The Pharmacokinetics and Clinical Efficacy of Oral Carprofen in the Laboratory Mouse

by

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THE PHARMACOKINETICS AND CLINICAL EFFICACY OF ORAL CARPROFEN IN THE LABORATORY MOUSE

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Provision of analgesia to laboratory mice can be challenging, and administration of analgesics via the water bottle is attractive in that it diminishes the time, training, skill, animal stress, and costs associated with other methods. The current study aimed to determine whether carprofen could be administered to mice via the drinking water by assessing stability, palatability, and clinical efficacy following ovarietomy. We demonstrated that both injectable meloxicam and carprofen are stable for 7 d in ambient light at room temperature, in dark at room temperature, and in dark at 4 °C. Carprofen is palatable to mice when administered via the drinking water, whereas meloxicam-medicated water is non-palatable. Mice drink an average of 18.4 mL of carprofen-medicated water / 100 g body weight / 24 h, and drink more in the dark phase compared to the light. Following a single oral gavage of 10 mg/kg carprofen, peak plasma concentration (20.3 +/- 2.4 µg/mL) occurs at 2 h post-administration. With access to a carprofen-medicated water bottle (10 mg/kg, 0.067 mg/mL), a comparable peak plasma concentration (17.0 +/- 2.9 µg/ml) is achieved following 12 to 24 h exposure. In order to assess clinical efficacy, mice received 10 or 20 mg/kg carprofen subcutaneously (immediately postoperative) or gained access to a carprofen-medicated water bottle for
24 h prior to ovariectomy. No significant behavioural changes were detected between treatment groups postoperatively. Following ovariectomy, pain was detected up to 1 h postoperatively, using facial grimacing as an indicator of pain. In comparison to control mice that did not undergo anesthesia or surgery, grimacing was reduced in duration in mice receiving 10 or 20 mg/kg carprofen subcutaneously or orally. However, no overall statistically significant difference could be detected between treatment groups using the Mouse Grimace Scale. Administration of 20 mg/kg carprofen subcutaneously or orally resulted in no significant difference in behavioural changes or facial grimacing compared to saline administration, which may be in part due to insufficient dose. The current study emphasizes the need for further evaluation of the clinical efficacy of nonsteroidal anti-inflammatory doses in mice prior to administration. Administration of carprofen via the water may be a feasible and efficacious method to administer analgesics to laboratory mice; however, further studies are required to determine optimal doses to achieve sufficient analgesia, particularly across a wide range of painful procedures.
DECLARATION OF WORK PERFORMED

All work reported in this thesis was performed by myself under the supervision of Dr. Patricia Turner, and the guidance of Dr. Robert Foster and the rest of my advisory committee: Dr. Ron Johnson, Dr. Brandon Lillie, and Dr. Lee Niel, with the following exceptions:

Dr. Patricia Turner collected blood samples for the pharmacokinetics study. Yu Gu (department of Biomedical Sciences, University of Guelph) analyzed meloxicam samples (HPLC) and Elizabeth Tor (CAHFS Toxicology Lab, UC Davis School of Veterinary Medicine) analyzed carprofen samples (HPLC). Kelsy Ervin (department of Psychology, University of Guelph) performed all surgeries (ovariectomies). Kristyn Hale, Sabina Romanescu, and Emily Vellekoop (student veterinarians, Ontario Veterinary College) scored mouse faces using the Mouse Grimace Scale. Dr. Michelle Edwards (Data Librarian, University of Guelph) provided statistical guidance and expertise.
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LIST OF ABBREVIATIONS

AUC  area under the concentration-time curve
Cl   apparent oral clearance
C_max peak plasma concentration
COX  cyclooxygenase
COX-1 cyclooxygenase-1
COX-2 cyclooxygenase-2
COX-3 cyclooxygenase-3
DP   prostaglandin D receptor
EP   prostaglandin E receptor
EP_1 prostaglandin E_1 receptor
EP_2 prostaglandin E_2 receptor
EP_3 prostaglandin E_3 receptor
EP_4 prostaglandin E_4 receptor
FACS Facial Action Coding System
FP   prostaglandin F receptor
HPLC high-performance liquid chromatography
IM   intramuscular
IP   prostacyclin receptor
IV   intravenous
LD_{50} median lethal dose
NSAID nonsteroidal anti-inflammatory
PGD  prostaglandin D
PGD_2 prostaglandin D_2
PGE  prostaglandin E
PGE_1 prostaglandin E_1
PGE_2 prostaglandin E_2
PGF  prostaglandin F
PGF_{1a} prostaglandin F_{1a}
PGF_{2a} prostaglandin F_{2a}
PGG_2 prostaglandin G_2
PGH₂  prostaglandin H₂
PGI  prostacyclin
PO  per os
PTGS1  prostaglandin-endoperoxide synthase 1
PTGS2  prostaglandin-endoperoxide synthase 2
SC  subcutaneous
t₁/₂  elimination half-life
Tₘₐₓ  time to peak plasma concentration
TP  thromboxane-A receptor
TTX-R  tetrodotoxin-resistant sodium channel
TTX-S  tetrodotoxin-sensitive sodium channel
TXA  thromboxane A
TXA₂  thromboxane A₂
Vd/F  apparent volume of distribution
GENERAL INTRODUCTION, HYPOTHESES AND OBJECTIVES

In 2011, approximately 1 million mice were used for biomedical research in Canada (CCAC, 2011). Providing analgesia to laboratory animals is required from both legal and ethical perspectives in order to mitigate pain and suffering. Little to no pharmacokinetic or clinical data exists for commonly used analgesics, and dose ranges and methods of administration are often extrapolated from other species. Small animal size and large numbers result in additional barriers that hinder one’s ability to provide laboratory mice with efficient, effective analgesia. These include, but are not limited to, time and effort for administration, cost of drugs and supplies, training of personnel, animal stress with handling, and pain identification.

Administration of drugs through the drinking water does not require the time, training, or skill that are required with other routes of administration. This method would also greatly diminish handling-associated stress, which occurs with other routes of administration (Hurst and West, 2010). To date, no published studies have assessed the pharmacokinetics or clinical efficacy of any nonsteroidal anti-inflammatory drugs when given through the drinking water. Should administration of carprofen and meloxicam through drinking water prove to be clinically efficacious, this may be employed as a scientifically proven method to provide sufficient analgesia to laboratory mice, in order to minimize pain and suffering in these animals wherever possible.
It is hypothesized that carprofen and meloxicam will be stable in drinking water over 7 d, that addition of these drugs will not alter normal water consumption, and that clinically efficacious plasma drug levels can be achieved via administration through the drinking water bottle. An additional hypothesis to be tested in this thesis is that behaviour scoring and the Mouse Grimace Scale (Langford et al., 2010) will be efficacious in identifying states of pain in mice, and will demonstrate that analgesia can be achieved via administration through the drinking water bottle.

The current study has four objectives: the first is to assess the stability of carprofen and meloxicam in water; the second is to evaluate their palatability, the third is to determine their oral pharmacokinetics when administered via the drinking water, and the fourth is to determine the clinical efficacy of oral carprofen in an ovariectomy pain model using the Mouse Grimace Scale and behaviour scoring.

Oral gavage of the drugs will determine the pharmacokinetic parameters of each drug via the oral route. Administration of the drugs through the drinking water will assess the palatability of the drugs, determination of pharmacokinetic parameters of each drug if administered through the drinking water, and determine if similar plasma drug concentrations can be attained by both methods of oral administration. Subcutaneous administration of the drug will allow clinical comparison of the two methods of administration. Assessment of clinical efficacy of carprofen will be achieved through behaviour scoring (Wright-Williams et al., 2007; Roughan et al., 2009; Leach et al.,
2012; Miller et al., 2012) and application of the Mouse Grimace Scale (Langford et al., 2010).
1.1. MAMMALIAN PAIN PHYSIOLOGY

1.1.1. Introduction

The International Association for the Study of Pain defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey and Bogduk, 1994). In comparison, nociception is defined as “the neural process of encoding noxious stimuli” (Merskey and Bogduk, 1994). From a purely physiologic standpoint, pain can be divided into neuropathic and nociceptive types, based upon the presence of disease (Treede et al., 2008). Neuropathic pain is the result of a pathologic process that affects the normal physiologic function of neurons. It is the result of a disease process or damage to the somatosensory system (Zimmerman, 2001; Treede et al., 2008; Cohen and Mao, 2014). Nociceptive pain occurs secondary to actual or threatened damage and is due to the activation of specialized peripheral nerve fibers called nociceptors (Dubin and Patapoutian, 2010). Neuropathic pain is typically chronic in nature since damage to nerves is typically present, whereas nociceptive pain is most often acute (Zimmerman, 2001; Treede et al., 2008; Cohen and Mao, 2014).

Nociceptor activation can occur through a variety of stimuli, including but not limited to temperature extremes, surgical trauma, hypoxia, and mechanical and chemical stimulation. The first part of this review will provide an overview of the pain pathway
and nociceptor physiology, and conclude with a focus on the molecular biology of inflammatory pain.

1.1.2. Pre-synapse

The nociceptor soma resides within the dorsal root ganglia, from which afferent nerve fibers (axons) extend away from the spinal cord and synapse with target organs. Afferent fibers are classified into different types based upon fiber myelination, and location of the synapse within the spinal cord. A-delta fibers synapse at Lamina I and V, peptidergic C-fibers synapse within Lamina I and Outer Lamina II, and non-peptidergic fibers synapse at inner Lamina II (Basbaum and Jessell, 2000; Braz et al., 2005).

1.1.3. Anatomy of the nociceptor

Sherrington first described the nociceptor in 1906 (cited by Burke, 2007), and while many of the complexities of the nervous system were still to be discovered, it was apparent that the nociceptor was of central importance in signal transduction to and from the central nervous system. Since this first, very basic description of the presence of neurons, knowledge of both the characteristics and capabilities of nociceptors has grown exponentially.

Two classes of nociceptors exist: medium diameter (lightly myelinated) A-delta fibers and small diameter (unmyelinated) C-fibers (Wall and Melzack, 1999). Axon diameter and myelination are directly associated with conduction velocity. A-delta fibers have a conduction velocity of approximately 5–30 m/s, and detect immediate, “fast” pain. A-
delta fibers can be further divided into Types I and II. Type I A-delta fibers have a low chemical and mechanical threshold but a high heat threshold. Type II fibers have a lower heat threshold but a high mechanical threshold. C-fibers have a conduction velocity of 0.4–1.4 m/s and detect secondary, “slow” pain (Djouhri and Lawson, 2004). Like A-delta fibers, the family of C-fibers is heterogeneous in that mechanical and heat sensitivity varies; however the majority of fibers possess a low heat threshold and high mechanical threshold in comparison to A-delta fibers (Treede, 1995). C-fibers can be further divided into peptidergic and non-peptidergic populations, based on the presence of the peptide neurotransmitter substance P (Julius and Basbaum, 2001; Basbaum et al., 2009). The A-delta and C-fibers travel from the dorsal root ganglion, through the dorsal root, and synapse within the dorsal horn of the spinal cord. Each axon synapses at a different anatomical position within the dorsal root of the spinal cord, which is believed to contribute to their varying roles in conduction of pain signals.

1.1.4. Activation of the nociceptor

The pain pathway begins with a stimulus that activates the nociceptor, such as extreme hot, cold, stretch, pressure, or the presence of endogenous inflammatory chemical mediators. The stimulus must surmount a threshold to depolarize the peripheral nerve terminals, and depolarization itself must then occur with sufficient amplitude and duration for nociceptor activation to arise (Basbaum and Jessell, 2000). Voltage-gated sodium, calcium, and potassium channels are present within the nociceptor cell membrane. Sodium channels are subtyped based upon their sensitivity (TTX-S) or resistance (TTX-R) to the puffer fish toxin tetrodotoxin (Lee and Ruben, 2008). A-delta
fibers possess only TTX-S channels whereas C-fibers express both TTX-S and TTX-R channels (Gold et al., 1996).

Activation of the aforementioned surface receptors results in an intracellular influx of sodium through voltage-gated sodium channels, which depolarizes the peripheral terminal (transduction) and results in release of neurotransmitters. The predominant neurotransmitter is glutamate, which is released at the synaptic cleft within the dorsal horn of the spinal cord (transmission). Neurotransmitter release results in propagation of pain signals via neurons up the dorsal horn of the spinal cord to the brainstem, thalamus, and midbrain (projection), where perception of pain takes place (Basbaum and Jessell, 2000; Dubin and Patapoutian, 2010).

Tissue injury results in the release of inflammatory mediators such as bradykinin, serotonin, interleukin-1β, tumour necrosis factor-α, and prostaglandins from cells that partake in the inflammatory response, including leukocytes, endothelial cells, and fibroblasts. These molecules can either decrease the activation threshold of the nociceptor, or directly activate the nociceptor itself (Julius and Basbaum, 2001). Lowering the activation threshold of sodium channels within the nociceptor membrane is believed to occur through the protein-kinase A pathway (England et al., 1996). Each prostaglandin interacts with a specific cell surface receptor.
1.1.5. Post-synapse

Post-synapse, information travels to the thalamus and brainstem via the spinothalamic and spinoreticulothalamic tracts, respectively. The activation of several cortical structures is responsible for the experience of pain. Synapses within the thalamus transmit information to the somatosensory cortex, and synapses within the brainstem at the parabrachial nucleus transmit information to the amygdala, cingulate cortex, and insular cortex. Synapses within the midbrain periaqueductal gray and rostral ventral medulla result in descending modulating output via efferent neurons (Basbaum and Jessell, 2000).
1.2. NONSTEROIDAL ANTI-INFLAMMATORY DRUGS AND PROSTAGLANDINS

1.2.1. Introduction

Prostaglandins were discovered in the 1930’s by Ulf Von Euler (Von Euler, 1936). Von Euler extracted the compound from human semen, and named it ‘prostaglandin’ because at the time he believed that it was secreted from the prostate gland. In 1964, an enzyme present within the vesicular glands of sheep was determined to participate in prostaglandin synthesis, and arachidonic acid was identified as the precursor of the reaction (Bergström et al., 1964; Van Dorp et al., 1964a; Van Dorp et al., 1964b); however, the exact identity or significance of this enzyme was unknown until 1976. In 1976, prostaglandin endoperoxide synthetase (cyclooxygenase-1) was isolated and purified from bovine (Miyamoto et al., 1976) and ovine (Hemler and Lands, 1976) vesicular glands. In 1971, 72 years after the release of Aspirin®, Vane proposed that NSAIDs, exhibited their anti-inflammatory effect through inhibition of an essential enzyme required for prostaglandins synthesis, later to be identified as cyclooxygenase (Vane, 1971). Van der Ouderaa et al. further characterized the cyclooxygenase-1 (COX-1) enzyme in 1977 (Van der Ouderaa et al., 1977) and Xie et al. discovered the second cyclooxygenase isoenzyme, cyclooxygenase-2 (COX-2), in 1991 (Xie et al., 1991).

1.2.2. Structure of cyclooxygenase isoenzymes

Cyclooxygenase an enzyme required for prostanoid synthesis exists as three isoenzymes:
COX-1, COX-2, and cyclooxygenase-3 (COX-3). COX-1 and COX-2 are encoded by the prostaglandin endoperoxide synthase 1 (PTGS1) and prostaglandin endoperoxide synthase 2 (PTGS2) genes, respectively (Yokoyama and Tanabe, 1989). COX-3 is encoded by PTGS1 as well, but is the result of variation in genomic splicing. At the present time, a functional COX-3 enzyme has been identified only in dogs while humans and mice have a nonfunctional variant due to a frameshift mutation (Chandrasekharan et al., 2002). An important difference between COX-1 and COX-2 exists at amino acid position 523 (Gierse et al., 1996), where the isoleucine in COX-1 is replaced with valine in COX-2. This replacement allows for access to a hydrophobic pocket, required for COX-2 inhibition (Kurumbail et al., 1996). Morita et al. (1995) demonstrated that COX-1 was predominantly cytoplasmic and associated with the endoplasmic reticulum, whereas COX-2 while also associated with the endoplasmic reticulum was equally associated with the nucleolemma. Historically, COX-1 was thought to be constitutively expressed, whereas COX-2 was believed to be induced by specific cellular processes or events (Xie et al., 1991). More recent research has suggested that COX-2 may also be constitutively expressed in certain mammalian tissues including gastrointestinal muscle (Porcher et al., 2002, 2004), and COX-1 expression may be unregulated under certain conditions (Lau et al., 2014).

COX-1 is present within all tissues to varying extents and comprised of 600 to 602 amino acids (species dependent). In comparison, COX-2 is expressed in specific tissues (Smith et al., 2000) and nascent COX-2 is comprised of 604 amino acids (Smith et al., 2000). The structure of bovine COX-1 was first published in 1994 (Picot et al., 1994),
followed by human (Luong et al., 1996) and murine COX-2 (Kurumbail et al., 1996). Both isomers contain three domains: a C-terminal catalytic domain, an N-terminal epidermal growth factor domain, and a membrane-binding domain. Both a heme and cyclooxygenase site is present within the catalytic domain. The heme site is responsible for the reduction of PGG$_2$ into PGH$_2$ and the cyclooxygenase site is responsible for the conversion of arachidonic acid to hydroperoxy endoperoxide prostaglandin G$_2$ (PGG$_2$).

### 1.2.3. Prostaglandins and inflammation

Prostanoids are involved in both physiologic and pathophysiologic processes, including homeostasis (Wallace and Tigley, 1995; Komhoff et al., 2000; Loftin et al., 2001, 2002), neoplasia (Loftin et al., 2002; Raz, 2002; Song et al., 2002), and inflammation (Langenbach et al., 1995; Loftin, 2002). Phospholipase A$_2$ converts cellular phospholipids into arachidonic acid. Arachidonic acid is converted to prostaglandin G$_2$ (PGG$_2$) and subsequently prostaglandin H$_2$ (PGH$_2$) via COX, which catalyzes the incorporation of two O$_2$ molecules into the arachidonic molecule and then reduces the molecule into its alcohol form. Thromboxane A$_2$ (TXA$_2$) synthase, prostaglandin D$_2$ (PGD$_2$) synthase, and prostaglandin E$_2$ (PGE$_2$) synthase convert PGH$_2$ into TXA$_2$, PGD$_2$, and PGE$_2$, respectively. Carbonyl reductase-I converts PGE$_2$ into prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) (Hamberg and Samuelsson, 1967; Kulmacz et al., 1994; Malkowski et al., 2000; Thuresson et al., 2001).

The receptors for prostaglandin D (PGD), prostaglandin E (PGE), prostaglandin F (PGF), prostacyclin (PGI), and thromboxane-A (TXA) are termed DP, EP, FP, IP, and TP,
respectively. EP receptors are the further divided into four subtypes, EP₁, EP₂, EP₃ and EP₄ (Kobayashi and Narumiya, 2002). Several studies have been conducted that demonstrate the pertinent role that PGE₂ and the EP receptors play in inflammation. EP₁ has been shown to participate in PGE₂-induced thermal hyperalgesia (Moriyama et al., 2005), EP₂ and EP₄ in paw swelling in a mouse model of arthritis (Honda et al., 2006), and EP₂ and EP₃ in the production of inflammatory exudate (Yuhki et al., 2004). EP₄ knockout mice show a marked decrease in clinical signs in an arthritic model, and a decrease in systemic interleukin-6 and interleukin-1 (McCoy et al., 2002). Prostaglandin production is low in normal (uninflamed) tissues, but increases significantly with the present of inflammatory cytokines. In order for prostaglandins to participate in inflammation-associated pain, prostaglandin production must be constant, as they act directly on peripheral sensory neurons and sites within the central nervous system (Funk, 2001).
1.3. PHARMACOLOGIC PROPERTIES OF MELOXICAM

1.3.1. Introduction

In addition to humans (Schmid et al., 1995), the pharmacokinetics of meloxicam has been studied in a variety of veterinary species including the horse (Toutain et al., 2004; Sinclair et al., 2006), cow (Mosher et al., 2011), goat (Shukla et al., 2007; Ingvast-Larsson et al., 2011), sheep (Shukla et al., 2007; Stock et al., 2013), llama (Kreuder et al., 2012), pig (Fosse et al., 2008, 2011), dog (Budsberg et al., 2002; Montoya et al., 2004; Yuan et al., 2009), donkey (Mahmood and Ashraf, 2011), chicken (Baert and De Backer, 2002). Long half-life and high bioavailability have made it an attractive candidate for use in exotic and non-domestic species as well, and pharmacokinetic parameters have been studied in the vulture (Naidoo et al., 2008), iguana (Divers, 2010), camel (Wasfi et al., 2012), red-tailed hawk (Lacasse et al., 2013), great horned owl (Lacasse et al., 2013), and koala (Kimble et al., 2013). For the purpose of this review, pharmacokinetic parameters in mice, rats, and rabbits will be contrasted and compared. Table 1-1 summarizes the pharmacokinetic parameters of meloxicam in the mouse, rat, and rabbit following a single oral dose, and exemplifies the interspecies variability that can exist.

1.3.2. Structure and physical properties

Meloxicam is a nonsteroidal anti-inflammatory drug (NSAID) with anti-inflammatory, analgesic, and antipyretic properties (Engelhardt, 1995). It is one of many compounds that comprise the enol-carboxamide class, which are characterized by a 4-hydroxy-1,2-benzothiazines-3-carboxamide 1,1-dioxide skeleton. Other members include droxicam,
isoxicam, lornoxicam, piroxicam, and tenoxicam (Salgado-Moran et al., 2013). It is a white crystalline powder with the molecular formula C_{14}H_{13}N_{3}O_{4}S_{2} and a molecular weight of 351.40 (Engelhardt, 1995; Davies and Skjodt, 1999).

1.3.3. Mechanism of action and toxicity

Decreased ulcerogenic potential and long half-life has made meloxicam a preferential candidate over other nonsteroidal anti-inflammatories in several domestic laboratory species for the last few decades (Engelhardt, 1995; Kawai, 1998; Laudanno et al., 2000). Anti-inflammatory action is thought to occur through selective inhibition of COX-2, resulting in a decrease in prostaglandin synthesis (Engelhardt, 1995). The safety margin has been attributed to selective COX-2 inhibition, and decreased bioavailability as plasma concentration increases above therapeutic levels (Toutain and Lees, 2004). Studies in domestic and non-domestic species have demonstrated a lack of gastrointestinal, hepatic, or renal toxicity at therapeutic doses (Goodman et al., 2009; Noble et al., 2012; Sinclair et al., 2012; Surdyk et al., 2013), and only mild changes at doses several-fold the therapeutic dose (Noble et al., 2012; Burukoglu et al., 2014). However, one must keep in mind acute drug reactions are still possible at any dose (Nakagawa et al., 2005; Niza et al., 2007). Turner et al. (2006) observed no clinical signs or biochemical abnormalities associated with toxicity following once-daily dosing of meloxicam at 1.5 mg/kg for 5 d in rabbits. Similarly, Delk et al., (2014) observed no clinical signs, biochemical, gross, or histopathological abnormalities associated with toxicity following once-daily dosing of meloxicam at 1.0 mg/kg for 29 d in rabbits.
1.3.4. **Bioavailability, absorption, distribution**

Following a single oral or intravenous (IV) dose in the mouse (10 mg/kg) or rat (1 mg/kg), area under the concentration-time curve (AUC) has been shown to be only slightly higher in the IV groups compared to oral, indicating a high bioavailability in both mice and rats (Busch et al., 1998). The bioavailability of meloxicam has not been studied in rabbits, but is presumed to be high based upon high bioavailability in other species (Schmid et al., 1995; Busch et al., 1998). Following a single oral dose to male and female mice, peak plasma concentrations were 18.1 and 20.7 mg/L and occurred at 0.7 and 0.6 h post-administration, respectively. In comparison, with a 10-fold lower dose, peak plasma concentrations in male and female rats following oral dosing with meloxicam were much lower (2.35, and 3.25 mg/L, respectively) with a longer time to peak plasma concentration (4.4 and 6.8 h, respectively). However, one must keep in mind that the AUC is proportional to dose if linear kinetics are followed. The peak plasma concentrations observed in the rat is comparable to that observed in mice, indicating bioavailability and clearance are similar in both species. In humans, the tablet formulation has been shown to be bioequivalent to the suspension formulation (Helmy and El Bedaiwy, 2013). A recent study (Delk et al., 2014) evaluated the oral pharmacokinetics of meloxicam in rabbits over 29 d, and peak plasma concentration plateaued at day 8 (0.81 ± 0.21 µg/mL). In general, tissue concentration is typically higher than that of plasma concentration, particularly when inflammation is present (Toutain and Lees, 2004).
1.3.5. Half-life and elimination

Following a single oral dose, the AUC is slightly higher in female compared to male rats, and is significantly higher in female compared to male rats following IV dosing (Busch et al., 1998). These findings are due to a significantly higher elimination half-life in female rats in comparison to male rats. Higher peak plasma concentration and longer elimination half-life in female rats in comparison to male rats is attributed to reduced levels of cytochrome P450 (Busch et al., 1998), which is required for meloxicam biotransformation in the rat. Approximately 60 - 65% of the total administered oral or IV dose was excreted in the urine of mice with the remainder being excreted in the feces after biliary excretion. In rats, approximately 70% of the total administered oral or IV dose was excreted in the urine, with the remainder excreted in the feces. Enterohepatic recycling was assessed in rats only, and in males was almost twice of that in females (Busch et al., 1998). Three meloxicam metabolites have been identified in the urine of rats (Engelhardt and Trummlitz, 1990), none of which were shown to be biologically active (Engelhardt and Trummlitz, 1990). In comparison, following a single oral dose in the rabbit (Turner et al., 2006), the elimination half-life is markedly longer, and approximately 1/10 that in the rat at a similar oral dose (Table 1-1).
1.4. PHARMACOLOGIC PROPERTIES OF CARPROFEN

1.4.1. Structure and physical properties

Carprofen is a propionic acid-based NSAID with anti-inflammatory, antipyretic, and analgesic properties. The molecular formula is $\text{C}_{15}\text{H}_{12}\text{ClNO}_2$ and it is a white, crystalline compound with a molecular weight of 273.72. Carprofen has a chiral center at the $\alpha$-carbon position, and exists as a racemic compound comprised of $R(-)$ and $S(\pm)$ enantiomers (Stoltenborg et al., 1981).

1.4.2. Mechanism of action and toxicity

Like other NSAIDs, it is believed that carprofen’s beneficial effects arise through variable and selective inhibition of COX isomers. While it is generally accepted that carprofen selectively inhibits the COX-2 isomer, this COX-2 selectivity has only been studied and subsequently demonstrated in dogs (Ricketts et al., 1998; Brideau et al., 2001; Streppa et al., 2002). Further, it should also be noted that these findings of COX-2 selectivity are inconsistent throughout the current literature (Kay-Mugford et al., 2000). In vitro studies have demonstrated that carprofen is non-selective for COX-2 in horses, and may be potentially selective for COX-2 in cats (Brideau et al., 2001).

The pharmacokinetics of carprofen have been evaluated in a number of species including the rat (Kemmerer et al., 1979; Iwakawa et al., 1991), cow (Lohuis et al., 1991), dog (McKellar et al., 1994), calf (Delatour et al., 1996a), cat (Taylor et al., 1996), sheep (Welsh, et al., 1992; Cheng et al., 2003), horse (Delatour et al., 1996b; Lees and Landoni,
2002; Mealey et al., 2004), rabbit (Hawkins et al., 2008), and human (Stoltenborg et al., 1981). Marked interspecies variability exists in the pharmacokinetics of carprofen, which has lent to difficulties in understanding the true mechanism of action and the stereospecific pharmacokinetics of this molecule. The pharmacokinetics of carprofen will be discussed, with emphasis on species variability.

1.4.3. Bioavailability, absorption, distribution

Like other NSAIDs, bioavailability of carprofen is high (Rubio et al., 1980; Hawkins et al., 2008). The plasma concentration of the S(+) enantiomer predominates after IV administration of racemic carprofen in the rat (Iwakawa et al., 1991) and rabbit (Hawkins et al., 2008), after SC administration in the rabbit (Hawkins et al., 2008), and after oral administration in humans (Stoltenborg et al., 1981). The plasma concentration of the R(-) enantiomer predominates after IV administration of racemic carprofen in the cow, (Lohuis et al., 1991), cat (Taylor et al., 1996), horse (Lees and Landoni, 2002), and sheep (Cheng et al., 2003), after SC administration in the cat, and after oral administration in dogs (Schmitt and Guentert, 1990).

Administration of IV racemic carprofen in the cat (Taylor et al., 1996), sheep (Cheng et al., 2003), calf (Delatour et al., 1996a), and horse (Lees and Landoni, 2002) results in a greater AUC for the R(-) enantiomer in comparison to the S(+) enantiomer, whereas the S(+) enantiomer predominates in the rabbit. SC administration of racemic carprofen in rabbits (Hawkins et al., 2008) results in a greater AUC for the S(+) enantiomer in comparison to the R(-) enantiomer, whereas the opposite holds true in the cat (Taylor et
al., 1996). In summary, marked interspecies variability exists in the pharmacokinetics of the individual enantiomers, which can have clinical repercussions dependent on which enantiomer is more clinically active in each species.

Interestingly, administration of 0.7 mg/kg carprofen via the IV or SC routes resulted in similar total AUCs (both enantiomers combined) in both cats (Taylor et al., 1996) and rabbits (Hawkins et al., 2008). However, administration of 0.7 mg/kg racemic carprofen IV to horses (Lees et al., 2002) resulted in an approximately 5-fold greater AUC. As mentioned, if bioavailability is assumed to be high in all species, then this may represent marked interspecies variation in elimination. In general, tissue concentration is typically higher than that of plasma concentration, particularly when inflammation is present (Toutain and Lees, 2004).

1.4.4. **Half-life and elimination**

Glucuronidation is required for excretion of carprofen from the body. Glucuronidation is catalyzed by enzymes of the uridine 5’-diphospho-glucuronosyltransferase family (Kuehl et al., 2005), which are found predominantly in the liver (Ohno and Nakajin, 2008). Maire-Gauthier et al. (1998) demonstrated that both species variability and stereoselectivity is present even in the process of carprofen glucuronidation. Rats exhibit a high degree of stereoselective glucuronidation compared to other species, and the R(-) enantiomer is glucuronidated at a faster rate in comparison to the S(+) enantiomer. Again, this may have clinical implications, dependent on which enantiomer is clinically active in the species, and whether this enantiomer is stereoselectively glucuronidated and excreted.
After IV administration of 0.7 mg/kg racemic carprofen to the cat (Taylor et al., 1996), half-life (mean ± SEM) was 15.5 ± 6.0 h. The same dosage administered to calves and cows (Delatour et al., 1996a; Lohuis et al., 1991) had a much greater half-life of 43.4 ± 2.3 and 30.7 ± 2.3 h.

Elimination of the R(-) enantiomer is greater than that of the S(+) enantiomer in the rat (Iwakawa et al., 1991) and rabbit (Hawkins et al., 2008). Mean residence time and elimination half-life of the R(-) enantiomer is greater than S(+) enantiomer after IV administration in the cow (Lohuis et al., 1991), cat (Taylor et al., 1996), horse (Lees and Landoni, 2002), and sheep (Cheng, 2003), after SC administration in cats (Taylor et al., 1996), and after oral administration in dogs (Schmitt and Guentert, 1990).

Stoltenborg et al. (1981) demonstrated in humans that excretion/elimination of the S(+) enantiomer in the urine and feces was slightly greater than the R(-) enantiomer. However, during this study the total amount of carprofen administered was not fully recovered in the feces and urine, so this may not be a true representation of the ratio of enantiomers that were excreted. A second study (Delatour et al., 1996b) demonstrated that the glucuronide conjugates of both enantiomers excreted in the urine in humans were close to racemic, due to a lack of stereoselective glucuronidation of carprofen in human plasma.

Delatour et al. (1996b) also demonstrated that the R(-) enantiomer predominated in the plasma of both the dog and horse, whereas the S(+) glucuronide predominated in bile and
urine, demonstrating stereoselective excretion of glucuronides. Priymenko et al. (1998) demonstrated in dogs that the R(-) enantiomer and S(+) enantiomers are both largely excreted in bile as glucuronide conjugates. After an IV 4 mg/kg dose, 74% of the R(-) enantiomer and 92% of the S(+) enantiomer were recovered in the bile once again demonstrating S(+) stereoselectivity of enterohepatic recycling.
1.5. ORAL DRUG ADMINISTRATION IN LABORATORY MICE

Several routes of drug administration exist for laboratory mice, including the following: oral, IV, intradermal, SC, intramuscular (IM), enteral, intranasal, intracerebral, inhalation, and epidural (Liu et al., 1970; Turner et al., 2011a). The method that is employed depends on the type of study being conducted, animal welfare considerations, and laboratory logistics.

Oral administration of drugs can be either involuntary (gavage) or voluntary (foodstuffs). Restraint is required for all methods of drug administration except voluntary oral. If restraint is necessary, methods to reduce handling stress include proper technique, experienced handlers, and gradual habituation to equipment or tools that may be used (Turner et al., 2011a). Gavage involves the placement of a small, curved stainless steel gavage needle with ball-tip into the esophagus and injection of the desired substance. Drugs can be incorporated into water (Ezell et al., 2012; Ingrao et al., 2013), wafers (Ferguson and Boctor, 2009), jellies (Zhang, 2011), gels (Overk et al., 2012), or pills (Walker et al., 2013). Most other methods of drug administration in the laboratory mouse require restraint. Several methods of restraint exist, but all carry the potential to cause stress and anxiety in the animal (Meijer et al., 2006; Cinelli et al., 2007; Hurst and West, 2010). Not only does this pose an animal welfare concern, but may also interfere with research data (Meijer et al., 2006; Cinelli et al., 2007; Turner et al., 2011a). Routine restraint has been shown to increase heart rate (Meijer et al., 2006a; Meijer et al., 2006b), body temperature (Meijer et al., 2006a; Meijer et al., 2006b), and serum corticosterone
(Miller and Chernoff, 1995), but can result in implantation failure or congenital malformations if performed during the peri-implantation period or organogenesis in the pregnant dam (Golub et al., 2004). Stress can also vary by type of restraint or injection. Heart rate increases significantly more following intraperitoneal injections compared to IM or SC injections (Meijer et al., 2006).

The small size of laboratory mice and the large numbers of animals that are generally required for individual research studies presents unique challenges when administering compounds via injection or oral gavage. Injections and gavage can be time consuming, and costs associated with staff or materials, such as syringes and needles, must also be taken into account. Appropriate restraint and injection also require training and skill, which ultimately increases costs further.

While voluntary oral administration of drug compounds is appealing, a major disadvantage is individual variability in food or water intake, and external factors that might influence food intake. External factors, such as pregnancy (Richard and Treyhorn, 1985; Makarova et al., 2010; Krasnow et al., 2011), pain (Tubbs et al., 2011), and chronic stress Jeong et al., 2013) can be associated with decreased food intake, whereas diseases such as diabetes mellitus have been associated with hyperphagia (Sindelar et al., 2002). The amount of drug consumed can be greatly influenced by these factors, and the amount of drug ingested can, therefore, vary tremendously between animals. Factors affecting water intake include temperature and humidity (Esher and Wolfe, 1979), genetics
(Silverstein, 1961), and disease states including those affecting the pituitary (Hummel, 1960).

Ultimately, several factors must be taken into account when choosing the most appropriate mode of compound administration for laboratory mice in any particular study. Voluntary oral administration is attractive compared to other methods as it does not involve stress, and requires less time, skill, and technical staff.
1.6. PAIN IDENTIFICATION IN LABORATORY MICE

1.6.1. Behaviour as an indicator of pain in laboratory rodents

Decreased food and water intake and overall locomotion were early behavioural indicators of pain recognized by scientists, researchers, and veterinarians in laboratory animals (Yoxall, 1978; Morton and Griffiths, 1985). Provision of analgesia resulted in amelioration of such changes and thus the efficacy of an analgesic could be assessed based upon its ability to return these parameters to within normal limits (Yoxall, 1978; Morton and Griffiths, 1985). Flecknell and Liles (1991) were the first to evaluate these parameters following a painful procedure in rats, and demonstrated a decrease in food and water consumption and locomotor activity. However, some analgesics can act as confounding factors. Opioids, for example, have been shown to increase food and water intake in rats (Sanger and McCarthy, 1981), independent of pain or analgesia.

Beynen et al. were one of the first groups to determine that pain and discomfort in mice (Beynen et al., 1987) and rats (Beynen et al., 1988) could be studied through behavioural observation. Beynen et al. (1987) studied gallstone-free and gallstone-bearing mice in order to determine a method to assess discomfort. They determined that, of several behavioural parameters assessed, only response to palpation of the right hypochondrium was significantly different between both groups. Squeaking and the magnitude of muscular contractions immediately following palpation characterized the response.
Behaviour scoring has since been proven as an effective means to identify postoperative pain in laboratory rats (Roughan and Flecknell, 2000, 2001, 2003) and mice (Wright-Williams et al., 2007). Since this time, behaviour has been used to assess the clinical efficacy of a variety of commonly used analgesics, including meloxicam (Roughan et al., 2004), carprofen (Roughan et al., 2004; Roughan and Flecknell, 2004), and buprenorphine (Roughan and Flecknell, 2004) in a variety of pain studies in the rat.

Following assessment of over 150 behavioural acts in mice, Roughan and Flecknell (2001) demonstrated that 'cat-like' back arching, horizontal stretching followed by abdominal writhing, and twitching while inactive were directly associated with the post-operative period, and were not associated with drug administration (and presumed to be pain-associated). In comparison, behavioural assessment by Wright-Williams et al., (2007) following vasectomy demonstrated the frequency of flinching, writhing, rear leg lift and press were likely pain-associated.

A recent study by Leach et al., (2012) assessed pain in mice following vasectomy using the following pain-associated behaviours: arch, circle, fall, flinch, press, rear leg lift, stagger, twitch and writhe, which were adapted from the previously discussed studies evaluating pain-associated behaviours in both mice (Wright-Williams et al., 2007) and rats (Roughan and Flecknell, 2001; Affaitati et al., 2002). Today, the use of ethograms to assess postoperative pain in the mouse and rat has not only been validated but is widely accepted in the field of laboratory animal science as one of the few objective indicators of pain in rodents.
1.6.2. Facial action as an indicator of pain in laboratory rodents

The facial action coding system (FACS) was developed in 1978 by Ekman and Friesen (cited by Ekman et al., 1980). It is defined by 44 action units, whereby one or several action units can characterize any human facial expression. The aim of the system was to characterize and define facial movements in a strict, anatomical context. By doing so, this would allow one to better understand the use of facial expression in human communication, specifically, through affective states. The system was then adapted to identify pain in neonates.

Like animals, the lack of ability of human neonates to verbally convey feelings of pain and discomfort led to a commonly held misconception that neonates did not feel pain to the same extent adults did (McGraw, 1941). This belief predominated for the better part of the 20th century, largely in part due to a paper by McGraw in 1941 which put forth the notion that the neonatal brain was underdeveloped incapable of experiencing pain similar to an adult. In the early 1980s, research advances gave insight into neonatal neuroanatomy and development, which implied neonates felt pain similar to their adult counterparts (Zisk, 2003). By the late 1980s, it was widely accepted that neonates felt pain and many studies attempting to quantify pain in children began to surface (Yaffa, 2003). Using the action units defined by the facial action coding system (FACS), early studies described specific facial movements associated with pain in neonates (Prkachin and Mercer, 1989; Prkachin, 1992).
The neonatal facial coding system is the most common pain scale using facial expressions for neonates (Srouji et al., 2010). It is comprised of 10 facial action units that were previously shown to be associated with pain in adults, and hypothesized to be associated with pain in neonates and infants (Grunau and Craig, 1987; Gruneau and Craig, 1990). These facial actions include the following: brow bulge, eye squeeze, deepening of the nasolabial furrow, opening of lips, vertical and horizontal mouth stretch, lip purse, taut tongue, and chin quiver. A score of ‘0’ is assigned if the facial action unit is not present, and a score of ‘1’ is assigned if the facial action unit is present.

In 2010, the Mogil lab published a new method to identify and quantify pain experienced by laboratory mice entitled the ‘Mouse Grimace Scale’ (Langford et al., 2010). Animals, like human neonates, are unable to verbally express pain or discomfort, which necessitated a quantitative measure of discomfort. The scale was based upon the identification of facial expressions or action units, assumed to be associated with states of pain or discomfort. Apart from behaviour scoring as previously discussed, no other methods existed whereby pain could be identified in laboratory mice in a clinical setting. The Mouse Grimace Scale is comprised of five facial ‘action units’, including orbital tightening, ear position, whisker change, nose bulge, and cheek bulge. Each action unit is assigned a score of 0 to 2, as the prominence of the facial expression increases. A score of ‘0’ is assigned if the facial expression is not present, ‘1’ if it is present to a moderate extent, and ‘2’ if it is obvious. The extent of the action units made was found to be proportional to the amount of pain or discomfort experienced.
The utility of the Mouse Grimace scale in assessing the clinical efficacy of commonly used analgesics has already been demonstrated. Similar facial grimace scales to identify pain have recently been developed in the rat (Sotocinal et al., 2011), rabbit (Keating et al., 2012), and horse (Dalla Costa et al., 2014). In 2012, Matsumiya et al. employed the Mouse Grimace Scale to assess the efficacy of a number of commonly used analgesics following laparotomy in female CD1 mice. In the placebo group, pain-associated grimacing was observed up to 36 h postoperatively. The ability of each analgesic (and dosage) to inhibit pain-induced facial grimacing was evaluated at 1 h postoperative only. Buprenorphine inhibited pain-induced facial grimacing in mice 1 h postoperatively at dosages equal to the current recommendations (0.05 and 0.1 mg/kg SC intraoperatively). Interestingly, carprofen was effective at 20 and 25 mg/kg; however, the current recommended dosage is up to 5-fold less (5 mg/kg SC every 24 h) (Flecknell, 2009).

The Mouse Grimace Scale is an attractive method for identifying pain in the laboratory mouse. While behaviour scoring is the current gold standard, benefits over behaviour scoring include ease of application and a shorter time commitment. Application of the Mouse Grimace scale has been shown to take less than 1/10th the amount of time required to perform behaviour scoring (Leach et al., 2012), and coders require minimal to no training (Matsumiya et al., 2010; Leach et al., 2012). Weaknesses include difficulties in visualization of faces in the dark phase (Matsumiya et al., 2010) and poor video quality resulting in inability to score certain facial action units (Leach et al., 2012). However, these can be overcome with refinement of the methodology and improved technology, and the Mouse Grimace Scale is still an attractive alternative to the current gold standard.
1.6.3. Challenges with pain assessment in laboratory mice

Pain identification and assessment in laboratory mice can be much more challenging than one might anticipate, and without sufficient knowledge and information regarding states of pain, distress, or discomfort of laboratory animals, provision of effective analgesia is challenging. Behaviour scoring has been shown to be effective in identifying pain in laboratory rats (Roughan and Flecknell, 2001, 2003) and mice (Wright-Williams et al., 2007) but the time and training required to carry out this technique makes behaviour scoring in a laboratory environment impractical. To overcome this hurdle, Roughan et al. (2009) investigated the use of the HomeCageScan device, automated behaviour recognition software, to identify a decrease in pain-related behavioural changes associated with the provision of analgesia. The use of this system allows for automated behavioural scoring, avoiding or diminishing the need for well-trained technical staff, as well as saving time and money. The HomeCageScan software resulted in generation of data similar to that obtained through manual scoring, and therefore proved to be a fast and effective method to assess pain-related behaviours in mice. However, in a study by Leach et al. (2012), while the HomeCageScan readily differentiated between pre and postoperative mice, the software had difficulty differentiating between postoperative states of animals with varying analgesia. It was hypothesized that this may have been due to the algorithm that the HomeCageScan currently employs, which is based upon general changes in normal behaviour as opposed to observation of more pain-specific behaviours, which occurs with manually behaviour scoring. The behavioural changes observed between pre and postoperative observations could have been due to postoperative pain,
but could have also been due to generalized stress secondary to the surgical procedure itself. With refinement, the use of the HomeCageScan software may eventually prove to be a practical, cost effective manner by which to assess the analgesic needs of laboratory rodents, thereby allowing provision of appropriate analgesia. However, limitations include a limited repertoire of pain–specific behaviours (Roughan et al., 2009).
Table 1-1. Pharmacokinetic parameters (mean) of meloxicam in the mouse, rat, and rabbit following a single oral dose

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Mouse(^1) (10 mg/kg)</th>
<th>Rat(^1) (1.0 mg/kg)</th>
<th>Rabbit(^2) (1.5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioavailability</td>
<td></td>
<td>94%</td>
<td>~ 95%</td>
<td>-</td>
</tr>
<tr>
<td>Area under the concentration-time curve (AUC)</td>
<td>mg*h/L</td>
<td>60.7</td>
<td>89.5</td>
<td>83.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>201.0</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elimination half-life (t(_{1/2}))</td>
<td>h</td>
<td>4.8</td>
<td>4.5</td>
<td>49.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>52.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.4</td>
</tr>
<tr>
<td>Peak plasma concentration (C(_{max}))</td>
<td>mg/L</td>
<td>18.1</td>
<td>20.7</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
</tbody>
</table>

\(^1\) Busch et al., 1998

\(^2\) Turner et al., 2006
CHAPTER TWO: AQUEOUS STABILITY AND ORAL PHARMACOKINETICS OF MELOXICAM AND CARPROFEN IN MALE C57BL/6 MICE

This chapter corresponds to the following manuscript: Ingrao JC, Johnson R, Tor E, Gu Y, Litman M, Turner PV. Aqueous stability and oral pharmacokinetics of meloxicam and carprofen in male C57BL/6 mice. J Am Assoc Lab Anim Sci. 2013; 52:553-559.

2.1. ABSTRACT

We found that carprofen and meloxicam under 3 environmental conditions (room temperature ambient light, room temperature dark, and 4 °C dark) remained stable for at least 7 d. We then evaluated the oral pharmacokinetics of meloxicam (20 mg/kg) and carprofen (10 mg/kg) in male C57BL/6 mice after oral gavage or administration in the drinking water. Mice did not drink meloxicam-medicated water but readily consumed carprofen-medicated water, consuming an average of 18.4 mL carprofen-medicated water per 100 g body weight daily; mice drank more during the dark phase than during the light phase. Plasma analyzed by HPLC (meloxicam) and tandem mass spectrometry (carprofen) revealed that the peak meloxicam and carprofen concentrations were 16.7 and 20.3 µg/mL and occurred at 4 and 2 h after oral gavage, respectively. Similar blood concentrations were achieved after 12 h access to the carprofen-medicated water bottle. At 24 h after oral gavage, the drugs were not detectable in plasma. Meloxicam plasma area under the concentration-time curve, elimination half-life, apparent volume of distribution, and apparent oral clearance were 160.4 ± 2.8 mg*h/L, 7.4 ± 2.0 h, 0.36 ± 0.07 L/kg, and 0.125 ± 0.003 mL/h × kg, respectively. Carprofen plasma area under the concentration-time curve, elimination half-life, apparent volume of distribution, and
apparent oral clearance were 160.8 ± 14.6 mg*h/L, 7.4 ± 4.6 h, 0.42 ± 0.12 L/kg, and 0.062 ± 0.005 mL/h × kg, respectively. No gross or microscopic evidence of toxicity was seen in any mouse. These findings indicate that carprofen can be administered in drinking water to mice and that medicated water bottles should be placed 12 to 24 h prior to painful procedures.
2.2. INTRODUCTION

The provision of appropriate and effective analgesia for research rodents is a constant challenge for veterinary care personnel (Anil et al., 2002; Karas, 2006; Turner et al., 2011a, 2011b). Several barriers exist that may hinder the administration of sufficient analgesia, including appropriate training for administering analgesics, the possibility for inducing handling stress, inter-strain and inter-individual variability in response to, and metabolism of, a given analgesic substance, and the different levels of analgesic required after any one of the numerous procedures or experimental conditions that any given research mouse may undergo (Flecknell, 2009). Furthermore, little information is available in the literature regarding the pharmacokinetics of commonly used nonsteroidal anti-inflammatory drugs (NSAIDs) in laboratory mice or after alternative routes of administration. Information regarding method of administration, dose ranges, and dosing intervals is often extrapolated from other species; however, the extent of interspecies variability is often unknown.

Carprofen is an NSAID derived from propionic acid and has anti-inflammatory, antipyretic, and analgesic properties. The compound has a chiral center at the $\alpha$-carbon position and exists as a racemic mixture composed of R (−) and S (+) enantiomers (Stoltenborg et al., 1981), which is a complicating factor in pharmacokinetic investigations. In all species studied to date, the R enantiomer is less potent than is the S enantiomer, and there is minimal conversion between enantiomers in vivo (Lees and Landoni, 2002). As with other NSAIDs, the positive pharmacologic effects of carprofen
arise through species-specific differential inhibition of COX isomers. The pharmacokinetics of carprofen have been evaluated in several mammalian species, including rats (Kemmerer et al., 1979; Iwakawa et al., 1991) cows (Lohuis et al., 1991; Delatour et al., 1996a), dogs (McKellar et al., 1994), cats (Taylor et al., 1996), sheep (Welsh et al., 1992; Cheng et al., 2003), horses (Lohuis et al., 1991; Soraci et al., 1995; Delatour et al., 1996b; Mealey et al., 2004), rabbits (Hawkins et al., 2008), and humans (Stoltenborg et al., 1981). Most of these studies evaluated pharmacokinetic parameters after intravenous (IV) or SC administration (McKellar et al., 1994; Soraci et al., 1995; Kay-Mugford et al., 2000; Clark et al., 2003; Ingvast-Laesson et al., 2011; Turner et al., 2006), whereas others evaluated the pharmacokinetics of carprofen after oral administration (Stoltenborg et al., 1981; McKellar et al., 1994). Evidence regarding the relative COX-2 selectivity of carprofen is inconsistent throughout the literature (Kay-Mugford et al., 2000), and in vitro studies have suggested that carprofen is a non-selective inhibitor of COX-2 in horses but a selective COX-2 inhibitor in cats (Brideau et al., 2001). These findings serve to further emphasize the potential for interspecies variability in the pharmacokinetics of carprofen and the need for species-specific dosage information.

Meloxicam is an enolic acid NSAID derivative that, similar to carprofen, has anti-inflammatory, analgesic, and antipyretic properties (Engelhardt et al., 1995). Its anti-inflammatory action is achieved through selective inhibition of COX-2, which may contribute to reducing the gastrointestinal side effects that may be seen with other NSAID. Meloxicam has a long half-life in many species, thereby supporting once-daily
administration and making the drug an attractive veterinary analgesic (Engelhardt et al., 1995). The pharmacokinetics of meloxicam have been evaluated in several mammalian species, including, but not limited to, rats (Schmid et al., 1995), horses (Toutain et al., 2004; Sinclair et al., 2006), cows (Mosher et al., 2012), goats (Shukla et al., 2007; Ingvast-Larsson et al., 2011), sheep (Shukla et al., 2007), pigs (Fosse et al., 2008, 2011), dogs (Budsberg et al., 2002; Montoya et al., 2004; Yuan et al., 2009), donkeys (Mahmood and Ashraf, 2011), chickens (Baert K and De Backer, 2002), humans (Schmid et al., 1995), and some exotic and wild species such as camels (Wasfi et al., 2012), iguanas (Divers et al., 2010), and vultures (Naidoo et al., 2008).

Both carprofen and meloxicam typically are administered to mice SC, but there are anecdotal reports of the provision of NSAIDs in the drinking water. This method of administration is an attractive option, because it potentially could ensure that adequate and stable plasma drug levels are achieved throughout a period of analgesic need and would remove the handling stress associated with SC injection or oral gavage (Meijer et al., 2006; Cinelli et al., 2007; Hurst and West, 2010). To date, no data have been published to support this route of administration for NSAIDs in mice. Before adopting this practice, it is important to determine whether meloxicam and carprofen are stable when diluted in water, whether rodents consume medicated water, and whether plasma drug levels obtained after this route of administration are comparable to those of other methods of administration. The current study evaluated the stability of carprofen and meloxicam in water under various environmental conditions and assessed the palatability and oral pharmacokinetics of both compounds when administered in the drinking water.
and by oral gavage. In addition, mice were evaluated for evidence of acute NSAID-related toxicity in gastrointestinal, hepatic, and renal tissues.
2.3. MATERIALS AND METHODS

2.3.1. Animals

Male 6 w old C57BL/6 mice (*Mus musculus*) were obtained from Charles River Laboratories (St Constant, PQ, Canada). Mice weighed 20.63 ± 1.47 g (mean ± SD) at study initiation and were housed in groups of 4 in standard polycarbonate cages on corncob bedding (Teklad Corn Cob Bedding, Harlan Teklad, WI, USA). All mice were housed in the Central Animal Facility at the University of Guelph, under a reverse 12:12 h light: dark cycle at 21 ± 1 °C and humidity 50.7 ± 3.7%. Mice were acclimated to the reverse 12:12 h light: dark cycle for 7 d prior to study initiation. Each cage contained a clear polycarbonate hut, a cotton nesting square, and a small handful of crimped paper. Food (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Teklad, WI, USA) and tap water were provided ad libitum. Vendor health surveillance reports indicated that animals were free from mouse adenovirus, mouse hepatitis virus, mouse parvoviruses, mouse rotavirus, mouse norovirus, Theiler murine encephalomyelitis virus, *Bordetella bronchiseptica*, *Citrobacter rodentium*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Salmonella* spp., *Helicobacter* spp., *Klebsiella* spp., *Pasteurella* spp., *Staphylococcus aureus*, and *Streptococcus* spp., ectoparasites, endoparasites, and enteric protozoa. The University of Guelph Animal Care Committee approved the animal use protocol, and the facility and procedures are in compliance with the Animals for Research Act of Ontario and the guidelines of the Canadian Council on Animal Care (CCAC, 1993).
2.3.2. Experimental design

For the pharmacokinetic study, 160 mice (n = 4 per cage) were randomized into 1 of 4 treatment groups (n = 40 per treatment group): 20 mg/kg meloxicam by oral gavage, 20 mg/kg meloxicam by water bottle, 10 mg/kg carprofen by oral gavage, or 10 mg/kg carprofen by water bottle. Oral gavage was used for comparison to an enteric mode of administration. Mice had access to a medicated water bottle from time 0 to 36 h. At 0, 5, 15, 30, and 60 min and 2, 4, 8, 12, 24, and 36 h after administration, 4 mice (one cage) were anesthetized by using isoflurane (Aerrane, Baxter, ON, Canada) in oxygen, and exsanguinated by cardiocentesis. Blood was collected into EDTA-(carprofen) or heparin-(meloxicam) coated tubes (Sarstedt, PQ, Canada).

2.3.3. Preparation of NSAID stability samples

To evaluate the stability of meloxicam when diluted, 2.34 mL of meloxicam (5 mg/mL, pH 8.0 – 9.7; Metacam injectable, Boehringer Ingelheim, ON, Canada) was added to 87.66 mL reverse-osmosis–purified water to yield a final solution concentration of 0.130 mg/mL. This solution was divided into 3 glass flasks, and one each was stored in ambient light at room temperature, dark (wrapped in foil) at room temperature, and 4 °C dark conditions. Darkness was achieved by wrapping flasks in foil. A 1 mL sample was collected from the bottom of each flask daily for 7 d and frozen at −80 °C until further analysis. Solutions were not stirred or agitated prior to sampling.

For carprofen solutions, 0.12 mL carprofen (50 mg/mL, pH 7 – 7.4; Rimadyl injectable, Pfizer Canada, PQ, Canada) was added to 89.88 mL of reverse-osmosis–purified water to
make a final solution concentration of 0.067 mg/mL. Solutions were stored and collected as described for meloxicam. Because of cost constraints, only samples from days 1, 3, and 7 after preparation were analyzed for carprofen concentration.

2.3.4. Preparation of NSAID dosing solutions

All mice were weighed 24 h prior to study initiation. Doses were calculated based on average body weight per group. For groups dosed by water bottle, dose concentrations were based on the assumption that mice would consume 15 mL per 100 g body weight every 24 h (Harkness et al., 2010). Seventeen bottles containing the NSAID in water were placed on cages at time 0; bottles containing untreated water were removed. The final solutions contained 0.13 mg/mL meloxicam or 0.067 mg/mL carprofen, with reverse-osmosis-purified water as the diluent. Room temperature and humidity were controlled and constant throughout the study in order to minimize effect on water intake. For oral gavage studies, mice were gavaged by using a volume of 5 mL/kg and a 22-gauge stainless steel gavage needle. All solutions were prepared immediately prior to administration. The final meloxicam solution had a mild, acrid odor, whereas the carprofen solution had no odor. Water consumption was calculated on a per-animal basis by subtracting the weight of the water bottle at the times of blood collection from the weight of the water bottle before drug administration. This value was then divided by the number of mice per cage.

2.3.5. Determination of plasma meloxicam concentration

After blood collection, samples were placed on ice immediately and then centrifuged to
separate plasma, which was frozen at −80 °C until further analysis. Meloxicam plasma concentrations were determined by HPLC. Briefly, samples were prepared by combining 100 µL plasma, 10 µL of the internal standard solution (piroxicam, 10 µg/mL in methanol; Sigma-Aldrich, PQ, Canada), 10 µL 1 N HCl, and 1 mL diethyl ether. The mixture was vortexed and centrifuged, and the upper layer was collected and evaporated under a constant flow of nitrogen. The residue was reconstituted in 100 µL of the mobile phase, and 50 µL was injected into the analytical column.

The Liquid Phase Separation and Assay Facility (Ontario Veterinary College, University of Guelph, ON, Canada) analyzed the samples. A chromatograph (Waters Alliance 2695, ON, Canada) with a photodiode array detector (model 2996, Waters) was connected to Empower 2 software (Waters) for data collection and processing. An analytical column (50 mm × 4.6 mm; inner diameter, 2.5 µm; Sunfire C18, Waters, Wexford, Ireland) was connected to a guard column (4 mm × 3.0 mm; Security Guard C18, Phenomenex, CA, USA), and isocratic chromatographic separation was conducted by using a mobile phase containing acetonitrile–water–acetic acid (60:40:1, v/v/v) at a flow rate of 1 mL/min.

Calibration curves were prepared by spiking 100 µL untreated (control) mouse plasma with 10 µL meloxicam (Sigma-Aldrich, ON, Canada) to create working solutions (5, 10, 25, 50, and 75 µg/mL) and 10 µL of piroxicam internal standard solution (10 µg/mL). The limit of detection was 0.25 µg/mL, on the basis of 3 times the signal-to-noise ratio at the time of analyte elution. The limit of quantitation for the assay was 0.5 µg/mL. Average recovery performed at 5 reference standard levels was 98.23 %
2.3.6. **Determination of plasma carprofen concentration**

After blood collection, samples were placed on ice, blood was centrifuged, and plasma was collected and frozen at −80 °C until further analysis. Prior to analysis, samples were prepared by extracting 0.1 mL plasma with 1 mL 1% ascorbic acid in 0.1 N HCl and 10 mL ethyl acetate. An aliquot (8 mL) of this extract was evaporated to dryness using a nitrogen evaporator (N-Evap Analytical Evaporator, Organomation Association, MA, USA) set at 60 °C. The residue was re-dissolved in 0.2 mL of the internal standard solution, 1 µg/mL d3-carprofen in 50% methanol:water (v/v; Toronto Research Chemicals, ON, Canada). The mixture was vortexed and then filtered through a 0.22-µm HPLC filter (Millipore, MA, USA) into a glass autosampler vial for analysis.

The extract was analyzed by using an HPLC system (Microm BioResources, CA, USA) coupled with a hybrid triple quadrupole–linear ion trap mass spectrometer (model 4000 Q TRAP, AB SciEx, ON, Canada). The HPLC column was a 20 × 2 × 3 mm C18 Column2 (Luna C18, Phenomenex, CA, USA), with a mobile phase containing 0.01 M ammonium acetate in 0.1% formic acid in water (A solution) and 0.01 M ammonium acetate in 0.1% formic acid in methanol (B solution) at a flow rate of 200 µL/min under a linear gradient of 50% B to 95% B over 7 min. The analysis for carprofen was completed by the California Animal Health and Food Safety Laboratory System Toxicology Laboratory (UC Davis School of Veterinary Medicine, CA, USA).

HPLC methods were validated prior to assay, and calibration curves were prepared by
spiking 1 mL untreated (control) mouse serum with 50 µL of 10 µg/mL carprofen standard (Toronto Research Chemicals, ON, Canada) and analyzed by using the method described. The limits of quantitation for the assay were 0.50 µg/mL and 1.3 µg/mL for drinking water and gavage samples, respectively. Recovery was 99% with 4% coefficient of variance (relative standard deviation).

2.3.7. **Microscopic evaluation of tissues**

After euthanasia by isoflurane–oxygen anesthesia and exsanguination, liver, lung, kidneys, stomach, and duodenum were removed from each animal and fixed in 10% buffered formalin. Tissues were routinely trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic evaluation. Slides were randomized and evaluated by a single blinded observer.

2.3.8. **Data analysis**

Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, IL). For all statistical analyses, only significant results are reported. Data is presented as mean ± SEM. Significance was set at \( p < 0.05 \). One-way ANOVA was used to determine significant differences in drug concentration in water between the 3 tested environmental conditions and by sampling day. A significant effect of day was evaluated further by using individual Student t-tests.

Determinations of maximal plasma meloxicam and carprofen concentrations and time to maximal concentration were determined by direct observation of data. Pharmacokinetic
parameters (plasma area under the concentration-time curve; elimination rate constant; elimination half-life; apparent volume of distribution; and apparent oral clearance) were determined using non-compartmental analyses. The area under the concentration-time curve (AUC) was calculated by using the trapezoidal rule and was extrapolated to infinity. The elimination rate constant was calculated by determining the rate of change (elimination) of the logarithmic linear regression of the plasma concentration–time curve. The elimination half-life was calculated by dividing 0.693 by the elimination constant. Apparent volume of distribution was calculated by dividing the dose of drug administered by the product of the elimination constant and the AUC (Craig and Stitzel, 2004). Note that the apparent volume of distribution is estimated as the volume of distribution divided by the bioavailability factor (from 0 to 1), because the exact amount of drug that enters the systemic circulation after oral administration is unknown (Toutain and Bousquet-Melou, 2004b). Apparent oral clearance was calculated by dividing the dose by the AUC (Craig and Stitzel, 2004).
2.4. RESULTS

Both injectable meloxicam and carprofen were stable for 7 d when diluted in reverse-osmosis–purified water and held under ambient light at room temperature, dark at room temperature, and dark at 4 °C (Figure 2-1). Analyte concentrations of solutions on a given day did not differ according to the environmental conditions under which the solutions were held or between days 1 and 7. Meloxicam concentration did not differ by day. There was a significant effect of day on carprofen concentration, which occurred between days 0 and 3 (p = 0.008) and days 0 and 7 (p = 0.004).

In general, mice consumed little or no meloxicam-treated drinking water over the 36 h test period (Figure 2-2a). In contrast, mice readily consumed carprofen-treated drinking water over the 36 h study, drinking an average of 18.4 mL per 100 g body weight every 24 h (Figure 2-2b). Mice consumed more carprofen-treated drinking water during the dark phase (17.5 mL per 100 g body weight every 24 h during 0 to 12 h and 24 to 36 h) than during the light phase (7.7 mL per 100 g body weight every 24 h during 12 to 24 h). Furthermore, mice drank more carprofen-treated drinking water during the first dark phase (14.6 mL per 100 g body weight over 12 h) than during the second dark phase (2.85 mL per 100 g body weight over 12 h).

After a single oral dose of 20 mg/kg meloxicam by gavage, the peak plasma concentration of 16.7 ± 0.4 µg/mL was achieved at 4 h after administration (Figure 2-3a). After a single oral dose of 10 mg/kg carprofen by gavage, the peak concentration of 20.3
± 2.4 µg/mL was reached at 2 h after administration, whereas the peak plasma concentration of 17.0 ± 2.9 µg/mL was achieved after 12 h of exposure to the carprofen-medicated water bottle (Figure 2-3b). For both NSAIDs, plasma concentrations after oral gavage rapidly declined to near undetectable levels (meloxicam, 1.3 ± 0.3 µg/mL; carprofen, 0 µg/mL) at 24 h after administration. The plasma carprofen concentration continued to increase with continued exposure to the medicated water bottle. Table 2-1 provides the pharmacokinetic parameters for meloxicam and carprofen after oral gavage.

The half-lives of both compounds were similar, the apparent volume of distribution of carprofen was slightly greater compared with that obtained for meloxicam, and the clearance of carprofen was half that of meloxicam (Table 2-1). There were no gross or microscopic changes indicative of NSAID-related toxicity in any tissue examined.
2.5. DISCUSSION

We have demonstrated that mice will readily consume carprofen-treated drinking water and that the maximal plasma concentration of 10 mg/kg doses at 2 h after gavage and at 12 h after the addition of treated water bottles to cages are similar. The plasma concentration of carprofen was more sustained when administered in the drinking water than by oral gavage. Dilutions of carprofen in water are stable for at least 7 d in the light or dark at room temperature, suggesting that this administration technique could readily be used in a vivarium as a replacement for individual dosing. Carprofen appeared to be stable in reverse-osmosis-purified water.

Mice did not consume injectable meloxicam when it was diluted in water, thus it is not suitable for administration in drinking water. We did not evaluate whether mice would consume the oral suspension of meloxicam when diluted in water because this route would be cost-prohibitive for providing analgesia to large numbers of mice on a study and because the commercially available oral suspensions are too dilute to reach effective concentrations when administered in the drinking water.

The current study evaluated total plasma concentration of carprofen, and as such, comments about the pharmacokinetic parameters obtained are somewhat limited. The comparative potency and metabolism of the R- and S- enantiomers of carprofen have not been reported for mice, and enantiomer concentrations were not evaluated in the current study. Within a given species, the ratio of the enantiomers is constant, but whether
enantiomer shifting occurs when carprofen is diluted in aqueous solutions is unknown (Maire-Gauthier et al., 1998). Meloxicam and carprofen appear to have similar pharmacokinetics in mice, but based on the total clearance rate, carprofen has a low to moderate blood clearance rate, indicating it is a suitable candidate for oral administration in this species (Toutain and Bousquet-Melou, 2004a). The rapid time to peak plasma concentration and modest half-life of carprofen in mice suggest that a dosing interval more frequent than once daily may be needed to maintain plasma drug levels. This conclusion has been indirectly supported by a recent study, which showed that the analgesic effect of carprofen at 20 mg/kg SC in mice after laparotomy was unlikely to last beyond 8 h (Matsumiya et al., 2012). Tissue drug levels and efficacious plasma drug levels are unknown for carprofen in mice and are required for the determination of an appropriate interval for oral dosing. The plasma drug concentration seen after the provision of carprofen-treated drinking water may indicate accumulation of carprofen over time, potentially related to overwhelming hepatic metabolism through repeated dosing or through enterohepatic recycling and accumulation. This is considerable for carprofen in other species (Priyenko et al., 1998; Clark et al., 2003). The total plasma concentration of carprofen continued to increase when the water bottle was left on the cage to 36 h. Although this increase may prompt concerns regarding potential toxicity, no gross or microscopic changes suggestive of acute toxicity were noted in any animal at any time point to suggest acute toxicity, and this increase may just reflect the maintenance of a therapeutic plasma carprofen concentration over a longer period with this method of dosing.
Given the stereoselective effects of the enantiomers, the total carprofen plasma levels we obtained likely overestimate the plasma levels of the more efficacious S(+) enantiomer. However, since these drugs are being compounded with water, it is unknown whether alterations in physicochemical properties are taking place. No studies have evaluated these changes which could, in theory, influence potency, absorption kinetics, or even result in production of toxic molecules. In addition, the effect of pH on chiral conversion of carprofen is unknown.

The LD50 for oral carprofen is 30 mg/kg daily in nulliparous rats and 19 mg/kg daily in lactating rats (McClain and Hoar, 1980). Similar to dogs, rats demonstrate significantly different carprofen pharmacokinetics than those in mice, with a terminal half-life as long as 40 h and with increased sensitivity to toxic effects in lactating animals (McClain et al., 1980). The LD50 for oral carprofen is not reported for mice. Additional studies are required to determine the extent of accumulation that would occur over time should mice receive access to carprofen-medicated water bottles for longer than 36 h. Pathologic changes should also be sought. In addition, correlative efficacy studies are required to determine therapeutic plasma carprofen concentration in mice.

Current knowledge about murine behaviour predicts that mice will drink more water during the dark phase than the light phase (Harkness et al., 2010), which was seen in the current study. Interestingly, significantly less carprofen-medicated water was consumed during the second dark phase than the first. The amount consumed during the second dark phase was also less than that consumed during the light phase. The reason for this
difference is unknown. Carprofen-treated drinking water may be very palatable to mice, and mice may have overhydrated during the first 12 h of access, resulting in lower consumption subsequently. Unaccounted leakage of bottles when they are inverted onto cage lids is typically minimal (a few drops) and is unlikely to account for the difference we noted between the two dark periods. The overall consumption was almost precisely that predicted over a 24-h period (15 mL/100 g body weight), suggesting that the difference in the volume of water consumed was not due to a sudden change in palatability. At our facility, carprofen-treated drinking water has been given to female mice with litters in an attempt to provide analgesia to pups prior to painful procedures, such as ear notching. Whether female mice or mice of different strains or ages consume the medicated water at a similar rate to that of the male mice in the current study, or whether carprofen concentration in the milk is similar to plasma levels are unknown. Additional studies are required to investigate whether carprofen-treated water is more palatable to mice than is non-medicated water, whether addition of carprofen to the water effects thirst, and whether mice of different strains or sex consume carprofen-treated water at similar rates.

The oral pharmacokinetics of meloxicam (10 mg/kg) in male and female mice have previously been investigated (Busch et al., 1998). Peak plasma concentrations of 18.1 and 20.7 mg-eq/L were achieved at 0.7 and 0.6 h after administration for male and female mice, respectively. This study achieved a similar peak plasma concentration; however, the time to peak plasma concentration was approximately 3 times that seen previously (Busch et al., 1998). Unlike the mice in the previous study, the mice were not fasted prior
to dosing, and this variation may account for much of the difference in time to peak plasma concentration. In addition, the study by Busch et al. (1998) used only female albino mice, whereas we used male mice of a different strain (C57BL/6), therefore sex or strain variation may be a factor. As expected, the AUC in the current study was much larger, given that the dose was twice that used in the earlier study (Busch et al., 1998). Importantly, the previous study demonstrated good (94%) oral bioavailability of the drug, indicating that the apparent clearance and volume of distribution values obtained in the current study likely are very close to the actual values. We evaluated the pharmacokinetics of meloxicam and carprofen after oral dosing. However, the clinical efficacy of the drugs given at these doses and by this route of administration has not yet been assessed. This information gap warrants further study.

In summary, both meloxicam and carprofen are stable in aqueous solutions when held for 7 d in dark, light, and cold environmental conditions. Single doses of meloxicam or carprofen in mice are cleared at rates that require greater than once-daily dosing to maintain therapeutic plasma drug levels. Drinking water containing carprofen (10 mg/kg) is palatable to mice, whereas aqueous solutions of injectable meloxicam (20 mg/kg) are highly unpalatable. To achieve peak plasma drug levels similar to those obtained after oral gavage, carprofen-containing water bottles should be placed on mouse cages at least 12 h prior to painful procedures.
Table 2-1. Pharmacokinetic parameters after oral gavage of meloxicam or carprofen to male C57BL/6 mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>Meloxicam* (20 mg/kg)</th>
<th>Carprofen* (10 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak plasma concentration ($C_{max}$)</td>
<td>µg/mL</td>
<td>16.7 ± 0.4</td>
<td>20.3 ± 2.4</td>
</tr>
<tr>
<td>Time to peak plasma concentration ($T_{max}$)</td>
<td>h</td>
<td>4.0 ± 0.0</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>Area under the concentration-time curve (AUC $_{0-\infty}$)</td>
<td>mg*h/L</td>
<td>160.4 ± 2.8</td>
<td>160.8 ± 14.6</td>
</tr>
<tr>
<td>Elimination half-life ($t_{1/2}$)</td>
<td>h$^{-1}$</td>
<td>7.4 ± 2.0</td>
<td>7.4 ± 4.6</td>
</tr>
<tr>
<td>Apparent volume of distribution (Vd/F)</td>
<td>L/kg</td>
<td>0.36 ± 0.07</td>
<td>0.42 ± 0.12</td>
</tr>
<tr>
<td>Apparent oral clearance (Cl)</td>
<td>mL/kg/h</td>
<td>0.125 ± 0.003</td>
<td>0.062 ± 0.005</td>
</tr>
</tbody>
</table>

* n = 4/group
Stability of injectable (A) meloxicam (0.130 mg/mL) and (B) carprofen (0.067 mg/mL) solutions diluted in reverse-osmosis-purified water and held in ambient light at room temperature, dark at room temperature, and in dark refrigerated (4 °C) environmental conditions for 7 d. Both NSAIDs were stable under all conditions tested for 7 d.

Figure 2-1. Concentration of NSAID in water over 7 d
Figure 2-2. Cumulative consumption of NSAID in water over 36 h

Mice readily consumed carprofen in water but refused to drink meloxicam in water. Mice drank an average of 18.4 mL carprofen solution per 100g body weight in 24 h. Mice consumed more water during the dark phase (0 to 12 h and 24 to 36 h in this study) than during the light phase. n = 40 per group.
Drug concentration of (A) meloxicam in plasma and (B) carprofen in plasma for 36 h following a single oral dose (gavage) or during constant dosing through the drinking water of 20 mg/kg meloxicam or 10 mg/kg carprofen. After 12 h of constant dosing, a plasma concentration similar to the peak concentration obtained following a single oral dose is obtained. n = 40 per group.
3.1. ABSTRACT

Providing analgesia to laboratory mice can be laborious, and may induce unwanted handling stress. Administration of carprofen via the water bottle is an attractive method to provide analgesia to laboratory mice as it eliminates many of the shortcomings associated with other methods of administration. The current study evaluated the clinical efficacy of carprofen administrated via the water bottle following ovariectomy. The Mouse Grimace Scale was used in parallel with behaviour scoring to determine the clinical efficacy of carprofen given via the water bottle and subcutaneously following ovariectomy. Mice received 10 or 20 mg/kg carprofen orally via a medicated water bottle over 24 h prior to surgery, 10 or 20 mg/kg carprofen subcutaneously immediately postoperative, or saline immediately postoperative. Mice were video recorded for 36 h postoperative. Video recordings were evaluated using behaviour scoring and the Mouse Grimace Scale. Neither the Mouse Grimace Scale nor behaviour scoring detected a significant main effect of treatment group. The duration of time spent performing behaviours varied between treatment groups at individual points over 36 h postoperative. All active behaviours (swim, dig, walk, climb, rear) increased following surgery up to the 36 h postoperative, all mice demonstrated pain-associated grimacing immediately postoperative.
Mice demonstrated pain-associated grimacing at 5, 15, 30, and 1 h following surgery in the saline-treated group, at 0, 5, 15, and 30 min following surgery in the 10 mg/kg subcutaneous and per os groups, and at 0, 5, and 15 min in the 20 mg/kg subcutaneous and per os groups. The lack of significant difference in pain-associated facial grimacing between treatment groups may be due to insufficient doses, or lack of sensitivity of the method of pain-identification used. The lack of clinical efficacy at the doses evaluated warrants additional studies to identify ideal doses of carprofen to achieve analgesia in the laboratory mouse.
3.2. INTRODUCTION

Reducing unnecessary suffering in laboratory animals is required in biomedical research. However, providing analgesia to laboratory mice presents several unique challenges. Their small size often necessitates full restraint which requires time, skill, and training. Handling stress alone has both welfare and research implications by altering physiologic parameters (Meijer et al., 2006; Cinelli et al., 2007; Hurst and West, 2010; O'Mahony et al., 2010). Experiments with mice often involve large numbers and means increased time, costs, and labor for researchers and laboratory personnel. These challenges may impede provision of complete, sufficient analgesia and can result in preventable states of pain and discomfort.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are given to mice to reduce pain and inflammation, but there is a lack of crucial pharmacokinetic and physiologic data. The dose and effect is extrapolated from other species, and modes of administration are presumed to be efficacious. There are anecdotal reports of administration of NSAIDs through the drinking water in laboratory rodents; however, no studies have demonstrated clinical efficacy.

Carprofen is a propionic acid based NSAID whose properties arise through selective cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibition (Ricketts et al., 1998; Kay-Mugford et al., 2000; Brideau et al., 2001; Streppa et al., 2002). The α-carbon in carprofen is a chiral center, resulting in a racemic mixture of R(-) and S(+).
enantiomers (Stoltenborg et al., 1981). The current recommendation for carprofen use in mice is subcutaneous (SC) administration, where doses range from 2 - 5 mg/kg every 12 to 24 h (Gaertner et al., 2008; Flecknell, 2009; Harkness et al., 2010; Quesenberry and Carpenter, 2012). However, Matsumiya et al. (2012) recently demonstrated a lack of clinical efficacy at doses less than 20 mg/kg SC.

Behavioural observations and assessment of pain-associated behaviours is the current gold standard for pain-assessment in mice. Specifically, pain composite scores, comprised of several individual pain-associated behaviours, are the current gold standard for pain identification in mice (Wright-Williams et al., 2007; Leach et al., 2012). However, behaviour can be altered by a variety of internal and external factors, which can confound behavioural observations. Confounding factors include: strain (Elmer et al., 1998; Mogil et al., 1999; Lariviere et al., 2002; Dickinson et al., 2009), drugs (Roughan and Flecknell, 2004), and surgical procedure (Roughan and Flecknell, 2001; Wright-Williams et al., 2007). In addition, behaviour scoring is laborious and time consuming. In contrast, the Mouse Grimace Scale is a relatively new alternative with reports of ease of application. Since the publication of the Mouse Grimace Scale in 2010 (Langford et al., 2010), facial grimace scales for pain identification have been developed in the rat (Sotocinal et al., 2011), rabbit (Keating et al., 2012), and horse (Dalla Costa et al., 2014).

Administration of carprofen in drinking water does not require handling of the mice, reduces stress and increases animal welfare (Hurst and West, 2010). Further, it does not require the time, training, or skill required with other routes of administration.
Administration of carprofen to mice through the drinking water (10 mg/kg) was shown to result in a peak plasma concentration following 12 to 24 h exposure to a medicated water bottle (Chapter 2: Ingrao et al., 2013), however clinical efficacy has not yet been demonstrated. The current study aimed to use behaviour scoring and the Mouse Grimace Scale to determine if administration of carprofen though the drinking water is clinically efficacious following ovariectomy.
3.3. MATERIALS AND METHODS

3.3.1. Animals

Female 12 w CD1 mice (*Mus musculus*) were obtained from Charles River Laboratories (St Constant, PQ, Canada). Mice weighed 26.28 ± 0.22 g (mean ± SD) at study initiation. All mice were housed in the Central Animal Facility at the University of Guelph, under a reverse 12:12 h light: dark cycle at 21 ± 1 °C and humidity 50.7 ± 3.7%. Food (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Teklad, WI, USA) and water (tap) were provided ad libitum. Prior to study initiation, mice were housed in groups of four in standard polycarbonate cages on corncob bedding (Teklad Corn Cob Bedding, Harlan Teklad, WI, USA). Each cage contained a clear polycarbonate hut, cotton nesting square, and a small handful of crimped paper. Mice were acclimated for at least 7 d prior to study initiation. Vendor health surveillance reports indicated that animals were free from mouse adenovirus, mouse hepatitis virus, mouse paroviruses, mouse rotavirus, mouse norovirus, Theiler murine encephalomyelitis virus, *Bordetella bronchiseptica*, *Citrobacter rodentium*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Salmonella* spp., *Helicobacter* spp., *Klebsiella* spp., *Pasteurella* spp., *Staphylococcus aureus*, and *Streptococcus* spp., ectoparasites, endoparasites, and enteric protozoa. The University of Guelph Animal Care Committee approved the animal use protocol prior to study initiation. The facility and procedures were in compliance with the Animals for Research Act of Ontario and the guidelines of the Canadian Council on Animal Care (CCAC, 1993).
3.3.2. Treatment groups and dosing

Cages of mice (n = 4) were randomly assigned to one of 7 treatment groups: 10 or 20 mg/kg carprofen SC, 10 or 20 mg/kg carprofen per os (PO) via water bottle, saline SC, baseline in dark (no anesthesia, no surgery), or baseline in light (no anesthesia, no surgery). A total of 8 mice (2 cages) were assigned per treatment group (56 mice total). Average weight per cage prior to surgery was used to calculate doses. Injections of SC carprofen were diluted with sterile saline from 50 mg/mL (stock concentration) to 1 mg/mL, and were given immediately after surgical staple placement while mice were still under general anesthesia. Mice receiving carprofen orally gained access to a medicated water bottle 24 h prior to surgery initiation. Room temperature and humidity were controlled and constant at 21 ± 1 °C and humidity of 50.7 ± 3.7%. Concentration of carprofen in water was 0.067 mg/mL for the group receiving 10 mg/kg, and 0.135 mg/mL for the group receiving 20 mg/kg. Oral dosing was calculated based on an average consumption of 15 mL of medicated water per 100 grams body weight over 24 h (Harkness et al., 2010; Ingrao et al., 2013).

3.3.3. Surgery

Surgeries took place during the first 3 h of the dark-phase. A single experienced surgeon performed all surgeries. Anesthesia was induced with isoflurane in oxygen at 5% and 2 L/min, and maintained at 2% and 0.75 L/min. The caudal dorsum was shaved and scrubbed with chlorhexidine solution. A 1-cm midline incision was made through the skin and muscle, and fat dissected away to allow for identification of the left ovary. Once the left ovary was identified, it was clamped with a Halsted Mosquito hemostat for
approximately 10 s, and transected. The ovarian pedicle was observed for bleeding prior to replacement into the abdominal cavity. The technique was repeated on the right side through the same skin incision. The skin incision was closed with a sterile surgical staple (9mm wound clips, MikRon Precision, CA, USA). There were no intraoperative complications. Each surgery took approximately 6 min, from time of induction to recovery. Mice were recovered in a cage with a surgical drape and heating pad (Snuggle Safe, Lenric C21 Ltd., West Sussex, UK), and moved to their home cage containing corncob bedding and a small handful of crimped paper once actively moving on all limbs. The home cage was then taken to a separate room for video recording, which began immediately. Time from recovery to start of video recording was approximately 2 min.

3.3.4. Recording

Video recording took place in clear, polycarbonate home cages. The camcorder was placed 15 cm from the front of the cage. Mice were video recorded continuously from 0 to 75 min, and for 15 min intervals at 2, 4, 8, 12, 24, and 36 h following surgery using a JVC GZ-E200 (JVC Americas Corp, NJ, USA) camcorder. When recording took place during the dark phase, a red light was used to visualize mice. Two additional baseline control groups (n = 8), which did not undergo anesthesia or surgery, were recorded for a 10 min period in the dark and light phases.

3.3.5. Behavioural observations

The ethogram was modified from that of Wright-Williams et al. (2007) (Table 3-1). Approximately 20 h of recordings (randomly chosen) were analyzed for development of
the ethogram. Behaviours that were not detected were removed from the ethogram. Inability to detect behaviours was due to poor video quality, particularly in the dark phase. The following behaviours could not be observed and were omitted from the ethogram: stagger, twitch, shake, flinch, press, rear leg lift (Wright-Williams et al., 2007). Behaviours were observed over 10 min periods at the following time points after surgery: 0, 10, 30 min and 1, 2, 4, 8, 12, 24, and 36 h. All video recordings were randomized prior to behaviour scoring. Observations were carried out with the Observer XT program (Version 9.0, Noldus Information Technology B.V., Wageningen, Netherlands) by an individual blinded as to treatment group and time point. All behaviours were mutually exclusive. Random duplication of observations was used to calculate intra-rater agreement. A Cohen’s Kappa determined an excellent intra-rater agreement of $\kappa = 0.98$ ($p < 0.0001$).

3.3.6. **Mouse Grimace Scale scoring**

A single image was captured from the video recordings for each mouse at the following time points following surgery: 0, 5, 15, 30 min and 1, 2, 4, 8, 12, 24, and 36 h. In order to eliminate behaviour bias, images were cropped prior to analysis so only the head was visible. All images were randomized prior to analysis. Scores were obtained from three observers who were blinded as to treatment group and time point. Observers were trained prior to scoring images. Training was comprised of studying the scale published by Langford et al. (2010). Observers were then given 50 random images to score. If at least 90% of scores were identical to those of the trainer (myself), they were given consent to score the images for this study. If less than 90% of the scores were identical to mine,
another study session was completed and a new random set of 50 images was assigned. This process was repeated until at least 90% of scores were identical. Each image was assigned a score of 0 to 2 for each of the four facial action units. A score of 0 was assigned if the facial action unit was not present, 1 if it was moderately present, and 2 if severe (Table 3-2). The original Mouse Grimace Scale developed by Langford et al. (2010) was comprised of 5 facial action units. However, ‘whisker change’ was not included in the current study since whiskers could not be consistently identified in the video recordings.

3.3.7. Data analysis

All statistical analyses were carried out using SPSS 21.0 software (SPSS Inc., IL, USA). Statistical analyses could be carried out in a variety of different manners, but the methodology chosen was based on a consensus of statisticians. For behaviour scoring, the Observer XT program provided the duration of time spent performing each behaviour during each 10 min period at each time point. A repeated measures ANOVA (general linear model) determined the effect of treatment and time on time spent performing each behaviour over 10 time points (described in 3.3.5). One-way ANOVAs determined the effect of treatment at individual time points, and the effect of time within groups. No corrections for multiple comparisons were applied due to the conservative nature of Tukey post-hoc tests. Significance was set at p < 0.05. Changes over individual time points were conducted for the active behaviours only, due to the low duration and frequency of individual behaviours observed. The surgical staple for a single mouse fell out between the 8 and 12 h time points. Observations from this point forward were omitted from analyses (3 observations total). A treatment-identifier was present in a single video
recording, and observations from this recording were omitted from analyses (10 observations total). Due to the similarities between behaviours and their low frequency, certain behaviours were combined for statistical analyses. The following behaviours were combined: rear and rear/lean, scratch head and scratch body, groom head and groom body.

A Mouse Grimace Scale score was calculated for each image as the mean of the four facial action unit scores. If an observer was unable to score a facial action unit due to poor image quality, no score was assigned and the mean was adjusted accordingly. Final mouse grimace scores were continuous, which allowed the use of parametric analyses (ANOVA). A one-way ANOVA demonstrated a significant main effect of observer (p < 0.0001), and Tukey’s post-hoc tests demonstrated a significant interaction with one of the three observers (“Observer 3”). Therefore, data obtained from “Observer 3” was omitted from further statistical analyses. Intra-rater agreement was calculated by random duplication of images. A Cohen’s Kappa determined very good intra-rater agreements of $\kappa = 0.89$ (p < 0.0001) (Observer 1) and $\kappa = 0.83$ (p < 0.0001) (Observer 2), and a good inter-rater agreement of $\kappa = 0.60$ (p < 0.0001). Mouse Grimace Scale scores were also obtained from baseline mice (no surgery, no anesthesia) in both the dark and light phases. Baseline scores (scores obtained from mice who did not undergo anesthesia or surgery in both the light or dark phase) were subtracted from Mouse Grimace Scale scores to obtain mean difference scores (Langford et al., 2010; Matsumiya et al., 2012). Mean difference scores were compared to a mean difference score of “0” (no pain), and a repeated measures ANOVA (general linear model) determined the effect of treatment and time on
mean difference score over 10 time points (described in 3.3.6). Significance was set at $p < 0.05$. 
3.4. RESULTS

3.4.1. Behavioural observations

A repeated measures analysis revealed a significant main effect of time for all behaviours except swim, a significant time*treatment interaction for walk, stand, climb, scratch, rear, and active behaviours, and no significant main effect of treatment on any behaviours (Table 3-3). One-way ANOVAs determined whether there was a significant difference between time spent performing each behaviour between treatment groups at each time point for behaviours which had a time*treatment interaction: walk (Figure 3-1), stand (Figure 3-2), climb (Figure 3-3), scratch (Figure 3-4), rear (Figure 3-5), and active (Figure 3-6). There was significant difference in duration performing each behaviour at specific time points (Figures 3-2 to 3-6), but none differed from the saline-treated group immediately following surgery (interpreted as being pain-associated). Further analyses were performed only on the active behaviours composite, due to the short duration and low frequency of individual behaviours. Time spent performing active behaviours was significant higher in the 10 mg/kg SC treatment group at 30 min postoperative, and at 4 h postoperative in both the 10 mg/kg SC and 20 mg/kg SC compared to the mice receiving carprofen via water bottle. Saline-treated animals spent a significantly shorter duration of time performing active behaviours at 2 h postoperative compared to the 20 mg/kg SC group only. Overall, active behaviour increased over time from 0 to 36 hours following surgery in all treatment groups (Figure 3-6).
There was a significant difference in total time spent performing the ‘walk’ behaviour at 2h between the saline-treated group and all other groups (F(4,33) = 4.922, 10 SC p = 0.029, 20 SC p = 0.022, 10 PO p = 0.005, 20 PO p = 0.011), at 8 h between the 20 SC treatment group and the 10 PO (F(4,30) = 4.157, p = 0.016) and 20 PO (F(4,30) = 4.157, p = 0.030) groups, and at 12 h between the 20 SC treatment group and the saline (F(4,34) = 3.363, p = 0.027) and 20 PO groups (F(4,34), p = 0.030).

There was a significant difference in total time spent performing the ‘stand’ behaviour at 30 min between the 10 SC and 20 PO treatment groups, (F(4,33) = 3.024, p = 0.047), at 2 h between the saline-treated group and all other groups (F(4,33) = 15.202, 10 SC p < 0.0001, 20 SC p < 0.0001, 10 PO p < 0.0001, 20 PO p = 0.001), and at 12 h between the 20 SC treatment group and all other groups (F (4,34) = 8.445, saline p < 0.0001, 10 SC p < 0.0001, 10 PO p = 0.002, 20 PO p = 0.004).

There was a significant difference in total time spent performing the ‘climb’ behaviour at 30 min between the 10 SC treatment group and all other groups (F(4,33) = 7.321, saline p = 0.001, 20 SC p = 0.001, 10 PO, p = 0.003, 20 PO p = 0.001), at 4 h between the 10 SC treatment group and 10 PO (F(4,32) = 4.762, p = 0.004) and 20 PO (F(4,32) = 4.762, p = 0.011) groups, and at 8 h between the 10 SC treatment group and 20 SC (F(4,30) = 3.415, p = 0.031) and 10 PO (F(4,30) = 3.415, p = 0.024) groups.

There was a significant difference in total time spent performing the ‘scratch’ behaviour at 1 h between the 20 SC and 10 PO treatment groups (F(4, 35) = 2.884, p = 0.038), at 2 h
between the 20 SC treatment group and the saline (F(4,35) = 3.506, p = 0.021), 10 PO
(F(4,30) = 3.415, p = 0.042), and 20 PO (F(4,30) = 3.415, p = 0.043) groups, and at 12 h
between the 20 SC and 10 PO treatment groups (p = 0.028).

There was a significant difference in total time spent performing the ‘rear’ behaviour at
30 min between the 10 SC treatment group and the saline (F(4, 35) = 3.336, p = 0.047)
and 20 PO (F(4, 35) = 3.336, p = 0.027) groups, and at 12 h between the saline and 20 SC
treatment groups (F(4,35) = 4.779, p = 0.001).

There was a significant difference in total time spent performing ‘active’ behaviours
(composite) at 30 min between the 10 SC treatment group and the saline (p < 0.0001), 20
SC (p = 0.003), 10 PO (p = 0.023) and 20 PO (p < 0.0001) groups, at 2 h between the
saline and 20 PO treatment groups (p = 0.016), at 4 h between the 10 PO treatment group
and at the 10 SC (p = 0.031) and 20 SC (p = 0.040) groups, and at 12 h between the 20
SC treatment group and the saline (p = 0.007) and 10 SC (p = 0.018) groups.

In the saline-treated group, there was a significant increase in active behaviour between
the 0 min time point and 24 h (F(9,66) =14.865, p < 0.0001) and 36 h (F(9,66) =14.865, p
= 0.010) time points, between the 2 h time point and 24 h (F(9,66) =14.865, p < 0.0001)
and 36 h (p < 0.0001) time points, between both the 10 and 30 min time points and the 4
(p = 0.008; p = 0.001), 8 (F(9,66) =14.865, p = 0.025; p = 0.001), 12 (F(9,66) =14.865, p
= 0.002; p < 0.0001), 24 (F(9,66) =14.865, p < 0.001), and 36 h (F(9,66) =14.865, p <
0.001) time points, and between the 2 h time point and 24 h (F(9,66) =14.865, p < 0.0001) and 36 h (F(9,66) =14.865, p < 0.0001) time points.

In the 10 mg/kg SC group, there was a significant increase in active behaviour between the 0 (F(9,64) = 4.796, p = 0.021), 10 (F(9,64) = 4.796, p < 0.0001), 30 min (F(9,64) = 4.796, p = 0.013) and 1 h (F(9,64) = 4.796, p = 0.002) time points and 4 h, and between the 10 min (F(9,64) = 4.796, p = 0.004) and 1 h (F(9,64) = 4.796, p = 0.036) time points and 8 h.

In the 20 mg/kg SC group, there was a significant increase in active behaviour between the 0, 10, 30 min, 1, and 2 h time points and 4 h (F(9,64) =10.385, p < 0.0001 for all), between the 0 (F(9,64) =10.385, p = 0.024), 10 (F(9,64) =10.385, p = 0.002), 30 min (F(9,64) =10.385, p < 0.0001), and 1 h (F(9,64) =10.385, p < 0.0001) time points and 24 h, and between the 12 h time point and 24 (F(9,64) =10.385, p = 0.004) and 36 h (F(9,64) =10.385, p < 0.0001).

In the 10 mg/kg PO group, there was a significant increase in active behaviour between the 10 (F(9,58) = 2.602, p = 0.021) and 30 min (F(9,58) = 2.602, p = 0.017) time points and 24 h.

In the 20 mg/kg PO group, there was a significant increase in active behaviour between the 10 (F(9,64) = 4.274, p = 0.048) and 30 min (F(9,64) = 4.274, p = 0.013) time points
and 24 h, and between the 10 (F(9,64) = 4.274, p = 0.021) and 30 min (F(9,64) = 4.274, p = 0.006) time points and 36 h.

3.4.2. Mouse Grimace Scale

A repeated measures analysis revealed a significant main effect of time, no significant main effect of treatment, and no time*treatment interactions on mean difference scores (all treatment groups combined) (Table 3-4a). A significant main effect of time on mean difference score was also present within each treatment group (Table 3-2b). In all treatment groups, mice demonstrated pain associated facial grimacing immediately following surgery. Mice demonstrated pain-associated grimacing for 1 h following surgery in the saline-treated group, for 30 min following surgery in the 10 mg/kg SC and PO groups, and for 15 min in the 20 mg/kg SC and PO groups.
3.5. DISCUSSION

The aim of this study was to evaluate the clinical efficacy of carprofen given in the drinking water and to compare it to SC administration and through application of behaviour scoring and the Mouse Grimace Scale. No significant difference between treatment groups was observed, and all mice initially demonstrated grimacing postoperatively. Langford et al. (2010) developed and standardized a facial coding system for mice (the Mouse Grimace Scale). Grimacing was not shown to be associated with anesthesia alone (sham control), and grimacing was related to stimulus intensity. Administration of acetaminophen also reduced grimacing significantly in an inflammatory pain model. Based on these observations, it was concluded grimacing was a direct result of pain.

Matsumiya et al. (2012) used the Mouse Grimace Scale to evaluate the ability of carprofen to inhibit postoperative facial grimacing in mice (CD1) following laparotomy, and significant inhibition was demonstrated in groups administered 20 and 25 mg/kg carprofen SC only (higher dosages were not evaluated). Similar to the current study, drugs were administered immediately following surgery, while animals were still under general anesthesia. Based on these findings, a lower (10 mg/kg) and similar (20 mg/kg) dosage was employed here on the assumption that the higher dose would completely eliminate (pain-associated) facial grimacing. CD1 mice of approximately the same age as the Matsumiya et al (2012) study were used. In this study, inhibition did not occur until a minimum of 15 min after surgery in either the SC or the PO treatment groups.
There are several possible explanations as to why mice grimaced. The first is that the drug required 15 min following SC administration to become clinically efficacious. This would not apply to the PO group though. Additional studies evaluating the pharmacokinetics of SC carprofen in mice are required to confirm this. A second explanation for facial grimacing is that ovariectomy resulted in pain greater than that caused by the laparotomy alone, and 20 mg/kg carprofen (SC or PO) was unable to substantially diminish immediate postoperative pain.

Residual gaseous anesthesia or stress (due to postoperative handling of mice or change in environment) may have also resulted in facial grimacing (not pain-associated) immediately following surgery. Matsumiya et al. (2012) evaluated the effects of gas anesthesia alone (isoflurane) on pain-associated grimacing in mice, and demonstrated it had no significant effect. However, only the 1 h postoperative time point was evaluated in that study. In this study, all treatment groups demonstrated pain-associated grimacing prior to the first hour postoperative. It is possible that residual isoflurane resulted in non-pain-associated facial grimacing immediately following surgery and was misinterpreted as a pain-associated change. In this study, there was significant grimacing (presumably pain-associated) for the first 30 min following surgery with carprofen-treated groups, and for the first 1 h following surgery with the saline-treated group. Based on the study by Matsumiya et al. (2012), we believe grimacing from 1 h onwards was not the result of gas anesthesia, and was ameliorated by administration of carprofen. Studies have also shown that behaviours commonly used to assess pain are not affected by the residual effects of
isoflurane gas anesthesia following surgery (Wright-Williams et al., 2007; Roughan et al., 2009), if behavioural observations commence once ataxia has subsided. No studies to date have evaluated whether stress alone affects or results in facial grimacing in mice.

Writhing is contortion of abdominal muscles, and is associated with pain in mice (Wright-Williams et al., 2007) and rats (Roughan and Flecknell 2000; Roughan and Flecknell 2002; Roughan and Flecknell 2004). There was no significant difference in writhing behaviour between any of the treatment groups (10 or 20 mg/kg SC or PO), or the saline injection only. Writhing was a component of the composite pain behaviours used by Leach et al., (2012) in a vasectomy (scrotal skin-incision) CD1 mouse pain model. Of all composite pain behaviours Leach et al. (2012) listed, only writhing was recorded in this study. Since the additional pain behaviours Leach et al. (2012) utilized to form the composite were not recorded in the current study, it is possible that writhing alone was not observed at a high enough frequency to be statistically significant or is not an appropriate measure of pain in ovariectomy (flank-incision) pain models. This is likely the case, given that individual behaviours are typically too infrequent to analyze individually, and analysis of the pain composite is the gold standard (Wright-Williams et al., 2007; Leach et al., 2012).

It is possible that mice that received a subcutaneous injection may have experienced some pain postoperatively due to the time required for therapeutic plasma drug concentration to be reached following injection. In comparison, mice that had access to a medicated water bottle may have not experienced any pain due to receiving preemptive
analgesia. In future studies, when comparison between drug administration via the water bottle and injectable methods are being made, injections should be given prior to surgery so as to minimize variability between groups and all subjects receive preemptive analgesia.

We did not include a control group undergoing gas anesthesia only (no surgery) to reduce the number of mice used in the experiments and to focus on comparing the effect of administration of carprofen in drinking water versus the current method of SC injection. Benefits to administration via the water bottle are numerous, including decreased time, cost, training, and handling-stress. Future studies are required to determine whether higher dosages than those evaluated would completely eliminate mouse grimacing (and presumably pain) following this procedure, and the effects of gas anesthesia on the Mouse Grimace Scale within the immediate postoperative period. Pain identification in the laboratory mouse is complex, with many potential confounding factors to consider.
<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swim</td>
<td>Forward motion, digging with forefeet into bedding</td>
</tr>
<tr>
<td>Dig</td>
<td>No forward motion, digging with forefeet into bedding</td>
</tr>
<tr>
<td>Writhe</td>
<td>Contortion of abdominal muscles</td>
</tr>
<tr>
<td>Wound lick</td>
<td>Chewing/licking surgical staple</td>
</tr>
<tr>
<td>Groom body</td>
<td>Grooming anywhere on body including limbs</td>
</tr>
<tr>
<td>Groom head</td>
<td>Grooming anywhere on head including face, head, ears</td>
</tr>
<tr>
<td>Walk</td>
<td>Forward motion, with at least two feet pushing body in any direction</td>
</tr>
<tr>
<td>Stand</td>
<td>No forward motion, at least three feet in contact with bottom of cage</td>
</tr>
<tr>
<td>Climb</td>
<td>Feet are not in contact with bottom of cage</td>
</tr>
<tr>
<td>Scratch body</td>
<td>Scratching anywhere on body including flank, limbs</td>
</tr>
<tr>
<td>Scratch head</td>
<td>Scratching anywhere on head including face, head, ears</td>
</tr>
<tr>
<td>Rear</td>
<td>Forefeet are not in contact with bottom of cage and glenohumeral joint is above coxofemoral joint. Forefeet are not in contact with wall of cage</td>
</tr>
<tr>
<td>Rear/Lean</td>
<td>Forefeet are not in contact with bottom of cage and glenohumeral joint is above coxofemoral joint. Forefeet are in contact with wall of cage</td>
</tr>
<tr>
<td>Active</td>
<td>Composite of all active behaviours (swim, dig, walk, climb, rear)</td>
</tr>
</tbody>
</table>

Modified from Wright-Williams et al., 2007
Table 3-2. Mouse Grimace Scale facial action units

<table>
<thead>
<tr>
<th></th>
<th>0 = Not Present</th>
<th>1 = Moderate</th>
<th>2 = Obvious/Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orbital Tightening</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Nose Bulge</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Cheek Bulge</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>Ear Position</td>
<td>![Image]</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

Adapted from Langford et al., 2010

Using the table as a guide, a score of 0 to 2 was assigned to each image for each of the four facial action units. Mouse Grimace Scale score is the mean of the four facial action unit scores.
The F- and p-values of a repeated measures analysis provide the main effects of time (36 h), treatment (10 mg/kg SC, 20 mg/kg SC, 10 mg/kg PO, 20 mg/kg PO, or saline SC), and time*treatment interactions on the duration of time spent performing each behaviour within a 10 min period at 10 time points following surgery. n = 8, + p < 0.05.

<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Effect of Time</th>
<th>Effect of Treatment</th>
<th>Time*Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swim</td>
<td>F(9,19) = 2.087, p = 0.085</td>
<td>F(4,27) = 2.507, p = 0.066</td>
<td>F(36,73) = 0.897, p = 0.633</td>
</tr>
<tr>
<td>Dig</td>
<td>F(9,16) = 4.284, p = 0.006+</td>
<td>F(4,24) = 0.314, p = 0.866</td>
<td>F(36,62) = 0.654, p = 0.914</td>
</tr>
<tr>
<td>Writhe</td>
<td>F(8,17) = 3.368, p = 0.017+</td>
<td>F(4,24) = 0.735, p = 0.577</td>
<td>F(32,64) = 0.553, p = 0.553</td>
</tr>
<tr>
<td>Wound lick</td>
<td>F(9,16) = 6.669, p = 0.001+</td>
<td>F(4,24) = 1.591, p = 0.209</td>
<td>F(36,62) = 0.691, p = 0.883</td>
</tr>
<tr>
<td>Groom</td>
<td>F(9,27) = 2.572, p = 0.028+</td>
<td>F(4,35) = 1.558, p = 0.207</td>
<td>F(36,103) = 1.479, p = 0.066</td>
</tr>
<tr>
<td>Walk</td>
<td>F(9,16) = 7.201, p &lt; 0.0001+</td>
<td>F(4,24) = 0.957, p = 0.449</td>
<td>F(36,62) = 2.560, p = 0.001+</td>
</tr>
<tr>
<td>Stand</td>
<td>F(9,16) = 18.590, p &lt; 0.0001+</td>
<td>F(4,24) = 2.060, p = 0.118</td>
<td>F(36,62) = 2.745, p &lt; 0.0001+</td>
</tr>
<tr>
<td>Climb</td>
<td>F(9,16) = 7.025, p &lt; 0.0001+</td>
<td>F(4,24) = 1.792, p = 0.168</td>
<td>F(36,62) = 1.707, p = 0.032+</td>
</tr>
<tr>
<td>Scratch</td>
<td>F(9,27) = 5.681, p &lt; 0.0001+</td>
<td>F(4,35) = 2.398, p = 0.069</td>
<td>F(36,103) = 1.707, p = 0.019+</td>
</tr>
<tr>
<td>Rear</td>
<td>F(9,27) = 8.062, p &lt; 0.0001+</td>
<td>F(4,35) = 0.654, p = 0.628</td>
<td>F(36,103) = 2.224, p = 0.001+</td>
</tr>
<tr>
<td>Active</td>
<td>F(9,27) = 14.103, p &lt; 0.0001+</td>
<td>F(4,35) = 1.227, p = 0.317</td>
<td>F(36,103) = 2.380, p &lt; 0.0001+</td>
</tr>
</tbody>
</table>
Figure 3-1. Total time performing ‘walk’ behaviour over 36 h following ovariectomy in mice treated with carprofen subcutaneously or in water, and in saline controls

Mice received 10 or 20 mg/kg carprofen subcutaneously (immediately following surgery), or orally via the drinking water (over 24 h prior to surgery). A significant difference in total time performing the behaviour between 2 or more treatment groups is denoted by an asterisk (*). All data is presented as mean ± SEM, * p < 0.05, n = 8 per treatment group.
Figure 3-2. Total time performing ‘stand’ behaviour over 36 h following ovariectomy in mice treated with carprofen subcutaneously or in water, and in saline controls

Mice received 10 or 20 mg/kg carprofen subcutaneously (immediately following surgery), or orally via the drinking water (over 24 h prior to surgery). A significant difference in total time performing the behaviour between 2 or more treatment groups is denoted by an asterisk (*). All data is presented as mean ± SEM, * p < 0.05, n = 8 per treatment group.
Mice received 10 or 20 mg/kg carprofen subcutaneously (immediately following surgery), or orally via the drinking water (over 24 h prior to surgery). A significant difference in total time performing the behaviour between 2 or more treatment groups is denoted by an asterisk (*). All data is presented as mean ± SEM, * p < 0.05, n = 8 per treatment group.
Figure 3-4. Total time performing ‘scratch’ behaviour over 36 h following ovariectomy in mice treated with carprofen subcutaneously or in water, and in saline controls

Mice received 10 or 20 mg/kg carprofen subcutaneously (immediately following surgery), or orally via the drinking water (over 24 h prior to surgery). A significant difference in total time performing the behaviour between 2 or more treatment groups is denoted by an asterisk (*). All data is presented as mean ± SEM, * p < 0.05, n = 8 per treatment group.
Figure 3-5. Total time performing ‘rear’ behaviour over 36 h following ovariectomy in mice treated with carprofen subcutaneously or in water, and in saline controls

Mice received 10 or 20 mg/kg carprofen subcutaneously (immediately following surgery), or orally via the drinking water (over 24 h prior to surgery). A significant difference in total time performing the behaviour between 2 or more treatment groups is denoted by an asterisk (*). All data is presented as mean ± SEM, * p < 0.05, n = 8 per treatment group.
Figure 3-6. Total time performing ‘active’ behaviours over 36 h following ovariectomy in mice treated with carprofen subcutaneously or in water, and in saline controls

Mice received 10 or 20 mg/kg carprofen subcutaneously (immediately following surgery), or orally via the drinking water (over 24 h prior to surgery). A significant difference in total time performing the behaviour between 2 or more treatment groups is denoted by an asterisk (*). All data is presented as mean ± SEM, * p < 0.05, n = 8 per treatment group.
Table 3-4a. Effect of time and treatment on mean difference score

<table>
<thead>
<tr>
<th>Effect of Time</th>
<th>Effect of Treatment</th>
<th>Time*Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>F(10,12) = 10.541, p &lt; 0.001⁺</td>
<td>F(4,21) = 0.511, p = 0.728</td>
<td>F(40,47) = 1.010, p = 0.484</td>
</tr>
</tbody>
</table>

The F- and p-values of a repeated measures analysis provide the main effects of time (36 h), treatment (10 SC, 20 SC, 10 PO, 20 PO, or saline), and time*treatment interactions on mean difference score at 10 time points following surgery. n = 8, ⁺p < 0.05.

Table 3-4b. Effect of time on mean difference score (by treatment group)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effect of Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>F(11,72) = 4.587, p &lt; 0.0001⁺</td>
</tr>
<tr>
<td>10 mg/kg SC</td>
<td>F(11,72) = 10.340, p &lt; 0.0001⁺</td>
</tr>
<tr>
<td>20 mg/kg SC</td>
<td>F(11,80) = 4.444, p &lt; 0.0001⁺</td>
</tr>
<tr>
<td>10 mg/kg PO</td>
<td>F(11,68) = 3.381, p &lt; 0.001⁺</td>
</tr>
<tr>
<td>20 mg/kg PO</td>
<td>F(11,80) = 7.037 p &lt; 0.0001⁺</td>
</tr>
</tbody>
</table>

The F- and p-values of a one-way ANOVA provide the main effect of time (36 h) on mean difference score at 10 time points following surgery within each treatment group. n = 8, ⁺p < 0.05.
Figure 3-7. Mean difference score over 36 h following ovariectomy (saline SC)

Significant mean difference scores were observed at 5 min (F(11,72) = 4.587, p = 0.014), 15 min (F(11,72) = 4.587, p = 0.011), 30 min (F(11,72) = 4.587, p = 0.015), and 1 h (F(11,72) = 4.587, p = 0.014) postoperative. Mice received saline SC immediately following surgery. Mean difference score is Mouse Grimace Scale score minus baseline (no anesthesia, no surgery, no treatment). Significance is calculated by comparison of mean difference score to a mean difference score of 0 (i.e. no difference between postoperative and baseline grimacing). All data is presented as mean ± SEM, * p < 0.05, n = 8.
Figure 3-8. Mean difference score over 36 h following ovariectomy (10 mg/kg carprofen SC)

Significant mean difference scores were observed at 0 min (F(11,79) = 10.340, p = 0.002), 5 min (F(11,79) = 10.340, p = 0.001), 15 min (F(11,79) = 10.340, p < 0.0001), and 30 min (F(11,79) = 10.340, p < 0.0001) postoperative. Mice received 10 mg/kg carprofen SC immediately following surgery. Mean difference score is Mouse Grimace Scale score minus baseline (no anesthesia, no surgery, no treatment). Significance is calculated by comparison of mean difference score to a mean difference score of 0 (i.e. no difference between postoperative and baseline grimacing). All data is presented as mean ± SEM, * p < 0.05, n = 8.
Figure 3-9. Mean difference score over 36 h following ovariectomy (20 mg/kg carprofen SC)

Significant mean difference scores were observed at 0 min (F(11,80) = 4.444, p = 0.025), 5 min (F(11,80) = 4.444, p = 0.001), and 15 min (F(11,80) = 4.444, p = 0.012) postoperative. Mice received 20 mg/kg carprofen SC immediately following surgery. Mean difference score is Mouse Grimace Scale score minus baseline (no anesthesia, no surgery, no treatment). Significance is calculated by comparison of mean difference score to a mean difference score of 0 (i.e. no difference between postoperative and baseline grimacing). All data is presented as mean ± SEM, * p < 0.05, n = 8.
**Figure 3-10. Mean difference score over 36 h following ovariectomy (10 mg/kg carprofen PO)**

Significant mean difference scores were observed at 5 min ($F(11,68) = 3.381, p = 0.006$) and 30 min ($F(11,68) = 3.381, p = 0.035$) postoperative. Mice received 10 mg/kg carprofen PO via the water bottle for 24 h prior to surgery. Mean difference score is Mouse Grimace Scale score minus baseline (no anesthesia, no surgery, no treatment). Significance is calculated by comparison of mean difference score to a mean difference score of 0 (i.e. no difference between postoperative and baseline grimacing). All data is presented as mean ± SEM, * $p < 0.05$, n = 8.
Figure 3-11. Mean difference score over 36 h following ovariectomy (20 mg/kg carprofen PO)

Significant mean difference score were observed at 0 min (F(11,80) = 7.037, p < 0.0001), 5 min (F(11,80) = 7.037, p = 0.001), and 15 min (F(11,80) = 7.037, p < 0.0001) postoperative. Mice received 20 mg/kg carprofen PO via the water bottle for 24 h prior to surgery. Mean difference score is Mouse Grimace Scale score minus baseline (no anesthesia, no surgery, no treatment). Significance is calculated by comparison of mean difference score to a mean difference score of 0 (i.e. no difference between postoperative and baseline grimacing). All data is presented as mean ± SEM, * p < 0.05, n = 8.
GENERAL DISCUSSION

In laboratory mice, common routes of drug administration include gavage, subcutaneous (SC), intramuscular (IM), and intraperitoneal injection. All require time, training, and can result in handling stress. Administration through the drinking water would reduce or eliminate many of these barriers, however no clinical data exists to prove analgesia is being provided. The current study set out to determine if sufficient analgesia could be obtained by administration of nonsteroidal anti-inflammatory drugs via the drinking water. To achieve this requires multiple steps. Both carprofen and meloxicam has to be stable in drinking water, oral administration of the medicated drinking water has to be palatable, plasma drug concentration needs to be adequate and finally, the drugs must be efficacious. The aims of the study were to 1) determine the stability of carprofen and meloxicam under 3 environmental conditions (room temperature ambient light, room temperature dark, and 4 °C dark) over a 7 d period, 2) determine the palatability of carprofen and meloxicam when given through the drinking water, 3) determine the oral pharmacokinetics of carprofen and meloxicam, and 4) assess the clinical efficacy of oral carprofen and meloxicam through application of the Mouse Grimace Scale and behaviour scoring.

The first part of the study demonstrated that meloxicam was an unsuitable candidate for oral administration via the drinking water, since mice were unwilling to readily consume medicated water. However, mice readily consumed carprofen-medicated water at a rate similar to that of un-medicated drinking water. The oral pharmacokinetic profile of both
drugs was determined, with the aim of determining optimal dosing intervals and extrapolating the approximate amount of time that would be required to administer the drug prior to a painful procedure. However, without a clinical correlate, this could not be determined. Pharmacokinetic parameters were determined but the clinical usefulness of these was questionable. The second study was done to determine the clinical efficacy of carprofen administered via the water bottle.

Carprofen is a chiral molecule comprised of two enantiomers, one clinically active and the other not. While total concentration of carprofen in plasma was measured, enantiomers were not separated or analyzed separately in this study. Enantiomeric conversion could occur in water over the 7 d period, resulting in the same total concentration, but a lower amount of the clinically active S(+) enantiomer. Previous studies have shown little to no conversion occurs in water, and the ultimate aim was to determine clinical efficacy irrespective of the form. If significant enantiomeric conversion occurred, medication of water would not be efficacious. In order to fully evaluate conversion in water, further studies are required to separate and analyze the individual enantiomers.

The palatability of carprofen and meloxicam using the intravenous (IV) solutions were determined. Oral suspensions are commercially available, however, we chose to use the IV solution since it is considerably cheaper. Use of the oral suspension to medicate water bottles would be cost prohibitive and impractical in a research or laboratory setting. Carprofen-medicated water was clear and odorless, whereas meloxicam-mediated water
was clear yellow and viscous. Meloxicam-medicated water was unpalatable, and thus not suitable for further consideration. However, given that meloxicam is an effective analgesic compound when administered enterally and parenterally to mice postoperatively (Wright-Williams et al., 2007; Flecknell, 2009; Roughan et al., 2009; Leach et al., 2012), evaluation of addition of a flavouring agent to medicated water to improve palatability may be worth pursuing, but requires further investigation.

Carprofen was administered via the water bottle to mice to determine clinical efficacy, and to extrapolate the therapeutic plasma drug concentration (in conjunction with the findings from the first study). Several obstacles were encountered. None of the carprofen-treated groups (SC or PO) demonstrated a significant difference in facial grimacing or behaviour postoperatively in comparison to the saline-treated group. The high-dose (20 mg/kg) SC treated group was expected to act as a positive control, where mice would not experience, or demonstrate, any pain. The saline SC group was expected to act as a negative control, with mice demonstrating pain since no analgesia was given. Surprisingly, no significant difference was observed between any treatment groups. In the mice receiving SC carprofen, this may have been due to the time at which point the drug was given. Since carprofen was given immediately postoperatively, by the time therapeutic plasma drug concentration was reached, detectable pain may have no longer been present. However, if this were the case, we would expect to see analgesia in the water bottle treated mice, since they had access to medicated water for 24 h. A more plausible explanation is that a therapeutic plasma drug concentration was not reached in any of the carprofen-treated animals. Further studies are therefore urgently required to
determine the therapeutic plasma drug concentration and clinical efficacy for carprofen in mice as this drug is widely used in the laboratory animal community and mice are not being provided with analgesia.

Studies show that mice demonstrate grimacing in painful situations (Langford et al., 2010). The intensity and duration of pain experienced by laboratory mice varies by experimental procedure and with both strain and sex (Mogil and Belknap, 1997; Wan et al., 2001; Banik et al., 2006; Mogil et al., 2011). Ovariectomy was the painful procedure used to determine the efficacy of medication here, but further studies using other procedures are required to determine its widespread applicability. Based on the findings here, the dose used may be far below what is necessary to prevent pain-associated grimacing in more painful procedures. Additional studies are required to assess the intensity and duration of pain in different experimental scenarios in a variety of strains/stocks of both male and female mice.

Behaviours used for statistical analysis in the current study were: swim, dig, writhe, wound lick, groom, walk, stand, climb, scratch, and rear. Swim, dig, and climb are easily observed, but have not been shown to be associated with pain in mice (Wright-Williams et al., 2007; Leach et al., 2012). Similar to the current study, previous behaviour studies in mice in a vasectomy pain model (Leach, 2012) have shown time spent wound licking increased following surgery, and grooming and rearing decreased increased (compared to preoperatively). However, similar to the current study, the study by Leach et al. (2012) did not detect any effect of treatment on wound licking, grooming, or rearing behaviours.
Writhe, flinch, hind leg lift, press, dig, and rear are associated with pain in the mouse (Wright-Williams et al., 2007). The composite pain score by Leach et al. (2012) was comprised of: arch, circle, fall, flinch, press, rear leg lift, stagger, twitch and writhe. Of these, only writhing was detected using our methodology. Dig and rear were frequently observed, but with no significant difference in time spent performing the behaviour between treatment groups, including the saline control. Ideally, inclusion of a preoperative group would have aided in determination of whether behaviours were potentially pain-associated or not. It was not anticipated that mice receiving 20 mg/kg SC would demonstrate pain, and this treatment group was to be used for this purpose. Writhing occurred too infrequently to be meaningful. This may have been due to inability to detect the behavior due to poor video quality, or limiting camera angle. Ideally, more than one camera would be used, placed at both the long and short side of the cage, which would allow for better visualization of behaviours.

Behaviour scoring did not mirror the results obtained from Mouse Grimace Scale scoring. A single study has compared the Mouse Grimace Scale and manual behavioural scoring (Leach et al., 2012). Leach et al. (2012) compared the two using a vasectomy model with mice receiving meloxicam (20 mg/kg SC, 30 min preoperative) or bupivacaine (5 mg/kg SC, intraoperative) and observed a high correlation. Several reasons may explain this discrepancy. The first is group size (n = 8). Small sample size may have led to insufficient power and type 2 error, however, similar sample sizes were used in previous studies (Wright-Williams et al., 2007; Leach et al., 2012) where this was not the case. The second is insensitivity of our observation methods due to poor quality of video
recordings, particularly in the dark phase. In the dark phase, the red light reduced the quality of the recordings, making small movements difficult to identify. In addition, the cameras were positioned along the short side of the cage, whereas convention is to place it along the long side. The increase in distance between the camera and the mouse (when they are at the back of the cage) may have resulted in a decreased ability to detect small movements or subtle behaviours. Lastly, three observers were used for recording of facial grimacing, but only one for behavioural observation, and observer bias may have been more significant than anticipated.

When administering drugs in the food or water to laboratory rodents, various factors can alter food or water intake, including physiologic changes, disease, and social dynamics. During pregnancy, and immediately following parturition, there is an increase in food consumption (Richard and Treyhorn, 1985; Makarova et al., 2010; Krasnow et al., 2011). Tubbs et al., (2011) showed that following partial hepatectomy with no analgesia, water intake did not decrease; however, food intake was reduced for 1 d following surgery. Hyperphagia can occur secondary to uncontrolled diabetes mellitus in the mouse and rat (Sindelar et al., 2002), and chronic restraint-associated stress has been shown to decrease food intake and modify food-intake related genes (Jeong et al., 2013). In contrast, social defeat stress is associated with an increase in food intake (Goto et al., 2014). Thus, when administering drugs via foodstuffs, many factors can disrupt normal food or water intake and subjects may not receive the desired dosage. An insufficient dosage of an analgesic has welfare implications as pain or discomfort results. Similarly, an overdose can result in toxicity. In the current study, there exists the possibility of variability in drug
consumption between animals, which would have affected plasma drug levels and the amount of analgesia experienced by the animal.

Male C57BL/6 mice were used for the pharmacokinetic portion of the study (chapter 2), as they have widespread use in biomedical research. A single sex was chosen to minimize confounding factors. Female CD1 mice were used for the clinical efficacy portion of the study (chapter 3) so the results could be compared with previous studies on pain and mouse grimace scoring (Leach et al., 2012; Matsumiya et al., 2012). In addition, we used animals that required ovariectomy prior to participation in another study. Combining the use of animals minimized the total number of animals used for the study and was more ethical than acquiring new C57BL/6 mice. Further studies are required to evaluate sex and strain differences in the pharmacologic profile of oral carprofen. In addition, we assumed water intake between both sexes and strains was similar. No studies have specifically evaluated water intake in these particular strains. Variability in water intake may have influenced plasma drug levels in the second portion of our study, resulting in different plasma drug levels than those expected based on the results in the first part of our study.

The goal of this study was to determine whether nonsteroidal anti-inflammatory drugs could be administered via the drinking water to mice and be clinically efficacious. We demonstrated that carprofen was a suitable candidate; it is stable over 7 d in both light and dark at room temperature and is readily consumed by mice at a rate similar to that of un-medicated water. In contrast, meloxicam was a poor candidate as mice refused to
drink meloxicam-medicated water. Oral pharmacokinetic parameters were determined, but a clinical correlate was required to extrapolate ideal dosing intervals and time to therapeutic plasma drug concentration. In the second study, mice received carprofen via the water bottle 24 h prior to ovariectomy, and we measured facial grimacing (Mouse Grimace Scale) and behavior as an indicator of pain. No significant difference was noted between treatment groups, including the saline-treated group. While carprofen appears to be an ideal candidate for oral administration, further studies are needed to determine the therapeutic plasma drug concentration in mice. Carprofen is a popular analgesic in laboratories and research settings, and current recommended doses are a small fraction of the doses used in this study. Further studies are both required and necessary to determine the ideal dose of carprofen in the laboratory mouse, at which point oral administration of the drinking water can be revisited as a viable, practical option.
SUMMARY AND CONCLUSIONS

1. Both injectable meloxicam and carprofen are stable for 7 d when diluted in reverse osmosis water and held at room temperature in ambient light, room temperature in dark, and at 4°C in dark.

2. Carprofen is palatable to mice when administered via the drinking water. Mice drink an average of 18.4 mL of carprofen-medicated water/100 g body weight/24 h, and drink more in the dark phase compared to the light. Meloxicam-medicated water is non-palatable and aversive to mice.

3. Following a single oral gavage dose of 10 mg/kg carprofen, peak plasma concentration (20.3 +/- 2.4 µg/mL) occurs at 2 h post-administration. With access to a carprofen-medicated water bottle (10 mg/kg, 0.067 mg/mL), a comparable peak plasma concentration (17.0 +/- 2.9 µg/ml) is achieved following 12 to 24 h exposure.

4. Following ovariectomy, there is no overall significant difference in the time spent performing certain behaviours (swim, dig, writhe, wound lick, groom, walk, stand, climb, scratch, rear) between carprofen-treated (10 or 20 mg/kg PO or SC) and saline-treated mice.
5. Following ovariectomy, active behaviours increase from 0 min to 36 h postoperative in all treatment groups, including the saline control treatment, and there is no significant difference in time spent performing postoperative active behaviours between carprofen-treated (10 or 20 mg/kg PO or SC) and saline-treated mice.

6. Following ovariectomy, mice receiving no analgesia (saline only) demonstrate pain-associated grimacing for 1 h. Facial grimacing is reduced to 30 min in groups receiving 10 mg/kg carprofen SC or PO, and to 15 min in groups receiving 20 mg/kg SC or PO.

7. While carprofen is a suitable candidate for oral administration via the drinking water, additional studies are required to determine the therapeutic plasma drug level of carprofen in mice so recommendations on dose and dosing interval can be made.
REFERENCES


Histamine Research Society, 44, 423.


TRPV1 unlike TRPV2 is restricted to a subset of mechanically insensitive cutaneous nociceptors responding to heat. J Pain 9, 298-308.


