr</td>
<td>0.09</td>
<td>N/A</td>
<td>0.11</td>
<td>0.11</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Tmax</td>
<td>hr</td>
<td>2.48</td>
<td>N/A</td>
<td>2.21</td>
<td>2.21</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>AUC</td>
<td>µg/mL *hr</td>
<td>90.08</td>
<td>N/A</td>
<td>71.05</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cmax</td>
<td>µg/mL</td>
<td>6.55</td>
<td>N/A</td>
<td>6.21</td>
<td>6.21</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ka T1/2</td>
<td>hr</td>
<td>0.63</td>
<td>N/A</td>
<td>0.60</td>
<td>0.60</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ke T1/2</td>
<td>hr</td>
<td>7.60</td>
<td>N/A</td>
<td>6.19</td>
<td>6.19</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 4.6: Carprofen S(+) enantiomer estimates following doses ranging from 2.2 to 4.4 mg/kg obtained in a model including healthy mongrel Hound and Beagle dogs in the data set (n=85 dogs).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Estimate</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ka</td>
<td>1/hr</td>
<td>1.57</td>
<td>575.58</td>
</tr>
<tr>
<td>V/F</td>
<td>mL/kg</td>
<td>138.00</td>
<td>29.85</td>
</tr>
<tr>
<td>Cl/F</td>
<td>mL/kg/hr</td>
<td>15.75</td>
<td>43.20</td>
</tr>
<tr>
<td>Tlag</td>
<td>hr</td>
<td>0.10*</td>
<td>325.40</td>
</tr>
<tr>
<td>Effect of Beagle Breed on Cl/F</td>
<td></td>
<td>0.53</td>
<td>N/A</td>
</tr>
<tr>
<td>Effect of OA on Cl/F</td>
<td></td>
<td>-0.25</td>
<td>N/A</td>
</tr>
<tr>
<td>Residual error</td>
<td>μg/mL</td>
<td>2.90</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 4.1: Plasma concentration versus time curves for carprofen enantiomers following once and twice daily oral administration in 73 dogs. The blue and red lines represent the population predicted fit for the R(-) and S(+) enantiomer, respectively.
a. R(-) enantiomer

b. S(+) enantiomer plot when OA is included as a covariate effect for Cl/F

c. S(+) enantiomer plot when OA is not included as a covariate effect for Cl/F

Figures 4.2.a, b, c: Population predicted concentrations versus individual concentrations for R(-) and S(+) carprofen enantiomers following carprofen administration in 73 dogs.
Figure 4.3. a, b: Individual predicted concentrations versus observed concentrations following carprofen administration in 73 dogs.
Figure 4.4. a, b, c: Scatter plots of individual data (red circles) versus population predictions (blue lines) for carprofen enantiomers in 73 dogs.

a. R(-) enantiomer

b. S(+) enantiomer when OA is included as a covariate effect for Cl/F.

c. S(+) enantiomer plot when OA is not included as a covariate effect for Cl/F.
Figure 4.5.a, b: Scatter plot of individual data (red circles) versus population predictions (blue lines) for S(+) carprofen enantiomers in 73 dogs, separated by dogs with OA and dogs without OA.
Figure 4.6.a, b: Visual predictive checks for the final models for R(-) and S(+) carprofen enantiomers based on 40 replicates, showing the observed (red) versus predicted quantiles (5th and 95th percent), and 5th and 95th percent confidence intervals around the predicted quantiles.

a. R(-) enantiomer

b. S(+) enantiomer
ABSTRACT

Measurement of unbound drug concentrations at their sites of action is necessary for accurate PK/PD modeling. The objective of this study was to determine the unbound concentration of carprofen in canine interstitial fluid (ISF) using in vivo ultrafiltration, and to compare pharmacokinetic parameters of free carprofen concentrations between inflamed and control tissue sites. We hypothesized that active concentrations of carprofen would exhibit different dispositions in ISF between inflamed versus normal tissues. Bilateral ultrafiltration probes were placed subcutaneously in 6 healthy Beagle dogs 12 hours prior to induction of inflammation. Two milliliters of either 2% carrageenan or saline control were injected subcutaneously at each probe site, 12 hours prior to intravenous carprofen (4 mg/kg) administration. Plasma and ISF samples were obtained at regular intervals for 72 hours, and carprofen concentrations were determined using HPLC. Prostaglandin E2 (PGE2) concentrations were quantified in ISF using ELISA. Unbound carprofen concentrations were higher in ISF compared to predicted unbound plasma drug concentrations. Concentrations were not significantly higher in inflamed ISF compared with control ISF. Compartmental
modeling was used to generate pharmacokinetic parameter estimates, which were not significantly different between sites. Terminal half-life (T½) was longer in the ISF compared with plasma. PGE$_2$ in ISF decreased following administration of carprofen. In vivo ultrafiltration is a reliable method to determine unbound carprofen in ISF, and that disposition of unbound drug into tissue is much higher than predicted from unbound drug concentration in plasma. However, concentrations and pharmacokinetic parameter estimates are not significantly different in inflamed versus un-inflamed tissues.
INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used class of analgesic drugs in both human and veterinary medicine, and are important in both the management of chronic pain as well as acute soft tissue injury and pain (Brune & Furst, 2007). Release of local inflammatory mediators following soft tissue injury contributes to the hallmark signs of inflammation: heat, pain, redness, swelling, and loss of function. Nonsteroidal anti-inflammatory drugs partially exert their analgesic effects via local inhibition of cyclooxygenase enzymes 1 and 2, which ultimately leads to decreased release of pro-inflammatory mediators (Vane, 1971; Brune & Furst, 2007). The ability to study NSAID distribution and anti-inflammatory effects directly at sites of action can improve not only our understanding of drug effects, but also to apply appropriate dosage regimens (Lees et al., 2004; Pelligand et al., 2012). This knowledge is critical in regards to NSAIDs, as plasma drug concentrations have not been correlated with therapeutic efficacy (Brune & Furst, 2007).

Carprofen, one of the most commonly administered NSAIDs in dogs, has been previously studied using models of inflammation such as carrageenan-injected tissues cages (McKellar et al., 1994; Lees et al., 1996; Brentnall et al., 2013). Tissue cages are surgically implanted artificial compartments that can be injected with inflammatory agents including carrageenan or lipopolysaccharide (Higgins et al., 1984; McKellar et al., 1994, Pelligand et al., 2012). Highly cellular fluid from these cages can be collected over time to study the distribution and effects of NSAIDs (Kirchner et al., 1997; Pelligand et al., 2014). While tissue cage models to study the PK-PD of NSAIDs have improved our knowledge of these drugs, they are not ideal. For example, surgery is required to both implant and remove the tissue cages. More importantly, sampling of fluids involves the collection of both protein-bound and
unbound drug concentrations, which is problematic as only the unbound drug is available to be pharmacologically active (Wright et al., 1996; Gonzalez et al., 2013). The investigators involved with the studies cited above acknowledge that the tissue cage may not provide a true depiction of the pharmacokinetics of NSAIDs in tissue sites because the tissue cage is an artificial environment that may affect drug distribution (Pelligand et al., 2014). One of the objectives of this study was to examine NSAID distribution in a more natural tissue environment.

Carprofen, like many NSAIDs, is highly protein bound at greater than 99% (Rimadyl®, Zoetis, New York, NY, USA). Previous studies have assessed total (bound + unbound) concentrations of carprofen from inflamed tissues using tissue cage models as described above (McKellar et al., 1994; Brentnall et al., 2013). A better understanding of the actions and distribution of active (protein-unbound) concentrations of carprofen in tissue sites could be gained by using a technique that samples protein-unbound drug. In vivo ultrafiltration (UF) is a minimally invasive method to determine unbound drug concentrations in the ISF, and produces similar results as in vivo microdialysis. The probe fibers are made of a semi-permeable membrane with a molecular weight cut-off of 30,000 Da. A vacutainer attached to the assembly passively aspirates fluid over time and allows for the collection of multiple samples without causing distress to the animal. The authors have previous experience using in vivo UF to collect antimicrobial drugs from tissue sites (Bidgood & Papich, 2002; Papich et al., 2010; Messenger et al., 2012), but to our knowledge this technique has never been applied to the collection of NSAIDs directly from ISF. The objective of this study was to utilize in vivo ultrafiltration to collect PGE₂ and protein-unbound carprofen directly from inflamed and healthy tissue sites in order to compare the distribution and residence of carprofen
concentrations in tissue sites with those of plasma, and to examine the anti-prostaglandin effects of carprofen directly at the tissue level. Our hypothesis was that unbound carprofen exposure in the inflamed tissue fluid would be higher than in plasma, and also higher than in the corresponding control tissue fluid.

MATERIALS AND METHODS

Animals

Six adult Beagle dogs weighing between 7.1 and 15.8 kg (mean 9.4 kg) and 1.6 to 6.8 (average 3.3) years of age were used in this study. Results of physical examination, complete blood counts, and serum chemistry panels were used to determine that the dogs were healthy prior to enrollment in the study. Dogs were housed at the North Carolina State University Laboratory Animal Resources facility and fed a maintenance diet. The study was reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University.

Study Design

Prospective research study.

Drug Administration and Blood Collection

Approximately 24 hours prior to drug administration, dogs were sedated using 2 mg/m² oral transmucosal detomidine gel (Detomidine Gel, Orion Pharmaceuticals, Finland) for intravenous catheter (Arrow, Becton Dickinson and Company, Franklin Lakes, NJ) placement into a jugular vein. Catheters were secured in place with suture and a light bandage. Catheters were flushed with sterile 0.9% saline to maintain patency. Blood samples were collected before (time 0) and 0.08, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 16, 24, 32, 40, 48, 60, and 72 hours after administration of carprofen. Upon collection, samples were transferred into
tubes containing lithium heparin as an anticoagulant and were immediately placed in ice. All samples were centrifuged at 1509 x g for 10 minutes within 60 minutes of collection. The plasma from centrifuged samples was separated and stored at -80°C until analysis within 30 days by HPLC.

**ISF fluid collection and inflammatory model**

Using sedation as described above for jugular catheter placement and one milliliter of 2% lidocaine for local anesthesia, bilateral UF probes (BASi systems, W. LaFayette, IN) were placed subcutaneously, on each side of the thorax and secured using nylon suture. Vacutainer tubes were attached to the external tubing of the probes to collect the fluid at the following time points: -12 h (12 hours after probe insertion), 0 (pretreatment and 12 hours after carrageenan injection), 2, 4, 6, 8, 12, 24, 32, 40, 48, 60 and 72 h after IV administration of carprofen. A time of at least 2 hours is recommended for recovery of microscopically normal blood flow to tissues after probe insertion (Stenken et al., 2010). The exact volume and weight of the fluid collected were recorded and averaged to calculate a lag-time for these samples. The fluid collected was immediately frozen at −80 °C until analysis by HPLC. Each dog received one injection of 2 mL of 2% sterile carrageenan (Viscarin; FMC biopolymers, Philadelphia, PA) subcutaneously at the site of probe placement, and 2 mL sterile saline as a control on the opposite side of the thorax 12 hours after UF probe placement; both injections were made via a pre-placed catheter in the same tissue plane as the UF probe. A pilot study indicated that this was the appropriate volume and concentration of carrageenan to induce mild but noticeable localized inflammation. The side (left or right) chosen for carrageenan injection was randomly assigned via coin toss. Animals were scored for pain following carrageenan administration at the time points utilized for ISF collection using a short form of the Glasgow
composite pain scale (Reid et al., 2007). Provision for rescue analgesia was made if pain scores were equal to or greater than 18 (out of 24 possible points), and consisted of buprenorphine at 0.03 mg/kg IV or IM.

**HPLC Drug Analysis**

Plasma samples were analyzed via high-performance liquid chromatography (HPLC) to determine the concentrations of carprofen in plasma and ISF using a method modified from previous studies (Priymenko et al., 1998). The HPLC system consisted of a quaternary solvent delivery system, an autosampler, and a fluorescence detector set at an emission wavelength of 310 nm and a detection wavelength of 375 nm (Agilent 1100 Series, Agilent Technologies, Wilmington, DE). Chromatograms were integrated with a computer program (Agilent Chemstation 2D software, Agilent Technologies, Wilmington, DE). A Zorbax Eclipse C18 column (Agilent Technologies, Wilmington, DE) was used for separation and was maintained at a constant temperature of 40°C. The mobile phase consisted of 55% 0.02 M potassium phosphate in water, adjusted to a pH of 3.0 with phosphoric acid, and 45% acetonitrile at a flow rate of 1 mL/min.

The analytic reference standard of carprofen, obtained from US Pharmacopeial Convention (USP, Rockville, MD), was used to prepare a stock solution in methanol that was made fresh daily when an analytical run was performed. All drug concentrations were determined from calibration curves made from pooled blank plasma collected from the experimental dogs. Phosphate buffered saline was used to create the ISF calibration curves due to limitations in blank ISF fluid volume. The calibration curve for plasma consisted of 6 standards that ranged between 0.1 μg/mL and 100 μg/mL and included a blank (0 μg/mL) sample, and for ISF included 6 standards ranging from 1 ng/mL to 10,000 ng/mL. The
calibration curves were accepted if the linear coefficient of determination ($r^2$) was $\geq 0.99$ and if the calibration curve concentrations could be back-calculated to within 15% of the true concentration of the standard. The limit of quantification (LOQ) for carprofen in plasma was 0.1 µg/mL, and was 1 ng/mL in ISF, based on a signal to noise ratio of at least 8:1. Average (SD) accuracy (%) and precision (% RSD) for the plasma assay was 9.43 +/- 3.70 and 5.58 +/- 4.15, respectively, and was 0.88 +/- 0.74 and 0.48 +/- 0.46 for the ISF assay. Accuracy and precision were calculated based on averages of 5 replicates of low, medium, and high concentrations of quality controls in blank matrix.

All plasma, ISF, calibration, quality-control, and blank-plasma samples were prepared in an identical manner, and fresh calibration curve standards and quality controls were made for runs made on separate days. One hundred twenty-five µL of sample was mixed with 125 µL pooled blank canine plasma, which was then acidified with 250 µL of 4% phosphoric acid in water (500 µL total volume) were loaded onto pre-conditioned solid phase extraction cartridges (Oasis HLB 1 mL, Waters Corporation, Milford, MA). After washing, samples were eluted with 1 mL methanol into glass tubes. The eluate was evaporated under air in a water bath at 40° C. Dried residue was reconstituted with 200 µL of HPLC mobile phase and vortexed briefly before transfer to the HPLC injection vial. The injection volume for plasma was 5 µL. All ISF samples were injected directly onto the system at a volume of 40 µL. Laboratory procedures were conducted in accordance with published guidelines (USP, 2014).

**Pharmacokinetic Analysis**

Plasma drug concentrations were plotted on linear and semi-logarithmic graphs for analysis and for visual assessment of the best model for pharmacokinetic analysis. Analysis of the curves and pharmacokinetic modeling were then performed by use of a commercial
pharmacokinetic program (Phoenix WinNonlin, Certara, St. Louis, MO). Compartmental analysis of the data was performed using a weighting factor of 1/(predicted Y)^2, where Y is the plasma concentration. Models were determined for best fit on the basis of visual analysis for goodness of fit, visual inspection of residual plots, and assessment of Aikake’s information criterion. Results for the CMAX and TMAX were taken directly from the data.

*Prostaglandin E2 analysis*

Prostaglandin E2 was quantified in ISF using a commercially available ELISA kit following manufacturer’s instructions (PGE2 EIA Kit Monoclonal, Cayman Chemicals, Ann Arbor, MI). Interstitial fluid was diluted using assay buffer in order to obtain concentrations in the linear range of the standard curve, and all samples were quantified in duplicate. The average value of the duplicate samples was used for analysis.

*Statistical analysis*

Data was tested for normality using a Shapiro-Wilk test. Prostaglandin E2 data were log-transformed for statistical analysis. All statistical analysis was performed using a commercially available software program (SigmaPlot Version 12.0, Systat Software, Inc., San Jose, CA). A paired t-test was used to compare unbound carprofen, PGE2 concentrations between ISF sampling sites at each time point, and pharmacokinetic parameter estimates between control and inflamed tissue sites. A repeated measures ANOVA with Bonferroni’s post-hoc analysis was used to compare PGE2 concentrations before and after carprofen administration for inflamed tissue sites, and an ANOVA on ranks with Dunn’s post-hoc analysis was used for the PGE2 concentrations in control tissue fluid as data was not normally distributed despite log transformation. Values of P < 0.05 were considered significant, post-hoc corrections were made to account for multiple comparisons with an adjusted p-value of
0.0038 considered significant. Descriptive statistics for pharmacokinetic parameters were obtained directly from the software program.

RESULTS

All animals completed the study without any adverse effects, and none of the subjects required rescue analgesia during the study period. Interstitial fluid was successfully collected from both control and inflamed tissue sites during the study, and although rare, occasionally there were missed samples from some dogs. Sample collections may have been incomplete because of occlusion of the external tubing secondary to kinking or the vacutainer may have been dislodged from the hub assembly by an active dog.

Pharmacokinetics

Carprofen was detected in ISF within 2 hours after IV injection and reached a maximum concentration (C\text{MAX}) of 11.7 (32.3%) ng/mL (geometric mean (CV\%)) at 7.0 (50.3\%) hours after administration in the inflamed tissue site. The C\text{MAX} in the control tissue site was slightly lower at 10.5 (25\%) ng/mL and occurred at 6.4 (55.9\%) hours after administration. The earliest time point sampled was 2 hours post-carprofen administration, which was necessary in order to collect enough volume of ISF for both HPLC and PGE\textsubscript{2} analyses. The time-concentration profile for the ISF data was fitted to a one compartment model with a lag time (Figure 5.1), while plasma data was fitted to a 2 compartment model (Figure 5.2). Concentration versus time data was available in 4/6 dogs for control tissue concentrations, and in 6/6 dogs for inflamed tissue concentrations of carprofen. Concentrations of carprofen in plasma were above the LOQ up to 60 hours post administration in 2 dogs, and up to the last time point (72 hours) in one dog. For both inflamed and control ISF, unbound carprofen concentrations were detected in 1 out of 6 dogs up to the last time point collected.
(72 hours post-carprofen administration). Concentrations of unbound carprofen in inflamed tissues were significantly greater than control tissue concentrations at approximately 19 and 35 hours post carprofen administration. Pharmacokinetic parameter estimates for plasma, control, and inflamed tissue fluids are reported in Tables 5.1 and 5.2. There was no difference in parameter estimates between the inflamed and control tissue sites.

**PGE2**

Prostaglandin E2 was quantified in both inflamed and control tissue sites and data are shown in Figure 5.3. There was a significant difference in PGE2 concentrations between control and inflamed tissue sites at every time point from 0 (time of carprofen administration and 12 hours after induction of inflammation) to 32 hours post-carprofen administration. Administration of carprofen resulted in a significant decrease in PGE2 concentrations at every time point compared to pre-drug administration in inflamed tissues from 4 hours post-carprofen administration to the end of the study period (72 hours). PGE2 concentrations in the control tissue sites were also significantly reduced by the administration of carprofen compared to baseline measurements from 4 to 24 hours post-drug administration. In one dog, PGE2 concentrations from the control tissue site began to increase at 40 hours post-carprofen administration and continued to rise until the end of the study. We suspect that the rise in PGE2 was caused by self-trauma of the probe site by the dog, which produced additional inflammation. This dog’s data is shown individually on Figure 5.3, as it was an outlier. The 60 hour sample was missing PGE2 data, thus only data from 40, 48, and 72 hours is represented.

**DISCUSSION**

The results of this study suggest that in vivo ultrafiltration is a reliable method to determine the unbound concentrations of carprofen in ISF. A goal of PK-PD modeling is to
be able to collect both unbound, active drug concentrations as well as biomarkers of efficacy, directly from the site of action. This technique refines previously reported techniques used to collect drug concentrations in tissue sites, including tissue cage models, because the disadvantage of those models was that they collected both protein bound and unbound drug molecules. Those methods are also more invasive and require surgery. Our dogs did not require surgery; the probes were placed using only mild sedation and local anesthetic. At the conclusion of our experiments, the probes were removed and the dogs were adopted as pets or returned to the research colony. In addition, other studies using tissue cages create an artificial environment (usually a hollow sphere or tube) that may not be optimal to represent normal drug influx and efflux to natural tissue. A recent publication discussed specific disadvantages directly related to the tissue cages, including different surface area geometry and lack of normal lymphatic drainage, both of which could affect drug movement in this tissue model (Pelligand et al., 2014). On the other hand, when tissue probes of the type used in this study are inserted, normal blood flow to tissues is restored within 2 hours (Stenken et al., 2010).

After IV administration, carprofen was rapidly detected in both the control (normal) and inflamed tissue sites, although concentrations did not reach maximal levels until 6-7 hours after administration. This T\text{MAX} was similar, although slightly less, than the T\text{MAX} previously reported by McKellar et al when carprofen was administered orally to Beagle dogs (McKellar et al., 1994). This study unexpectedly found that concentrations of carprofen in normal (uninflamed) ISF was not significantly different than those collected from inflamed tissue fluid at most of the time points studied. However, there were some limitations in the study to consider when interpreting this finding. For example, the power of this study was low given the variation in the concentration data between dogs. A post-hoc power analysis applied to the
data would have required approximately 25 dogs to be enrolled in the study in order to achieve a desired power of 0.8; therefore, future studies should be designed using a larger number of animals to account for the expected large variability and improve the power of the study.

Despite the limitation in power in the current study, our results are similar to previous work on the tissue penetration of carprofen in dogs. McKellar and colleagues (1994) also found that concentrations of both carprofen enantiomers in the transudate, which would reflect uninflamed tissue, increased over the 24-hour study period; however, in inflamed exudate the concentrations of both enantiomers were highest at 6 and 10 hours post administration. Their data is somewhat limited because samples were obtained from only 2 dogs during a 24 hour time period and the results were highly variable. Visual inspection of Figure 1 shows the similarity in unbound carprofen concentrations from the two sites. AUC ratios of unbound carprofen between inflamed and control tissues sites were approximately 1:1. This finding was contrary to our hypothesis and previously held beliefs that there is greater exposure of some NSAIDs at sites of inflammation compared to healthy tissue (McKellar et al., 1994; Brune & Furst, 2007; Lees et al., 2004). Generally, acidic, highly protein-bound NSAIDs distribute unequally in the body. Carprofen, a propionic acid (pKa 4.4) with high plasma protein binding might be expected to have limited distribution in normal non-inflamed tissues sites, but would preferentially distribute to a higher extent to inflamed sites where protein escapes the capillaries because of diminished microvascular integrity. Greater exposure in inflamed sites has been attributed to either to physicochemical properties of drugs such as NSAIDs that are weak acids, or secondary to increased blood flow, increased vascular permeability and extravasation of albumin and other proteins (McKellar et al., 1994; Lees et al., 2004).
Additionally it has been hypothesized that a more acidic tissue pH could lower the plasma protein binding of NSAIDs, such that there would be more unbound drug at sites of inflammation. Tissue fluid pH was measured in our study (data not reported) and was similar between inflamed and control sites over time in the dogs. We quantified only the unbound carprofen concentrations from tissue sites and found there was no difference in tissue distribution secondary to inflammation and believe that tissue pH was not a factor influencing carprofen distribution in these dogs. Moreover, with the pKa of carprofen being 4.4 it should be insensitive to mild changes in pH above or below the physiological pH range of 7.2-7.4.

The tissue concentrations of carprofen collected via ultrafiltration were present for much longer than what would be predicted based on plasma concentrations (Figure 1), which emphasizes the relevance of sampling directly from the site of action versus plasma sampling as a surrogate marker for biophase drug concentrations. The concentration of unbound carprofen in ISF was higher than that predicted from the unbound drug concentrations in plasma, which is in agreement with the conclusions by other investigators that plasma concentrations do not accurately reflect or predict NSAID concentrations in tissues (McKellar et al., 1994; Brune & Furst, 2007). These findings support the importance of PK-PD study designs that utilize systemic and effect-site drug concentrations, coupled with biological effects for model input (Derendorf & Meibohm, 1999; Lees et al., 2004).

Data was variable among dogs, especially in the PK parameter estimates from inflamed tissue sites, with CV% exceeding 50% for some parameters and AUC exceeding 70%. There was essentially a bimodal distribution in the estimates from inflamed tissue sites. Three of the dogs eliminated carprofen quickly and concentrations in tissue were not detected in these dogs after 32-40 hours, whereas the other 3 dogs had detectable concentrations out to 60-72 hours.
The AUC values were more than doubled those of the others in the latter group of dogs, which contributed to the high AUC CV% reported. These differences are difficult to explain, as plasma PK estimates were not necessarily different between these two subgroups of dogs, however our results are consistent with the large variability in tissue concentrations of carprofen previously reported by McKellar and colleagues (1994).

The plasma pharmacokinetics of IV carprofen estimated in this study were similar to previous reports where total (R and S) carprofen has been quantified (Schmitt & Guentert, 1990; McKellar et al., 1990). However, carprofen is a racemic mixture and the S (+) enantiomer is responsible for the majority of the anti-inflammatory activity (Lees et al., 2004). An enantioselective assay is preferred, but the ISF carprofen concentrations reported here represent the sum of both enantiomers because of the sample size limitation for our derivitization method. A previous report from a study in dogs showed slightly greater penetration of the R (-) enantiomer into both tissue cage exudate and transudate (McKellar et al., 1994). But that study measured total (bound and unbound) drug concentrations, and the sample size was small (n = 2). Studies in other species have reported slightly higher concentrations of the R (-) enantiomer versus the S (+) enantiomer of carprofen at effect sites, such as normal synovial fluid or tissue cages (Lees et al., 1996; Armstrong et al., 1999; Brentnall et al., 2013).

The previously referenced tissue cage study performed in dogs found no significant reduction in PGE2 concentrations in inflammatory exudate following carprofen administration, which is in contrast to our findings (McKellar et al., 1994). Other investigators have reported a decrease in PGE2 concentrations in carprofen-treated cattle compared to placebo-treated cattle, but the results were statistically significant at only some time points (Brentnall et al.,
Both of these studies utilized carrageenan-stimulated tissue cages. In the present study, carprofen administration significantly decreased PGE$_2$ concentrations in inflamed tissue fluids from 4 hours after administration throughout the duration of the study period (72 hours), indicative of a rapid onset and prolonged duration of action directly at tissue sites of injury. The carrageenan model of inflammation is short-acting, lasting approximately 24-36 hours when injected into tissue cages (Kirchner et al., 1997; Pelligand et al., 2012). Therefore, it is possible that PGE$_2$ concentrations would not have been elevated beyond 36 hours in our study, even if carprofen had not been administered. Preliminary studies from our laboratory support the duration of inflammation following subcutaneous carrageenan injection as being approximately 36 hours in Beagle dogs, based on significantly elevated PGE$_2$ in inflamed tissue fluid compared to saline control when no carprofen was administered (K. Messenger, unpublished data). The administration of carprofen also significantly reduced PGE$_2$ concentrations in the control tissue site at time points from 4 to 24 hours, compared to baseline. The insertion of ultrafiltration probes 24 hours prior to the injection of carrageenan induced some degree of inflammation, although minimal compared to carrageenan as seen in Figure 5.3. Studies with microdialysis have documented a temporary local inflammatory response induced by the insertion of the dialysis probes (Stenken et al., 2010). Concentrations of PGE$_2$ in completely normal canine tissue fluid are unknown, but data from a study where no anti-inflammatory drugs were administered to dogs showed that PGE$_2$ concentrations peaked between 6 and 8 hours after probe insertion, with an average value of 5580 pg/mL, which decreased to values of approximately 700 pg/mL at 24 hours after insertion and remained less than 500 pg/mL for a 72 hour period (K. Messenger, unpublished data). In the present study,
PGE$_2$ concentrations were less than 200 pg/mL within 4 hours of carprofen of administration (Figure 5.3).

Pre-clinical studies to assess the PK-PD properties of NSAIDs are needed to screen drugs for development and predict effective dosing regimens. We have demonstrated that in vivo ultrafiltration is a reliable method for such studies. It has the advantage of being less invasive than surgically implanting artificial tissue cages and collects only the unbound (active) drug concentrations. Any pharmacokinetic model system should ideally assess the unbound, pharmacologically active drug concentration at the tissue site because the plasma drug concentrations of highly bound NSAIDs do not relate well to activity at the target (inflamed) tissue (Brune & Furst, 2007). The technique we described allows for simultaneous measurement of plasma drug concentration with the unbound concentrations in tissue fluids, which reflects the diffusion from the plasma to tissue site. In conclusion, the results of this study demonstrate prolonged drug concentrations of carprofen in tissues, despite a relatively short plasma half-life. In addition we have demonstrated the feasibility of using in vivo ultrafiltration to study the PK-PD of NSAIDs in dogs, which ultimately is a refinement of the currently utilized PK-PD models.
REFERENCES


Table 5.1: Pharmacokinetic parameter estimates for unbound carprofen in inflamed and control interstitial fluid. Legend: Geo Mean, geometric mean; CV%, coefficient of variation for the geo mean; k01, absorption rate; k10, elimination rate; and corresponding half-lives (t ½); AUC, area-under-the-curve for the plasma concentration vs time profile; CMAX, maximum concentration after oral administration; TMAX, time to peak concentration. *Indicates that values were taken directly from the data. Estimates are based on a 1-compartment model with a lag time. There was no significant difference between the parameter estimates for control versus inflamed sites.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Inflamed ISF</th>
<th></th>
<th>Control ISF</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geo Mean</td>
<td>CV%</td>
<td></td>
<td>Geo Mean</td>
<td>CV%</td>
</tr>
<tr>
<td>AUC</td>
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<td>71.86</td>
<td>278.52</td>
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<tr>
<td>CMAX*</td>
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<td>11.70</td>
<td>32.30</td>
<td>10.50</td>
<td>25.00</td>
</tr>
<tr>
<td>k01</td>
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<td>52.90</td>
<td>0.31</td>
<td>132.75</td>
</tr>
<tr>
<td>k01 t½</td>
<td>hr</td>
<td>1.61</td>
<td>52.90</td>
<td>2.20</td>
<td>132.75</td>
</tr>
<tr>
<td>k10</td>
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<td>41.72</td>
<td>0.06</td>
<td>53.31</td>
</tr>
<tr>
<td>k10 t½</td>
<td>hr</td>
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<td>41.72</td>
<td>11.58</td>
<td>53.31</td>
</tr>
<tr>
<td>TMAX*</td>
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</table>
Table 5.2: Pharmacokinetic parameter estimates for total (bound + unbound) carprofen in plasma. Legend: Geo Mean, geometric mean; CV%, coefficient of variation for the geo mean; A and B, intercept terms; α and β, distribution and elimination rate constants; α t½ and β t½, distribution and elimination half lives; k10, k12, k21, microdistribution rate constants; Vss, volume of distribution at steady state; Cl, total body clearance; MRT, mean residence time; AUC, area under the concentration-time curve; V1, volume of the central compartment. Estimates are based on a two-compartment model.

<table>
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<tr>
<td>α</td>
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<td>α t½</td>
<td>hr</td>
<td>1.63</td>
<td>25.13</td>
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<tr>
<td>AUC</td>
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<tr>
<td>AUMC</td>
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<tr>
<td>B</td>
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<tr>
<td>Vss</td>
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</tr>
<tr>
<td>k12</td>
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<td>61.30</td>
</tr>
<tr>
<td>k21</td>
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</tr>
<tr>
<td>V1</td>
<td>mL/kg</td>
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</table>
Figure 5.1: Mean (SD) of carprofen free drug concentrations in plasma, and control (n=4) and inflamed (n=6) interstitial fluid of dogs. * Indicates that values were significantly (p<0.05) higher in inflamed tissue compared to control.
Figure 5.2: Plasma concentrations (mean +/- SD) of total (protein-bound and unbound) carprofen from 6 dogs.
Figure 5.3: Mean ± SD PGE₂ (pg/mL) in control and interstitial fluid collected via in vivo ultrafiltration. * Indicates that inflamed PGE₂ concentrations were significantly (p<0.0038) lower compared to baseline (time 0).
6. SUMMARY AND FUTURE DIRECTIONS

Carprofen is the most frequently used NSAID in dogs in the United States and many other countries. As such, it is important to evaluate clinical questions involving this compound in vivo and in the intended patient population whenever possible, rather than speculating on potential outcomes using data derived from in vitro experiments or healthy research animals. The work presented in this dissertation sought to investigate potential factors that could influence the pharmacokinetics of carprofen in dogs, as a possible mechanism to explain the variability in clinical response as well as risk for adverse effects. Although there have been numerous studies involving the effects and pharmacokinetics of carprofen in dogs, none have investigated the factors that might account for variation in pharmacokinetics in the intended treatment population. Ultimately, we found very few factors significantly affected the pharmacokinetics of carprofen in dogs, supporting the widespread use and general safety of this important drug in veterinary medicine.

The first two studies focused on plasma pharmacokinetics of carprofen enantiomers in dogs. The importance of an enantiospecific assay for carprofen lies in differences in the pharmacokinetics and pharmacodynamics of each enantiomer, with substantially greater anti-inflammatory activity for the S(+) enantiomer compared to the R(-) enantiomer, despite carprofen only being available in a racemic formulation. The factor investigated in the first study was a potential drug-drug interaction through a protein-binding displacement effect. In vitro studies confirmed that a drug displacement interaction does occur with carprofen enantiomers and the highly protein-bound antimicrobial cefovecin in canine plasma. When assessed in vivo in dogs, the clearance of the R(-) enantiomer of carprofen, but not the S(+) enantiomer, was significantly increased. However, the R(-)
enantiomer does not exhibit significant anti-inflammatory activity and therefore there is no clinical relevance of the increased clearance of this enantiomer. An important limitation of this study was that total plasma concentrations of carprofen were assessed and measuring the free drug concentrations would provide more information on the possibility of a drug displacement interaction.

The use of population pharmacokinetics to assess patient-specific factors that might influence the pharmacokinetics of carprofen in dogs proved to be useful for the identification of possible differences in clinical dogs compared to healthy research dogs. Overall, dogs in this study exhibited a lower apparent clearance than previously reported for carprofen. In addition, in dogs with osteoarthritis, this value was even less. The clinical relevance of this finding is not fully elucidated at this time, as none of the dogs in this study experienced adverse drug effects. While similar results have been reported for other NSAIDs, the mechanism remains unclear. While possible mechanisms have been proposed, such as inhibition of hepatic metabolizing enzymes by inflammatory mediators, no specific investigations have been performed in dogs. Also, there was a wide range in plasma drug concentrations in the dogs in this study, but we were unable to correlate these with specific factors. Our study supports that hypothesis that severe adverse drug effects following carprofen administration are idiosyncratic in nature and cannot be predicted. The Beagle dogs used in our studies had higher apparent clearance estimates for carprofen enantiomers, which is consistent with the previous literature on carprofen pharmacokinetics in this breed. These differences are concerning because Beagles are commonly used by drug companies, both human and veterinary, to study the basic pharmacokinetics and pharmacodynamics of new drugs. Differences between breeds that
could result in toxicity may not be discovered until clinical trials, or even post-marketing surveillance, which not only results in large economic losses but also in potentially unnecessary patient morbidity and mortality. The finding of different clearance values in Beagles warrants future work (discussed below) to further understand the mechanism behind these differences.

Because only the protein-unbound concentrations of NSAIDs are pharmacologically active, we wanted to investigate methods to collect these concentrations in vivo, rather than extrapolating based on plasma drug concentrations. We utilized ultrafiltration, which has been previously established as a method to collect unbound concentrations of antimicrobials in dogs, to collect carprofen directly from the interstitial fluid in dogs (Bidgood & Papich, 2002). This work is the first report on the use of ultrafiltration for the collection of NSAIDs in any species. We extended the methods to simultaneously collect and measure PGE2 as a biomarker of the anti-inflammatory activity of carprofen. The important results of this study were that plasma carprofen concentrations and pharmacokinetics do not reflect the concentrations and pharmacokinetics in tissues. The presence and activity of carprofen in canine tissue fluid appears to be present far longer than one would predict based on plasma pharmacokinetics. A single dose of carprofen may provide benefits for far longer than 24 hours in some animals, and repeated doses could result in accumulation in tissue; further studies are needed to confirm these speculations. Concurrently, we assessed the effects of inflammation on the tissue distribution of carprofen, and found that carprofen distributes equally to inflamed and normal (healthy) tissues. This result was surprising, and should be investigated further, perhaps with a larger number of animals to confirm these results as our study was performed in only 6 dogs.
These results could have important implications for both efficacy and toxicity of carprofen. In addition, our method establishes a novel method of studying the pharmacokinetic and pharmacodynamic activity of anti-inflammatory drugs, and could be used for testing new compounds.

The studies performed here open many interesting avenues for future work. The different methods used here, including NLME modeling and in vivo ultrafiltration, can be applied together in studies to assess NSAID distribution and effects at tissue sites under different conditions in dogs. Our tissue study assessed concentrations of carprofen after a single injection, but repeating this study using oral carprofen at steady state, and comparing with other chronically administered oral NSAIDs, would allow us to assess the extent, if any, to which drug accumulation occurs in tissue sites. Local NSAID accumulation, and the demonstration of efficacy, could lead to changes in dose recommendations where perhaps less frequent dosing is required.

This refined model can also be applied to numerous other species; for example, we are currently undertaking a study utilizing in vivo ultrafiltration to compare plasma and tissue concentrations, and anti-inflammatory activity, of the NSAID flunixin in a surgical incision model in healthy calves. This study will allow us to further investigate the utility and clinical relevance of this methodology applied to the study of NSAIDs and other anti-inflammatory drugs in naturally-occurring models of pain and inflammation.

In vivo ultrafiltration could be combined with in vivo protein binding displacement studies. In the work presented, we investigated a single drug displacement interaction for carprofen using one clinically relevant drug in dogs (cefovecin). However, other highly protein bound drugs with narrow therapeutic indices may result in clinically relevant drug
interactions, or such interactions may change the pharmacodynamics of carprofen. Ultrafiltration would allow us to better investigate the both amount and activity of plasma unbound drug concentrations, which was a limitation of the work reported here.

Lastly, in vivo ultrafiltration can be applied to the study of novel tissue biomarkers of inflammation and pain. We have already performed a pilot study involving the collection of pro-and anti-inflammatory cytokines such as interleukins -6, -8, and -10 utilizing this technique and have obtained promising results. Because small peptides are easily filtered through the probe membranes, fluid samples collected with this technique can be further analyzed for novel biomarkers using proteomic approaches, an area we are also interested in pursuing as novel therapeutics are developed to treat pain and inflammation.

The findings in the population pharmacokinetics study raise several important questions regarding carprofen metabolism and breed-specific and disease-specific differences. Very little work has been done to assess the effects of inflammation on hepatic CYP enzymes in dogs, however there is evidence to suggest inflammatory mediators inhibit CYPs (Aitken et al., 2006). Elucidating these effects in dogs could have very important clinical implications in drug therapy for inflammatory diseases. Breed effects and differences in metabolic enzymes, specifically in Beagle dogs, would have widespread implication in the pharmaceutical industry where Beagles are the most commonly utilized pre-clinical large animal models. Genetic differences accounting for drug metabolism are critical to identify; in a worst-case scenario, these differences could result in serious adverse drug events resulting in the removal of a product from the market. There are important challenges associated with these studies, including costs as well as limitations in our understanding of the roles of CYP enzymes in canine drug metabolism (Martinez et
al., 2013). However, as our knowledge gaps become smaller and technology improves for canine genomics and drug metabolism, these studies will become feasible.
REFERENCES

