Evaluation of a Transdermal Drug Delivery System for Veterinary Patients

by

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Biomedical Sciences

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ABSTRACT

EVALUATION OF A TRANSDERMAL DRUG DELIVERY SYSTEM FOR VETERINARY PATIENTS

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University of Guelph, 2014

Advisor: Dr. Ronald Johnson

Canine osteoarthritis (OA) is a progressive degenerative disease often leading to inflammation, pain and disuse of the affected joint. Meloxicam, commonly used to manage canine OA, is given as oral tablets or suspensions, or intravenous (IV) or subcutaneous (SC) injections. However, these routes are associated with decreased owner compliance and adverse drug reactions (ADRs) with chronic use. delivra™ uses the transdermal delivery route to transport meloxicam. Meloxicam compounded in delivra™ (TDM) was investigated to evaluate i) penetration of meloxicam into canine synovial fluid (SF) and plasma, ii) stability of TDM and iii) anti-inflammatory and analgesic properties of TDM. Results demonstrated that delivra™ was able to transport meloxicam to canine SF and plasma at comparable levels to oral Metacam® suspension and meloxicam was stable in delivra™ for up to 2 months. However, initial work with TDM did not demonstrate significant anti-inflammatory or analgesic effects in a rat model of inflammation.

KEYWORDS: Canine osteoarthritis, meloxicam, transdermal drug delivery, synovial
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DELCA garnATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in the body of this thesis was performed by me.

All statistical analyses were performed with the assistance of William Sears (Population Medicine, University of Guelph). A compound chemist employed by Delivra Inc. formulated all delivra cream samples used for my thesis work. Dr. Saad Enouri assisted with experimental protocols used in Chapters 1 and 3. Dr. Thomas Gibson and Amanda Hathway collected study samples used in Chapter 1. Dr. Heather Knych (UC Davis School of Veterinary Medicine) analyzed samples collected in Chapter 1. Dr. David Baranowski (Delivra Inc.) assisted with the paw tissue processing and biomarker analyses in Chapter 3. Dr. Christopher Pinelli assisted with the preparation of paw tissue samples for histopathological analyses in Chapter 3. Dr. Patricia Turner provided histopathological scores for paw tissue samples in Chapter 3. Yu Gu (Biomedical Sciences, University of Guelph) performed high performance liquid chromatography (HPLC) analysis on delivra™ cream samples for Chapters 1 and 2.
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<td>CRD</td>
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<td>GMP</td>
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<td>Hematoxylin and eosin</td>
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<td>Intravenous</td>
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<td>Metalloproteinase (s)</td>
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<td>NHP</td>
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<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>NO</td>
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<td>NPN</td>
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<td>NSAID (s)</td>
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<tr>
<td>OA</td>
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<td>OVC</td>
<td>Ontario veterinary college</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PD</td>
<td>Pharmacodynamic</td>
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<td>PG (s)</td>
<td>Prostaglandin (s)</td>
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<td>PGE$_2$</td>
<td>Prostaglandin E$_2$</td>
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<td>PGI$_2$</td>
<td>Prostaglandin I$_2$</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetic</td>
</tr>
<tr>
<td>PLO</td>
<td>Pluronic lecithin organogel</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>RB</td>
<td>Running buffer</td>
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<td>Relative humidity</td>
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<td>Ribonucleic acid</td>
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<td>Room temperature</td>
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<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative real-time polymerase chain reaction</td>
</tr>
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<td>RTNL</td>
<td>Room temperature no light</td>
</tr>
<tr>
<td>RTL</td>
<td>Room temperature with light</td>
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<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
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<tr>
<td>SF</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>SG</td>
<td>Specific gravity</td>
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<tr>
<td>TAE</td>
<td>Tris acetate ethylenediaminetetraacetic acid</td>
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<tr>
<td>TDA</td>
<td>ASU compounded in delivra™ base</td>
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<td>Diclofenac compounded in delivra™ base</td>
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<td>TE</td>
<td>Tris-EDTA</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TPR</td>
<td>Temperature, pulse and respiration</td>
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<tr>
<td>Tx (s)</td>
<td>Thromboxane (s)</td>
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<td>University of Prince Edward Island</td>
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<td>Abbreviation</td>
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<td>-----------</td>
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<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VDD</td>
<td>Veterinary drugs directorate</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VICH</td>
<td>International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products</td>
</tr>
<tr>
<td>VNDS</td>
<td>Veterinary new drug submission</td>
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INTRODUCTION

1.0 Osteoarthritis

1.1 Overview of canine osteoarthritis

Canine osteoarthritis (OA) is a major concern in veterinary medicine, with a prevalence of about 20% in the canine population over 1 year of age (Hedhammar et al., 1979; Hegemann et al., 2002; Johnson et al., 1994; Moore et al., 2001; Roush et al., 2002). It is a progressive degenerative disease involving changes in various joint structures including subchondral bone, articular cartilage, synovial membrane, as well as associated ligaments and periarticular structures (Doig et al., 2000; Pelletier et al., 2001). While it can affect any joint, OA usually occurs in synovial joints, such as the hip or stifle, and is classified as either primary OA or secondary OA. Defective articular cartilage structure results in primary OA and typically presents in multiple joints. Secondary OA which is more commonly seen in veterinary medicine, occurs secondary to other joint diseases, or as a result of stresses placed on normal, healthy articular cartilage (Bennett et al., 1995). Many risk factors have been identified including genetics, hormonal factors, congenital and developmental abnormalities for primary OA, and obesity or other inflammatory, traumatic, or metabolic diseases for secondary OA (Chikanza et al., 2000).

While the exact mediators and mechanisms leading to OA development and progression are not fully understood, it is currently suggested that OA develops when the catabolic processes on cartilage exceed the anabolic processes leading to a net loss of cartilage in the joint (Dean et al., 1989; Dijkstra et al., 1995). Since articular cartilage functions to absorb shock and decrease friction between articulating bones (Vaughan-
Scott et al., 1997), this loss can result in bone-on-bone contact, remodeling of subchondral bone, mobility impairment, inflammation, and pain in the affected joint (Hegemann et al., 2002). Additionally, it has been suggested that during the development of OA, the synovial membrane lining the synovial joints contributes cell wall phospholipids that act as precursors for the formation of arachidonic acid (AA), which are then converted to prostaglandins (PGs) and leukotrienes (LTs) by the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes respectively (Vaughan-Scott et al., 1997).

Although inflammation is not present in all OA cases, many inflammatory mediators such as interleukins (ILs) and metalloproteinases (MMPs) have been recently shown to play important roles in its progression. For example, in an experimental model of canine OA investigators found a significantly increased concentration of the proinflammatory mediators interleukin 6 (IL-6) and TNFα in the synovial fluid of affected joints compared with control joints (Venn et al., 1993). Thus, inflammation and pain have become major areas of focus for OA management.

1.2 Current management of canine osteoarthritis

There is currently no cure for canine OA, however many management strategies have been developed that are targeted at reducing the associated inflammation and pain and increasing or maintaining joint strength and mobility (KuKanich et al., 2012). Recently, a multimodal approach to OA management has been suggested which involves a combination of non-pharmaceutical and pharmaceutical modalities. The most notable benefit to this approach is the potential reduction in the required pharmaceutical dose, therefore possibly reducing adverse drug reactions (ADRs). A direct comparison of
multimodal and monomodal therapies, however, has not yet been performed (Lascelles, 2007).

Non-pharmaceutical management strategies include weight loss, acupuncture, nutraceuticals, and low-level laser therapy (Rychel, 2010). Nutraceuticals such as avocado soybean unsaponifiables (ASU) (Altinel et al., 2007; Boileau et al., 2009), glucosamine and chondroitin (Canapp Jr et al., 1999), polysulfated glycosaminoglycans (GAGs) (Sevalla et al., 2000), and omega-3 fatty acids (Bauer, 2007; Budsberg et al., 2006; Vandeweerd et al., 2012), have recently been suggested to have anti-inflammatory, analgesic and chondroprotective effects. However these treatments have not yet been fully assessed in clinical trials. Pharmaceutical treatments include non-steroidal anti-inflammatory drugs (NSAIDs) alone, or in conjunction with opioids, N-methyl-D-aspartate (NMDA) antagonists, tricyclic antidepressants, anticonvulsants and calcium and sodium channel blockers (Lascelles, 2007). These combinational therapies offer a potential synergistic effect with NSAIDs, allowing for a reduction in dose required for each drug.

2.0 Non-steroidal anti-inflammatory drugs

In Canada, many NSAIDs are currently approved for use in canines including: ketoprofen, meloxicam, carprofen, phenylbutazone, tolfenamic acid, vedaprofen, deracoxib, firocoxib and robenacoxib (Health Canada, 2013a). Ketoprofen is available as an intramuscular (IM), intravenous (IV) or subcutaneous (SC) 10 mg/mL injectable solution. Meloxicam is available as an oral 1.5 g/L or 1.5 mg/mL suspension, an oral 1 mg or 2.5 mg chewable tablet, or an IV or SC 5 mg/mL injectable solution. Carprofen is available as an oral 25 mg, 75 mg or 100 mg tablet. Phenylbutazone is available as an IV
200 mg/mL injectable solution. Tolfenamic acid is available as an IM or SC 40 mg/mL injectable solution or as an oral 6 mg, 20 mg or 60 mg tablet. Vedaprofen is available as an oral 5 mg/mL gel. Deracoxib is available as an oral 25 mg, 75 mg or 100 mg tablet. Firocoxib is available as an oral 57 mg or 227 mg chewable tablet. Finally robenacoxib is available as an oral 5 mg, 10 mg, 20 mg or 40 mg tablet. Is this information useful or should we take it out of the thesis?.....You can leave it [RJ]

2.1 NSAID pharmacodynamics

NSAIDs are the most frequently used analgesic class in veterinary medicine (Lascelles et al., 2005b) and the most commonly used pharmaceutical for the management of canine OA (Johnston et al., 2008). They have anti-inflammatory, analgesic, and antipyretic effects (Montoya et al., 2004) and act mainly by reversibly inhibiting the COX enzymes, through competitive inhibition of the AA active site on COX, thus inhibiting the production of PGs and thromboxanes (Txs) (MacPhail, 2000; Smith et al., 2000; Thuresson et al., 2001; Vane et al., 1998; Vane, 1971). NSAIDs were originally thought to act peripherally to inhibit inflammation and pain, however recent data suggests that they may also have a central mechanism of action contributing to their analgesic effects through inhibition of PG synthesis in the spinal cord (Budsberg, 1999; Dolan et al., 2003; Dolan et al., 2000; Malmberg et al., 1992; Smith et al., 1998). NSAIDs have also been shown to directly inhibit functions carried out by neutrophils such as adhesion, aggregation, chemotaxis, degranulation and superoxide anion generation (Abramson et al., 1989; Johnston et al., 1997; Kaplan et al., 1984). In addition, NSAIDs may have a direct effect on the joint (Pelletier et al., 2004). In human osteoarthritic synoviocytes, the inhibition of COX-2 has been shown to result in a
significant inhibition of nitric oxide (NO) induced cell death (Jovanovic et al., 2002),
which could contribute to a potential reduction in disease progression. In another study,
in synovial fluid (SF) of canines with naturally occurring hip OA, expression of both
COX isoforms has been shown to be up-regulated (Lascelles et al., 2009).

2.2 Cyclooxygenase enzymes

The COX enzyme exists as three isoforms: COX-1, COX-2 and the recently
discovered COX-3. They are all responsible for catalyzing the conversion of cell
membrane derived AA to PGs and TxA₂ (Blikslager et al., 2005). COX-1 is constitutively
expressed in most tissues (Blikslager et al., 2005; Crofford, 1997; Dubois et al., 1998;
Smith et al., 1994; Vane et al., 1995) and therefore has classically been termed the
beneficial or ‘good’ COX isoform. The COX-2 isoform was found to be synthesized by
inflammatory cells and macrophages (Papich, 2008), and is inducible locally and
centrally (Beiche et al., 1996; Laird et al., 1997; Yamamoto et al., 1996) by various pro-
inflammatory factors including cytokines, hormones, and growth factors at sites of
inflammation (Blikslager et al., 2005; Dubois et al., 1998; Hutchison, 2004; Vane et al.,
1995; Willoughby et al., 2000) and was therefore termed the ‘bad’ COX isoform.
However, contradicting evidence has suggested that both COX isoforms are constitutive
and inducible in some tissues (Dubois et al., 1998; Guan et al., 1997; Harris et al., 1994;
Lascelles et al., 2009; Papich, 2008; Wooten et al., 2008; Wooten et al., 2010). COX-3
has been recently found in the cerebral cortex of the canine (KuKanich et al., 2012) and
has been said to be involved in pain and fever control at the level of the central nervous
system (CNS), specifically the brain (Blikslager et al., 2005).
Due to a difference in primary amino acid structures between COX-1 and COX-2, specifically a substitution of isoleucine in COX-1 to valine in COX-2 at positions 523 and 434 of the active site (Kurumbail et al., 1996), the binding pocket on the COX-2 isoform is larger than that of COX-1. As a result the traditional small NSAIDs fit into both the COX-1 and COX-2 binding sites and are thus termed nonselective COX inhibitors, whereas COX-2 selective NSAIDs will fit into the large COX-2 active site but not COX-1. COX-3 appears to be a splice variant of the COX-1 isoform and originates from the same COX-1 gene (Chandrasekharan et al., 2002; Schwab et al., 2003).

2.3 Prostaglandins

PGs are a group of fatty acids that play many important autocrine and paracrine roles in both healthy and diseased states (Dubois et al., 1998). As mentioned above, COX converts AA to PGs, specifically PGG_2 and then PGH_2. PGH_2 is then converted to a variety of eicosanoids: PGE_2, PGD_2, PGF_2α, PGI_2 and TxA_2 depending on the downstream enzymes present.

In platelets COX-1 converts PGH_2 to TxA_2, which is important in stimulating platelet aggregation (Dubois et al., 1998; KuKanich et al., 2012). PGs produced by COX-1 are also constitutively active in a healthy gastrointestinal tract (GIT) (Wallace et al., 2000; Wilson et al., 2004) promoting mucus and bicarbonate secretion (Chu et al., 1999; Garner et al., 1983) which protect the stomach lining from damage by its acidic contents, maintaining the pH gradient at the gastric mucosal surface (Baumgartner et al., 2004), and reducing gastric epithelial permeability (Takezono et al., 2004).

COX-2 derived PGs have been shown have negative effects through their involvement in inflammatory, pyrogenic, and pain inducing activities. In contrast, they
have also been shown to have beneficial and protective effects (Konturek et al., 2005), and are important in the maintenance of blood flow during times of GI damage (Kotani et al., 2006) for example following irritant exposure (Gretzer et al., 2001), ischemia (Maricic et al., 1999) or aspirin induced inhibition of COX-1 (Davies et al., 1997). COX-2 deficient mice were shown to have an increased susceptibility to NSAID induced GI injury compared with control mice (Langenbach et al., 1995) and had an impaired ability to resolve inflammation (Wallace et al., 1998). It was also demonstrated that COX-2 expression was increased in inflamed gastric mucosa and its products were important in mucosal defense through increased PGE$_2$ production, angiogenesis and vascular endothelial growth factor (VEGF) production (Souza et al., 2003; Wooten et al., 2010).

In both the above-mentioned COX-1 and COX-2 GI effects, PGE$_2$, and to a lesser extent, PGI$_2$, are the main PGs responsible (Simmons et al., 2004). PGE$_2$ and PGI$_2$ produced by COX-2 have also been shown to be important in the kidney by maintaining renal perfusion, stimulating renin release and maintaining glomerular filtration rate (GFR) during volume depletion, hypotension, hypovolemic or hyponatremic states in dogs (Gambaro et al., 2003; Khan et al., 1998; KuKanich et al., 2012; Opgenorth et al., 1987; Osborn et al., 1984; Rodriguez et al., 2000; Simmons et al., 2004).

Evidence suggests that COX-2 derived PGs also have proinflammatory effects in the early stage of inflammation and anti-inflammatory effects in the later resolution stages (Gilroy et al., 1999). As proinflammatory mediators they synergize with other mediators to promote vascular permeability, potentiate the effects of other proinflammatory agents and initiate and perpetuate chemotaxis and hyperalgesia (Adams, 2001; Robinson, 1988). Additionally, in a rat adjuvant model of arthritis, it was shown
that an increase in COX-2 expression in inflamed joint tissues leads to the increase in PG synthesis detected in those joints (Anderson et al., 1996).

COX-2 derived PGs are said to have anti-inflammatory properties during resolution of the acute inflammatory response (Gilroy et al., 1999). They have been shown to inhibit mast cell derived histamine release, TNFα and platelet activating factor (Hogaboam et al., 1993), macrophage derived TNFα and IL-1 (Kunkel et al., 1986a; Kunkel et al., 1985; Kunkel et al., 1986b) and neutrophil derived LTB₄ and IL-8 (Gonzalez-Crussi et al., 1983; Haurand et al., 1989; Wertheim et al., 1993). Synthesis of COX-2 derived PGs is increased following GI injury or stress, which is thought to promote anti-inflammatory effects through increased blood flow, and reduction of leukocyte adherence and activation (Wallace, 2008).

Studies performed in both human and animal models suggest a contradictory role of PGs on bone metabolism. PGs have been reported to both increase bone formation and stimulate bone resorption (Kawaguchi et al., 1995; Pilbeam, 1996). In addition, COX-2 has been suggested to be crucial to bone remodeling in response to acute stress (Pilbeam et al., 1997).

In the brain, it has been suggested that COX-2 plays a role in neural development and adaptation, as supported by its activation corresponding to environmental imprinting (Kaufmann et al., 1997). It has also been shown that in endothelial cells within the brain, activation of COX-2 leads to a similar cascade of events as that of the fever response (Cao et al., 1997; Matsumura et al., 1997), namely production of PGs that act on the temperature sensing neurons.
Finally, COX-2 derived PGE\textsubscript{2} and PGI\textsubscript{2} enhance nociceptive stimuli produced by mediators resulting in hyperalgesia and allodynia (Lees \textit{et al.}, 2004b), and enhancing sensitivity of pain receptors to painful stimuli (Dray \textit{et al.}, 1996; Simmons \textit{et al.}, 2004). These PGs are thought to play a central role in pain induction, through pain transmission in the spinal cord (Yamamoto \textit{et al.}, 1996) where COX-2 is constitutively located (Malmberg \textit{et al.}, 1992; Nishiyama, 2006). In one study, meloxicam, a preferential COX-2 inhibitor, was administered systemically and was able to inhibit inflammation related pain locally, but had no effect centrally (Laird \textit{et al.}, 1997). Conversely in another study, a different selective COX-2 inhibitor was able to inhibit a central pain response, but not local (Yamamoto \textit{et al.}, 1996). As such, an important role for COX-2 in both central and peripheral pain transmission has been suggested.

2.4 \textit{NSAID cyclooxygenase selectivity}

NSAIDs all inhibit COX enzymes, although the extent of COX-1 and COX-2 inhibition varies depending on the specific drug and the dose. While some NSAIDs are COX-2 preferential or COX-2 selective, all NSAIDs become nonselective COX inhibitors at a very high dose (Lees \textit{et al.}, 2004a; Wolfe \textit{et al.}, 1999). As mentioned previously, COX-1 plays an important role in Tx synthesis by platelets; therefore inhibition of serum TxA\textsubscript{2} synthesis is measured in blood samples for determination of COX-1 inhibition. On the other hand, COX-2 is highly expressed at sites of inflammation, thus inhibition of PGE\textsubscript{2} synthesis in inflammatory exudate, specifically from stimulated leukocytes or macrophages, is used to determine COX-2 inhibition of NSAIDs (Lees, 2003; Papich, 2008). The degree of COX-1 and COX-2 inhibition of an NSAID is reported as a ratio of COX-1:COX-2 inhibition (Curry \textit{et al.}, 2005). In general,
nonselective NSAIDs are suggested to be those that have an inhibition ratio of about 1. COX-2 preferential NSAIDs are suggested to have 5 to 100 times greater COX-2 inhibition compared with COX-1, and COX-2 selective NSAIDs have greater than 100 times COX-2 inhibitory activity (Riviere et al., 2013). It has been suggested that NSAID related ADRs occur due to the inhibition of the COX-1 isoform and the anti-inflammatory, analgesic and antipyretic effects of NSAIDs occur as a result of selective COX-2 inhibition (Kore, 1990; Lascelles et al., 2005a; Monteiro-Steagall et al., 2013). This has led to considerable research being conducted on COX-2 selective or COX-2 preferential NSAIDs, with COX-1:COX-2 ratios >1.0 (Curry et al., 2005), in order to potentially reduce NSAID related ADRs. It is important to note that the COX-1 sparing concept refers only to reducing the potential GI adverse effects in a healthy state and does not relate to the reduction of NSAID related renal or hepatic ADRs, or effects on impaired GI systems (Mattia et al., 2004). COX-2 selectivity also has no association with efficacy of COX-2 isoform inhibition, but rather refers to the potential sparing of COX-1 inhibition by the NSAID (KuKanich et al., 2012).

Since the COX-1 isoform has classically been associated with maintaining physiologic functions, it was suggested that COX-1 sparing NSAIDs would produce fewer NSAID related adverse events (Aragon et al., 2007; Budsberg, 1999; Lascelles et al., 2005a; Vane DSc et al., 1998). Some studies have demonstrated a significant reduction in the risk of GI effects with selective COX-2 inhibitors in vivo (Blikslager et al., 2005; Luna et al., 2007; Moore et al., 2006; Nishihara et al., 2001; Reimer et al., 1999). However, there is some contradictory evidence suggesting that COX-2 selective
NSAIDs can still produce adverse GI events in canines (Lascelles et al., 2005a; Lascelles et al., 2005b; Luna et al., 2007; Moreau et al., 2005; Zeino et al., 2010).

Additionally, numerous inconsistencies in COX selectivity have been demonstrated that are a result of different species, assays, and research labs and as such, it has been recommended that COX isoform selectivity be determined in vivo in the target population (KuKanich et al., 2012).

2.5 NSAID pharmacokinetics

NSAIDs have been reported to have good parenteral and enteral bioavailability (50-100% depending on the drug and species), experience a high degree of protein binding (>90%), accumulate in inflamed tissues and are cleared slowly from inflammatory exudate (Lees, 2003; Lees et al., 2004b; Papich, 2008). The portion of NSAIDs in the plasma that is unbound to protein is considered to be active (Papich, 2008; Toutain et al., 2002). Typically drug plasma half-lives and clearance rate, along with other pharmacokinetic (PK) characteristics, are used to estimate the duration of action of a drug. However, due to their high protein binding, accumulation and slow clearance from inflamed tissues, the NSAID PKs do not correlate well with, and are poor indicators of their anti-inflammatory and analgesic effects (Lees, 2003; Lees et al., 2004b). NSAIDs are cleared primarily via hepatic elimination by secretion in the bile, conjugation and metabolic reactions. A smaller fraction of NSAIDs may also be eliminated through renal excretion (Lees, 2009). The clearance rate for each NSAID varies and can be affected by species, age, and presence of hepatic or renal disease. Therefore, older canines, or those with renal or hepatic insufficiencies may experience a decreased rate of NSAID
elimination or increased half-life, and can be at an increased risk for NSAID related ADRs.

2.6 NSAID adverse drug reactions

While the canine is the most frequently reported species to experience NSAID related ADRs, the true incidence of ADRs after long-term use of NSAIDs in normal and OA dogs is currently unknown (Budsberg, 1999; Lees et al., 2004b). NSAIDs have been associated with various ADRs affecting the GIT, kidney, liver, bone and platelet function in canines (Lascelles et al., 2005a; Lemke et al., 2002; Luna et al., 2007; Mansa et al., 2007; McMillan et al., 2011; Reimer et al., 1999; Zhang et al., 2002).

Among all of the NSAID related ADRs, GI damage or disturbance is the most commonly reported NSAID related ADR (Cunningham et al., 1994; Monteiro-Steagall et al., 2013; Papich, 2008). This can occur by two main mechanisms: direct irritation of the mucosa and/or indirectly as a result of PG inhibition. Many NSAIDs are weakly acidic (KuKanich et al., 2012) which makes them more lipophilic when they are in the acidic stomach environment. Therefore they are better able to diffuse through the gastric mucosa and cause direct GI irritation of the mucosal lining when administered orally. Since NSAIDs are excreted primarily into the bile, their acidic nature may cause further GI damage upon secretion, regardless of the route of administration (KuKanich et al., 2012). By inhibiting PG synthesis, specifically PGE₂ and PGI₂, NSAIDs are indirectly inhibiting the protective effect on the GI mucosa leading to decreased cytoprotection, blood flow, mucus synthesis and mucosal cell turnover and repair (Budsberg, 1999; Konturek et al., 2005; KuKanich et al., 2012; Whittle et al., 1984; Whittle, 2004; Wolfe et al., 1999). Other mechanisms of GI damage include increased production of LTs,
alterations in ion channel conductance, inhibition of PGI$_2$, and inhibition of aspirin triggered lipoxin (ATL) (KuKanich et al., 2012).

It is thought that in a healthy canine NSAIDs have little effect on the kidney since renal PG synthesis is relatively low (Johnston et al., 1997; Lobetti et al., 2000). However, PGs, specifically those produced by COX-2 (Lascelles et al., 2005b; Simmons et al., 2004), become critical to the maintenance of normal renal function during times of renal insufficiency such as hypovolemia, dehydration and hypotension (Gambaro et al., 2003; Knights et al., 2005). As such, geriatric, working or otherwise renally compromised dogs may be at an increased risk for renal ADRs.

ADRs affecting the liver are proposed to be idiosyncratic and unrelated to the COX selectivity profile of NSAID used (Lascelles et al., 2005b; Mansa et al., 2007; Reymond et al., 2012) and as such, any NSAID has the potential to cause hepatic ADRs (Lascelles, 2013; Lee, 2003).

NSAIDs lead to an inhibition of TxA$_2$ synthesis in platelets, causing impaired platelet function, specifically platelet aggregation (KuKanich et al., 2012). There has also been a recent investigation into the role of COX in bone healing and repair that has demonstrated a critical role for COX-2 in the formation and healing of bone. In this study, addition of PGE$_2$ was able to completely rescue the defect in osteogenesis that is required for bone healing. Thus, it is through inhibition of COX-2 and, therefore, PGE$_2$ formation that NSAIDs are thought to potentially impair normal bone healing and repair (Ochi et al., 2011; Zhang et al., 2002).
3.0 Meloxicam

3.1 Overview of meloxicam use in canines

Meloxicam, an enolic acid derivative of the oxicam class, is a COX-2 preferential NSAID as determined by in vitro studies (Brideau et al., 2001; Budsberg, 1999; Hawkey, 1999; Kay-Mugford et al., 2000; Laudanno et al., 2000; MacPhail, 2000). It was one of the first preferential COX-2 inhibitors commercially developed (Churchill et al., 1996; Engelhardt et al., 1996a; Engelhardt et al., 1996b; Ogino et al., 1996; Pairet et al., 1996) and in 2001 was approved for use in canines to treat inflammation and pain in Canada (Doig et al., 2000). It is also approved for use in veterinary medicine in the United States of America (USA) and European Union (EU). It is a potent PG synthesis inhibitor and has been shown to be antipyretic, anti-inflammatory and analgesic (Budsberg, 1999; Engelhardt et al., 1995). Meloxicam is approved for administration in canines enterally as an oral chewable tablet or suspension, or parenterally as an IV or SC injection in Canada.

3.2 Meloxicam pharmacokinetics

Meloxicam, similar to many other NSAIDs, is highly protein bound and therefore distributes mainly in the extracellular fluid compartment. As such, plasma levels of meloxicam do not directly reflect its in vivo effects (Busch et al., 1998; Lascelles et al., 1998; Musser et al., 1998; Schmitt et al., 1990). Meloxicam is also highly ionized at physiological pH (Busch et al., 1998; Schmitt et al., 1990). Meloxicam has a shorter half-life compared to other members of the oxicam class due to its unique methyl group in its thiazolyl functional group that, when broken down, undergoes rapid removal from the body (Busch et al., 1998).
When administered orally, meloxicam undergoes significant enterohepatic recirculation, hepatic metabolism (Wallace, 2003) and has a delayed onset of action (Busch et al., 1998; Euller-Ziegler et al., 2001), reaching a maximum plasma concentration 7 or 8 hours after dosing in dogs (Busch et al., 1998; Plumb, 2002). A PK study showed that meloxicam, when administered subcutaneously in canines, reaches maximum plasma concentration 2.5 hours after administration and its plasma half-life in canines is 24 hours following oral, IV or SC administration (Busch et al., 1998; Höglund et al., 2002; Poulsen et al., 1999), therefore allowing a once a day dosing regimen with any of these dosing methods.

### 3.3 Meloxicam pharmacodynamics

Meloxicam has been shown to be an effective anti-inflammatory agent in canine models of synovial joint inflammation (van Bree et al., 1994), with the ability to decrease leukocyte numbers (Vaughan-Scott et al., 1997) as well as decrease inflammatory and nociceptive PG synthesis (Rinder et al., 2002). As well, meloxicam has proven efficacy in significantly improving clinical signs of naturally occurring (Davies et al., 1999; Doig et al., 2000; Peterson et al., 2004) and experimental canine OA (Cross et al., 1997; Rainsford et al., 1999; van Bree et al., 1994). Long-term meloxicam treatment for 30 and 60 days was also able to restore ground reaction force, an objective measurement of lameness, back to normal in naturally occurring arthritic dogs without causing ADRs (Moreau et al., 2003).

### 3.4 Meloxicam adverse drug reactions in canines

Meloxicam related ADRs in canines have been well studied (Doig et al., 2000; Innes et al., 2010; Luna et al., 2007; Moreau et al., 2003; Nell et al., 2002). Short-term
studies in healthy (Crandell et al., 2004; Fusellier et al., 2008) and hypotensive canines
(Boström et al., 2006) and long-term studies in healthy canines (Luna et al., 2007) have
demonstrated a lack of renal ADRs. As such, meloxicam has been associated with
reduced NSAID related renal ADRs compared to traditional nonselective NSAIDs
(Engelhardt et al., 1996b). While meloxicam has been reported to have some GI toxicity
at higher doses (Enberg et al., 2006; Reed, 2002), meloxicam is generally considered to
have a safer GI profile (Doig et al., 2000; Jones et al., 2002) compared with nonselective
NSAIDs. In some studies, meloxicam has been associated with clinical signs of GI
ADRs, however these are reported to be mild, transient (Nell et al., 2002) and relatively
infrequent (Forsyth et al., 1998; Moreau et al., 2003). When compared with COX-2
selective NSAIDs, short-term meloxicam treatment resulted in a similar GI safety profile
supporting its use in canines (Wooten et al., 2009). In a long-term study of meloxicam
administration, a lack of significant changes in serum biochemistry was reported,
suggesting a lack of hepatic ADRs (Luna et al., 2007). Meloxicam was also shown to
have no effect on cartilage proteoglycan production and cartilage destructive cytokine
production at doses sufficient to cause inhibition of PG synthesis (Bassleer et al., 1997;
Rainsford et al., 1997).

Development of transdermal meloxicam formulations are currently under way to
provide an alternative to the currently available parenteral routes of administration (Bevis
et al., 1996; Gupta et al., 2002). A recent PK study (Yuan et al., 2009) was conducted
comparing meloxicam levels achieved in plasma and SF of beagle dogs following
transdermal meloxicam delivery in a gel at a dose of 1.25 mg/kg and oral meloxicam
delivery as a tablet at a dose of 0.31 mg/kg. This study demonstrated that transdermal
meloxicam administration leads to 20% higher SF levels than plasma levels of meloxicam. Additionally, the plasma levels of meloxicam were lower and the SF levels were eight times higher than that achieved by the oral route of administration. However, the variability in meloxicam levels for the transdermal route of administration was much larger than for oral administration (Yuan et al., 2009). Based on SF meloxicam levels measured 8, 12, 24, 48, 72 and 96 hours and plasma meloxicam levels measured 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 72 and 96 hours following meloxicam administration, this research group suggested that the majority of meloxicam delivered by the gel formulation entered the body via direct penetration across the skin and concentration in the target site rather than from systemic circulation, suggesting an overall reduced systemic exposure to meloxicam. This observation has been made in other studies (Hui et al., 2001; Singh et al., 1994; Suh et al., 1997). If this local delivery of meloxicam to the SF is true, then the gel formulation used in this study is acting as a topical drug delivery system rather than a true transdermal delivery system.

4.0 Veterinary drug delivery

Veterinary medicine is a continuing area of pharmacologic interest and advancement, as can be seen based on the number of recently approved veterinary drugs (Philips, 2007). In general, drug products developed for use in companion animals such as canines are designed with not only efficacy, but also ease of administration, reduction in ADRs and convenience in mind (Horspool, 2013). Since there is no cure for canine OA, management of the disease is long-term. It is, therefore, important that the drug is safe over the long-term and that owner compliance is high. While there are very few studies investigating owner compliance, it has been suggested that decreasing dosing
frequency and increasing ease of administration of the drug can maximize owner compliance (Horspool, 2013). Thus, these are important factors that have been considered in developing treatments for canine OA and other chronic diseases.

4.1 **Enteral administration**

Oral dosage forms take the enteral route into the body, entering the GIT where they are absorbed, then circulating through the hepatic portal vein to the liver where they are typically metabolized and released into general systemic circulation via the hepatic veins and transported to the target tissues. This initial metabolism by the liver is termed the hepatic first pass effect, and it can significantly decrease the bioavailability of the drug (Turner *et al.*, 2011).

The benefits of oral dosing include affordability, and the relative ease of dosing. Oral administration is not painful for canines and, if combined with food, may be received quite willingly by the patient (Turner *et al.*, 2011). However administration with food may decrease absorption of some drugs in the GIT (Turner *et al.*, 2011). Additionally, if an incorrect dosage has been given, the owner or veterinarian can induce vomiting shortly after administration to remove the drug from the body, thus preventing overdose. Oral dosage forms are also typically more affordable compared to injectable formulations (Turner *et al.*, 2011).

With treatments that require dosing once or multiple times per day, owner compliance may not be perfect even with the oral dosage form. In addition, some dogs may be difficult to dose orally, either spitting out the drug or biting the person administering the drug. Due to the exposure of the GIT to the drug, there is also an increased risk of direct injury, for example with drugs that are very acidic. As discussed
earlier, drugs administered via this mode are subject to the hepatic first pass effect, which can significantly decrease drug bioavailability. Enzymes within the GIT and liver may inactivate the drug, or produce active metabolites that can have adverse effects. Having to undergo hepatic metabolism may also delay the onset of action of the drug. Drug absorption characteristics such as hydrophobic or hydrophilic polarity need to be considered and may exclude some drug candidates from being used, since drug entry into the systemic circulation depends on absorption across the GIT (Turner et al., 2011). Finally, enteral routes of drug delivery can be associated with very erratic absorption characteristics, with plasma levels spiking drastically following administration.

4.2 Parenteral administration

Currently, the only parenteral routes of meloxicam administration approved for use in veterinary medicine in Canada are IV or SC injectable forms of meloxicam (Metacam®) (Health Canada, 2013a). These modes introduce the drug directly into the body where it circulates to the target tissue. Both of these delivery routes require aseptic preparation of the skin. SC injection is performed by injecting the drug into the fatty tissue just under the epidermis and dermis of the patient, from which point it is thought to be taken up by macromolecules and enter the bloodstream, however the exact mechanism of uptake is not fully understood (Kagan et al., 2007). With IV injection, the drug is administered directly into the systemic circulation. Both forms of injected drugs do not enter the hepatic portal circulation before the systemic circulation and therefore are not subject to the “hepatic first pass” effect.

Benefits of the parenteral route of administration include better control of plasma levels of drug, greater drug bioavailability and, with the IV route, a rapid onset of action.
PK studies have demonstrated a much smaller spike in plasma levels of drug following SC compared with enteral administration, thus reducing the risk of toxic drug effects. SC routes of administration can act as a drug reservoir, allowing for depot formation and sustained release of drug, thus reducing required dosing frequency while delaying onset of action compared to the IV route (Horspool, 2013; Turner et al., 2011). As well, due to the avoidance of hepatic metabolism, drug bioavailability is significantly greater and the drug effects can occur rapidly after dosing compared to oral routes. This higher level of control of plasma drug levels and rapid action is especially beneficial during emergencies or surgical procedures.

The injectable routes can be associated with numerous drawbacks including difficulty of administration, discomfort for the patient due to local pain or irritation, higher cost (Horspool, 2013) and doses that cannot be removed once administered. Injecting a drug subcutaneously or intravenously is a more technically difficult task for the average pet owner compared with oral dosing. Inadequate aseptic technique can lead to serious complications such as septicemia (Turner et al., 2011). Since a needle is required to penetrate the skin, these dosage forms are more painful for the patient and would likely be poorly tolerated for chronic conditions compared to oral administration. Additionally, once the drug has been administered it cannot be retrieved and therefore poses an increased risk of overdose or ADRs compared to oral administration. Finally, some drugs are administered as a prodrug, which need to undergo some form of metabolism to be converted to the active form. This typically occurs via hepatic metabolism, thus if a drug is administered parenterally it may not become activated as rapidly or completely compared to oral administration. Novel transdermal drug delivery
systems, without the drawbacks of injections, are currently being investigated due to the benefits of parenteral administration over the oral route.

5.0 Transdermal drug delivery

5.1 Anatomy of the skin

The skin functions to prevent toxicants and potentially harmful microbes from entering the body as well as prevent water loss from the body. As such, it has a very low permeability. The stratum corneum is the outermost layer of skin that is primarily responsible for the impermeability characteristic of the skin (Blank, 1964; Prausnitz et al., 2004). The stratum corneum is made up of a thin matrix of lipids and keratinous corneocytes that are covalently bound making it highly lipophilic (Naik et al., 2000). The top layers are desquamated every 2 to 4 weeks and injured regions are rapidly repaired to maintain the integrity of this barrier (Prausnitz et al., 2004).

Beneath the stratum corneum lies the avascular epidermis, followed by the dermis, which contains capillary beds that supply the dermis and epidermis (Prausnitz et al., 2008). Hair follicles are located in the dermis and deeper hypodermis and have associated apocrine sweat glands and sebaceous glands. The hypodermis is mostly fatty tissue and is also termed the SC layer, lying just above the skeletal muscle (Pavletic, 1991).

5.2 Overview of transdermal drug delivery

Transdermal drug delivery is a form of parenteral drug administration that has been recently investigated for use in veterinary medicine. A drug is typically compounded in a delivery system, for example a cream, ointment, or patch matrix and is transported across the skin where it accumulates or enters the systemic blood circulation.
from the dermis to reach distant target tissues. The transdermal drug product may also pass to the SC tissues directly through hair follicles (Otberg et al., 2008; Prausnitz et al., 2004).

Often, the terms transdermal and topical drug delivery are incorrectly used synonymously. Topical drug delivery refers to local delivery of a drug by absorption through the skin, with its effects occurring directly at the site of application. In this form of delivery, the majority of the drug typically does not enter the systemic circulation, thus reducing potential systemic effects of the drug. On the other hand, transdermal drug delivery is achieved by absorption of the drug through the skin followed by transport of the drug to the target site by the systemic circulation. Unlike topical formulations, transdermal drug formulations can be applied to a region with a large surface area that more readily absorbs the drug, and then have their effects at distant sites.

Typically transdermal drug products produce consistent plasma drug levels over time (Beetge et al., 2000), however this route is associated with higher variability between individuals. This mode of administration is usually non-invasive and pain free for the subject and has been suggested to have good patient compliance in animals (Guy et al., 1987; Prausnitz et al., 2008; Willis-Goulet et al., 2003). Additionally, this route can provide sustained release of drugs, especially for those drugs with short half-lives allowing for a potential reduction in dosing frequency and less wide swings in peaks and troughs (Berba et al., 1991; Naik et al., 2000). Dosing can be interrupted by removing the patch, since the drug reservoir is external to the body (Guy et al., 1987; Prausnitz et al., 2004; Turner et al., 2011). As with other parenteral routes, transdermal drug delivery avoids the hepatic-first pass effect, improving bioavailability of the administered drug.
compared with the enteral route of administration (Prausnitz et al., 2008). Avoidance of
the enteral route also protects the patient from direct GI injury that may occur with
exposure to some drugs (Bachhav et al., 2010), however transdermals can still cause GI
problems indirectly. Transdermal delivery systems may also be less expensive than other
parenteral modes of administration (Prausnitz et al., 2008). However unlike the IV route
of administration, some transdermal drugs can undergo biotransformation by microbes on
the skin surface or enzymes within the epidermis, thus allowing some prodrugs to be
administered by this mode (Bucks, 1984; Guy et al., 1987).

5.3 Drawbacks of transdermal drug delivery

Transdermal drug products can become painful or irritating to the patient if
caucistic, acidic or otherwise irritating materials are used. Additionally, chronic
administration of materials to the same area of skin may induce a sensitization effect
(Murphy et al., 2000). The onset of action of transdermally delivered drugs is delayed as
compared with injectable formulations, primarily due to the time required for the drug
product to pass the stratum corneum. As well, due to the nature of the stratum corneum it
is difficult to transport large hydrophilic drugs transdermally (Prausnitz et al., 2004).
Since biotransformation can occur by this route, unwanted metabolites of the parent drug
may be formed, causing unknown or detrimental adverse effects (Guy et al., 1987).
Finally, transdermal formulations administered to canines have been associated with
higher inter individual variability in plasma drug levels compared to the oral route of
administration (Yuan et al., 2009), which can pose challenges when trying to achieve
desired plasma drug levels transdermally.
5.4 Transdermal drug formulation

Successful delivery of the drug across the skin depends on properties of the drug, skin to be penetrated and the transdermal delivery system (Guy et al., 1987). Such factors include surface area and thickness of the skin where the compound is applied, concentration and lipid solubility of the drug and the delivery system, and duration of contact between the skin and drug compound (Turner et al., 2011). Ideal drugs for transport across the skin have a low molecular mass, high lipophilicity and are required at low therapeutic doses (Guy et al., 2003; Prausnitz et al., 2004). These drugs also ideally have some hydrophilicity in order to be released from the stratum corneum into the more hydrophilic epidermal layer and subsequent systemic circulation.

Transdermal delivery systems can be delivered in a reservoir system such as a pouch or patch, or dispersed in a polymer (Shah et al., 1992). Transdernals can also be applied topically as creams, ointments, gels, sprays, pastes, suspensions, lotions, foams, aerosols or solutions, which can then drive drug molecules through the skin layers and into the systemic circulation (Prausnitz et al., 2008). Briefly, creams are typically defined as oil-in-water semisolid emulsions or aqueous dispersions of fatty acids or alcohols. Emulsions are immiscible mixtures of two or more liquids that are stabilized by emulsifying agents to prevent separation. Foams are emulsions that have been packaged into special devices that dispense the liquid as an aerated product. Gels are semisolid suspensions of organic or inorganic molecules in a liquid. Lotions refer to viscous emulsions that are similar to creams. Ointments are semisolids that are typically mostly hydrocarbons, waxes or polyols. Pastes are stiff semisolids and sprays are liquids that
have been formed into discrete droplets by means of a specialized nozzle (Ueda et al., 2009).

Recently, various enhancement techniques, such as chemical enhancers, have been investigated with the goal of improving transport across the skin for drugs lacking these characteristics (Uster, 1990). Chemical skin permeability enhancers are aimed at reversibly increasing stratum corneum permeability or providing a stronger driving force for transport while avoiding injury to underlying tissues (Prausnitz et al., 2008). Examples of chemical enhancers include liposomes, solvents and surfactants. Liposomes form a spherical transport vesicle with a lipid bilayer around hydrophilic drug molecules and have been suggested to enhance drug transport into the skin (Kogan et al., 2006; Toutou et al., 2010). Lipophilic drug molecules can readily diffuse into the stratum corneum or be transported, integrated in the lipid bilayer, by liposomes. However lipophilic drug molecules are required to have some hydrophilic properties in order to diffuse deeper into the epidermis and systemic circulation (Guy et al., 1987). Solvents and surfactants are designed to increase the permeability of the stratum corneum by removing lipids, therefore disrupting the molecular structure (Prausnitz et al., 2008).

Iontophoresis and electroporation are techniques that utilize electric fields to transport drug molecules. They are generally not painful for the individual and the rate of drug delivery can be controlled (Prausnitz et al., 2004). Iontophoresis uses a small current whereas electroporation requires high-voltage pulses. Finally, ultrasound uses sound frequencies to deliver drugs transdermally by altering the skin barrier thermally, chemically, and mechanically (Naik et al., 2000). The main drawback of these enhancers
is increased skin irritation due to enhanced permeability and disruption of skin structure (Lashmar et al., 1989).

5.5 delivra™ as a transdermal drug delivery system

LivRelief™ has developed a novel transdermal delivery system called delivra™. delivra™ is an organic cream made up of a proprietary combination of plant based oils and waxes that form liposomes to transdermally transport lipid soluble and aqueous drugs into the body. The delivra™ base has been suggested to have anti-fungal, anti-bacterial and anti-inflammatory properties and is currently approved for human use in Canada and the United States. In clinical trials performed by LivCorp Inc., delivra™ was shown to effectively transport an NSAID, naproxen, across multiple layers of skin to the underlying musculature, suggesting a potential use for this base as a transdermal delivery system (LivCorp Inc., 2011).

6.0 Veterinary drug approval

In Canada, the approval of veterinary drug formulations is regulated by Health Canada, specifically through the veterinary drugs directorate (VDD). The VDD is a national and international collaboration of many organizations with Health Canada and is responsible for ensuring the safety, quality and effectiveness of approved veterinary drugs (Health Canada, 2013b). Through the VDD, Health Canada provides many suggested guidelines and policies that drug sponsors can follow in order to ensure their drug complies with requirements of the Food and Drug Regulations. Additionally, some guidelines recommended by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), a shared program between the European Union (EU), United States of America (USA) and
Japan, have been implemented by Health Canada. As guidelines, these documents can be subject to some flexibility at the discretion of the drug sponsor, as long as changes can be scientifically justified.

An application for a new veterinary drug formulation is termed a veterinary new drug submission (VNDS). Information required for a VNDS includes a master volume, manufacturing and quality control, animal safety, efficacy, human safety and environmental impact. A component of the manufacturing and quality control section involves information about the stability of the drug formulation with changing environmental conditions such as temperature, humidity and light. This data would then be used to help determine an appropriate shelf life for the final product. In the animal safety portion of the application, it is suggested that drug sponsors include information based on laboratory animals or scientific literature, if available, to determine PK and PD information such as the toxic effects in the target animal species. Finally, clinical efficacy of the drug product in the target animal species would support the efficacy portion of the VNDS.
RATIONALE

Canine OA is a common disease in veterinary medicine with no current cure. Management is daily and long-term and has, therefore, been associated with decreased owner compliance and occurrence of drug related ADRs. Meloxicam is a commonly used NSAID to manage the clinical signs of canine OA. It is administered orally or as an injection and has been reported to have a long half-life allowing for once daily dosing and good tolerability in canines. However meloxicam related ADRs have been reported and are suggested to occur as a result of high systemic drug levels and may be affected by route of administration, such as through direct irritation of the GI mucosa. Additionally, both enteral and parenteral routes of administration have numerous drawbacks that decrease owner compliance including cost, ease of administration, and route associated adverse effects.

Transdermal drug delivery has recently been investigated to address these disadvantages. LivCorp Inc. has developed a plant-based natural health product called delivra™ into which various drugs can be compounded. In human and in vitro studies, LivCorp Inc. has found that the delivra™ cream base was able to transport an NSAID, naproxen, through various layers of skin. delivra™ is currently approved for use as a natural health product in human medicine in Canada and the United States.

Due to its success in human medicine, and the potential benefits to veterinary patients of transdermal drug delivery, delivra™ is currently being investigated for use in veterinary medicine. It is suggested that with a transdermal meloxicam formulation owner compliance may be increased due to its ease of administration compared to other available routes. Meloxicam related ADRs might also be decreased by direct and indirect
mechanisms making it a better candidate for long-term use to treat canine OA. In order for the delivra™ base to be useful in veterinary medicine it would need to be approved by Health Canada, since the final drug product would be a new veterinary drug formulation. As such, LivCorp Inc. would need to file a VNDS to the VDD of Health Canada. Therefore, one of the objectives of this research is to investigate the delivra™ base as a possible transdermal delivery system for meloxicam for use in veterinary medicine. In order to most closely resemble the veterinary drug approval process, these experiments are focused on addressing some of the technical sections of the VNDS, specifically the manufacturing and quality control, animal safety, and efficacy (proof of concept) sections.

**Overall hypothesis:**

Meloxicam compounded in delivra™ will be stable, while being safe and efficacious in experimental animal models.

**Objectives:**

To test the above hypothesis I will complete three main objectives:

1. Evaluation of meloxicam levels in SF and plasma in research dogs when administered in a delivra™ compounded formulation
2. To evaluate the in vitro stability of meloxicam when compounded in delivra™
3. Evaluation of the anti-inflammatory and analgesic properties of meloxicam when compounded in delivra™ in a rat paw inflammation model
CHAPTER 1 – EVALUATION OF MELOXICAM LEVELS IN CANINE SYNOVIAL FLUID AND PLASMA WHEN COMPOUNDED IN DELIVRA
Introduction

The management of canine osteoarthritis poses a great challenge to many veterinarians and pet owners, as it is a chronic condition for which there is no cure. Pharmaceutical management usually involves NSAIDs, especially those of the COX-2 selective or preferential classification (Monteiro-Steagall et al., 2013). These NSAIDs are able to block the larger COX-2 active site, however, they cannot fit into the smaller COX-1 active site (Kurumbail et al., 1996). Therefore the COX-1 isoform is spared while maintaining the efficacy of COX-2 inhibition. COX-1 is constitutive in most tissues including the GIT, platelets and kidneys (Smith et al., 1994; Wooten et al., 2008). Contrary to this, COX-2 has been shown to be induced at sites of inflammation (Papich, 2008) and increased in naturally occurring OA joints of canines (Lascelles et al., 2009). As such, COX-2 preferential and selective NSAIDs were developed to manage canine OA and reduce the risk of GI ADRs that occur with nonselective NSAIDs, which are suggested to be due to inhibition of the constitutive COX-1 isoform (Monteiro-Steagall et al., 2013). However, recent studies have outlined physiological and pathological roles for both COX isoforms (Dubois et al., 1998; Guan et al., 1997; Harris et al., 1994; Lascelles et al., 2009; Papich, 2008; Wooten et al., 2008; Wooten et al., 2010). This recent finding may provide a possible explanation for the ADRs that have been reported even with oral administration of COX-2 inhibitors.

Meloxicam is a COX-2 preferential inhibitor, as determined by in vitro studies (Kay-Mugford et al., 2000), and was one of the first COX-2 preferential NSAIDs to be approved for the management of chronic and acute pain and inflammation in veterinary medicine (Doig et al., 2000). In canine experimental models of joint inflammation, it has
been demonstrated to be an effective anti-inflammatory agent, reducing leukocyte numbers in the affected joint (van Bree et al., 1994). Additionally, meloxicam has been shown to significantly reduce clinical signs of lameness in naturally occurring OA joints of canines (Davies et al., 1999; Doig et al., 2000; Moreau et al., 2003). Meloxicam has a plasma half-life in canines of 24 hours (Höglund et al., 2002), making it a good choice for once daily dosing, and therefore a good treatment option for chronic administration to dogs with OA.

It has been suggested that canines are the most frequent species with NSAID related ADRs (Khan et al., 2012) and that NSAID administration is the main factor that predisposes a dog to serious GI ulceration (Monteiro-Steagall et al., 2013). Additionally, GI ADRs are reported to be the main factor that limits the ability of a patient to use NSAIDs (Cunningham et al., 1994). Therefore, reduction in NSAID related GI ADRs in canines would be desirable when developing disease management protocols, especially for chronic illnesses. Meloxicam has demonstrated good renal tolerability in short-term and long-term studies in healthy dogs (Boström et al., 2006; Crandell et al., 2004; Fusellier et al., 2008; Luna et al., 2007). It has been suggested that stronger COX-2 selectivity will result in a lower risk of GI ADRs due to more COX-1 sparing. However, as discussed earlier, COX-2 has been recently shown to play physiological roles. While sparing of the COX-1 isoform may theoretically greatly reduce the incidence of ADRs, they still occur even with COX-2 inhibitors. Additionally, in one study, the GI safety of long-term administration of meloxicam was compared with carprofen and ketoprofen in healthy dogs (Forsyth et al., 1998). This study demonstrated no significant differences in GI ADRs between COX-2 preferential (meloxicam), COX-2 selective (carprofen) and
COX-1 selective (ketoprofen) NSAIDs. This finding supports the lack of significant
differences in GI safety that has been reported in a short term study in healthy dogs
(Wooten et al., 2009). While meloxicam is considered to have a good GI safety profile, it
has been shown that at high doses, meloxicam can produce toxic GI effects such as
ulceration and perforation (Enberg et al., 2006; Reed, 2002). Additionally, some studies
have reported mild GI effects in some dogs with long-term clinical doses of meloxicam
(Doig et al., 2000; Forsyth et al., 1998; Jones et al., 2002), which may worsen over time.

Meloxicam is currently available in enteral or parenteral dosage forms (Health
Canada, 2013a). As an enteral drug, it is given as an oral suspension or chewable tablet. It
can also be administered parenterally as an IV or SC injection. Due to the technical
difficulty with injections, higher cost, and discomfort for the dog, the enteral route of
administration is currently the most suitable option for the average pet owner. However
this route also has several drawbacks including erratic absorption characteristics, GI
irritation and difficulty of administration for some patients. The GI irritation is suggested
to occur directly, as a result of direct GI contact, and indirectly, as a result of PG
synthesis inhibition. NSAIDs are classified as weak acids and are more lipophilic
(unionized) in an acid environment. Therefore they are able to diffuse more readily
through the stomach wall, which can cause direct irritation of the GI mucosa (Konturek et
al., 2005; Whittle, 2004; Wolfe et al., 1999). It has been suggested that with the use of
parenteral delivery systems, direct contact of the GI mucosa with meloxicam is avoided,
thus potentially reducing direct adverse GI effects. Additionally, transdermal routes do
not use injections and, therefore, would allow for pain free parenteral dosing while still
providing the other benefits of parenteral delivery including more consistent plasma levels and avoidance of the hepatic first pass effect.

Some studies have investigated transdermal delivery systems for NSAIDs (Beetge et al., 2000; Yuan et al., 2009). In the study by Yuan et al, investigators compared their transdermal meloxicam formulation with meloxicam oral tablets in dogs and sampled plasma and SF. For the transdermal formulation they found SF meloxicam levels were about 8 fold higher than plasma levels. On the other hand SF meloxicam levels were 20% lower than plasma levels for the oral dosage form. Since COX-2 has been recently suggested to be constitutive in some tissues such as the GIT, bone, and in the kidney, where it is especially important during compromised renal states (Khan et al., 1998; Pilbeam et al., 1997; Wallace et al., 2000), concentration of meloxicam in the target site may allow for decreased systemic ADRs associated with systemic COX-2 inhibition. However, the investigators also found a much larger variability in meloxicam concentration for the transdermal dosage form, which can be a potential drawback in transdermal drug delivery.

LivCorp Inc. has developed a transdermal delivery system for use in human medicine. This delivery system is called delivra™ and utilizes plant based oils and waxes that form liposomes around drug molecules to enhance delivery across the skin layers. In this study, meloxicam was compounded in the delivra™ base and a comparison of SF and plasma meloxicam levels was made between oral suspension (Metacam®) and transdermal meloxicam cream (delivra™) delivery routes.
The objectives of this study were:

1) To evaluate meloxicam levels in SF of stifle joints in the canine after oral and transdermal treatment
2) Evaluate meloxicam levels in plasma in the canine after oral and transdermal treatment
3) Evaluate meloxicam related systemic and local ADRs in the canine when administered orally and transdermally

The hypothesis of the current study is that the delivra™ base will deliver meloxicam at equal or greater levels to the SF, while achieving equal or lower levels of plasma meloxicam when compared with the veterinary approved oral suspension formulation (Metacam®) in healthy research canines with minimal to no ADRs.

**Materials and methods**

**Animals**

The following study was conducted according to Canadian Council on Animal Care (CCAC) guidelines and following animal use protocol (AUP) approval by the University of Guelph Animal Care Committee (ACC). Research dogs (n=6, mongrels, Marshall BioResources, New York, USA), 3 male and 3 female, aged 6-24 months, weighing 18-25 kg were used in this study. Dogs were deemed healthy after physical examination and analysis of results of serum biochemistry, complete blood count (CBC), and urine specific gravity (SG) samples. Prior to the start of the study, clinical assessments of both stifle joints, including gait analysis and palpation of joint area, were performed by Dr. Thomas Gibson (DACVS, Clinical Studies, OVC) to ensure joints were normal on orthopedic examination. Serum biochemistry profile and urine SG were
repeated following last treatment day in both phases, to identify any changes that may have occurred with meloxicam administration. Daily temperature, pulse, and respiration (TPR), as well as any clinical signs of adverse reactions to meloxicam were monitored for each dog during both phases of the study, and up to 7 days post last treatment day in each phase.

**Preparation of meloxicam compounded in transdermal vehicle base**

Meloxicam oral suspension for dogs (Metacam®, 1.5 mg/mL, Boehringer Ingelheim, Missouri, USA) was purchased from the OVC pharmacy.

Meloxicam compounded in the transdermal delivra™ cream (50 mg/g, LivCorp Inc., Ontario, Canada) was prepared by a compound chemist in Quebec Canada. Due to the proprietary nature of the cream composition, much of the preparation process of the delivery vehicle cannot be disclosed. The ingredients of the base are as follows: argania spinosa oil, beeswax (bleached), benzoic acid, black currant seed oil, calendula officinalis, capryl glycol, catearyl olivate, cetyl palmitate, dehydroacetic acid, dromiceius, rutin, ethoxydiglycol, glycerin, inulin lauryl carbamate, isopropyl palmitate, lotus, melaleuca alternifolia leaf oil, peucedanum ostruthium, phenoxyethanol, potassium citrate, potassium sorbate, purified water, rubus idaeus, sambucus nigra fruit extract, sodium hydroxide, sorbitan palmitate, sorbitan olivate, xanthan gum, and sodium phytate. Briefly, delivra™ is a plant-based oil in water emulsion that is prepared at 75°C. Meloxicam was pre-solubilised in ethoxydiglycol and added during the cooling process at 40°C.
Preparation of canine stifles

Canine stifle joints were randomly selected for treatment in phase 1 and the contralateral stifle was used in phase 2. Stifle joints were shaved prior to baseline sample collection in both phases of the study. A plastic octagonal template was used to make an outline on the stifle joint for transdermal cream application and sample collection. The template was drawn on the lateral side of the stifle, caudal to the patella between the condyles of the femur and tibia.

Treatment application

The study was conducted as a 2-phase, balanced, randomized study. Dogs were randomized to either oral meloxicam suspension (Metacam®) or transdermal meloxicam in delivra™ cream (TDM) treatment groups. Metacam® was dosed according to the label, at a loading dose of 0.2 mg/kg body weight (BW) on day 1, followed by 0.1 mg/kg BW every 24 hours for another five days for a total of six treatments. TDM was dosed at 1.0 mg/kg BW every 24 hours for six days. The TDM was applied to the centre of the octagonal outline drawn on the stifle and rubbed onto the skin by hand in a circular manner for two minutes. Following the completion of phase 1 of the study, a 21-day washout period was observed, and then the dogs were crossed over in phase 2 of the study receiving the remaining treatment and using the contralateral stifle joint. During the study, Elizabethan collars and/or BiteNot® (when Elizabethan collars were not tolerated) were placed on dogs to prevent licking of the stifle area.

Sample collection

Samples of SF, plasma, and stifle skin biopsies were collected prior to treatment and 8 hours post last dosing in each phase. Blood samples were collected from the
cephalic vein, while the dogs were conscious. Blood was placed in 5 mL heparin tubes (BD Vacutainer®, New Jersey, USA) on ice and spun down for plasma collection. Following plasma collection, dogs were sedated with an IV injection of dexmedetomidine (Dexdomitor®, Zoetis, Kirkland, QC, 0.5 mg/mL at a dose of 20 µg/kg in phase 1 and 10 µg/kg in phase 2) and butorphanol (Torbugesic®, Zoetis, Kirkland, QC, 10 mg/mL at a dose of 0.2 mg/kg). Stifles were aseptically prepared with Germi-Stat® (Germiphene Corp., Ontario, Canada), isopropyl alcohol and Baxedin® (Omega Laboratories Ltd., Quebec, Canada). The stifle was flexed, and SF collected by standard arthrocentesis, using a 1.5” 22 gauge needle and 3mL tuberculin syringe. Arthrocentesis was performed blinded to treatment groups. Two skin biopsy samples of the treatment area skin were also obtained using a 5mm biopsy punch. Sedation was reversed with atipemazole (Antisedan®, Zoetis, Kirkland, QC, 5 mg/mL at a dose of 20 µg/kg), following sample collection. The SF and plasma samples were stored at -80°C until analyzed for meloxicam concentration using mass spectrometry by a third-party (Dr. Heather Knych, University of California Davis School of Veterinary Medicine). Meloxicam concentration was determined blinded to the treatment group.

Statistical analysis

This study was conducted as a randomized, blinded, cross-over study. In addition to the treatment effect, the statistical analysis included a carry over effect, random effect of dog (a blocking factor) and a period effect. The model included all variables initially, then non-significant terms (P>0.1) were removed from the model; the analyses reduced to 2-sample t-tests. After testing for equality of variances, it was determined that the comparison of plasma meloxicam levels would be performed using the Satterthwaite
model with unequal variance, whereas the SF comparison used a pooled-variance estimate in a standard 2-sample t-test. Residual analyses suggest that ANOVA assumptions were adequately met for the SF data. The plasma data exhibited unequal variance, which was accounted for in the model, but otherwise ANOVA assumptions appeared to be satisfied.

**Results**

**Plasma meloxicam levels**

Plasma meloxicam levels for each canine following both phases of oral Metacam® and TDM treatments are listed in Table 1. Overall plasma mean±SE meloxicam concentrations for both oral Metacam® and TDM treatment groups are listed in Table 3 and shown in Figure 1. Prior to the start of the study, the mean plasma meloxicam levels in both oral and TDM treatment groups was 0 ng/mL. Following the washout period, and prior to dosing in phase 2, the mean plasma meloxicam level in the oral treatment group was 2.52±0.69 ng/mL and in the TDM treatment group was 0.83±0.26 ng/mL. These values were not significantly different (P=0.6290). After six days of treatment, the mean plasma meloxicam concentration for the oral treatment group (n=6) was 812.4±220.1 ng/mL and for the TDM treatment group (n=6) was 962.7±704.9 ng/mL. There was no significant difference in mean plasma meloxicam concentrations between the oral and the TDM treatment groups (p=0.6360). However, there was a significant difference in variance between the oral and TDM treatment groups (p=0.0232).
SF meloxicam levels

SF meloxicam levels for each canine following both phases of oral Metacam® and TDM treatments are listed in Table 2. Overall SF mean meloxicam concentrations ± SE for both oral Metacam® and TDM treatment groups are listed in Table 4 and shown in Figure 2. The mean SF meloxicam level in both oral and TDM treatment groups was 0 ng/mL at the start of the study and following the washout period. The mean SF meloxicam concentration for the oral treatment group was 430.1±185.8 ng/mL and for the TDM treatment group was 484.9±365.9 ng/mL after six days of treatment. There was no significant difference in mean SF meloxicam levels between oral and TDM treatment groups (p=0.7525). There was also no significant difference in variance between oral and transdermal treatment groups (p=0.1632).

Meloxicam related ADRs

Due to financial restrictions, stifle skin biopsy samples have not yet been analyzed histologically for meloxicam-related skin effects. However, clinical examination of the TDM application site revealed no obvious evidence of adverse reactions to TDM.

Discussion

It has been suggested, based on therapeutic meloxicam concentrations in horses, that a plasma concentration of about 100 ng/mL of meloxicam should be sufficient for clinical effects in canines (Yuan et al., 2009). In this study, meloxicam concentrations in the plasma greatly exceeded this suggested level and as such, it is likely that meloxicam delivered in delivra™ would be clinically efficacious. Additionally, the delivra™ base was able to transport meloxicam to the SF and plasma at comparable levels to the current
veterinary approved oral Metacam® formulation in healthy research dogs, providing further support for its potential use as a clinically effective veterinary transdermal delivery system.

In this study, there was a significantly higher variability in plasma meloxicam concentrations for the TDM treatment group compared with the oral treatment group, which has been demonstrated in other studies (Yuan et al., 2009). This increased variability of meloxicam levels is one challenge in developing transdermal drug formulations. During both phases of the study dogs wore Elizabethan collars or a BiteNot® to prevent licking of the stifle area. However, throughout each phase some of the dogs were able to remove the collars and as a result, had access to the stifle for short periods of time. Therefore, dogs in the TDM treatment group may have been able to lick off some of the TDM treatment that was applied, which, acting as a partial oral dose, could potentially increase perceived plasma levels of meloxicam and therefore increase the variability for this group. However based on subjective observation during treatment administration, the majority of TDM appears to have been thoroughly absorbed in the two minutes of treatment application. Therefore any licking of the stifle and oral absorption of the TDM treatment that might have occurred subsequently would likely have contributed minimally to plasma or SF levels of meloxicam.

Variability in meloxicam levels associated with the TDM group could also result from variation in the amount of meloxicam given to each dog. The TDM formulation was administered based on a mg/kg dosing regimen, however if the formulation was not homogenous, this calculation may result in different doses of meloxicam being administered to each dog. In formulating a transdermal drug delivery system, LivCorp
Inc. has compared the effects of formulation techniques on the variability in drug concentration within a batch of cream. They found that there was significantly lower variability when the drug was added to the components of the delivra™ base and then prepared, rather than when the drug was added to the pre-formulated delivra™ base. In this study the TDM formulation was made by adding meloxicam to the components of the delivra™ base. Therefore the TDM formulation used in this study is thought to be homogenous and likely did not contribute to the variability in meloxicam levels measured in the TDM treatment group.

During SF sample collection, any contamination with blood could cause variability in detected SF meloxicam levels. If contamination occurred in orally or TDM treated dog samples, the SF sample may demonstrate higher than correct meloxicam levels. Additionally, once part of the SF sample is deemed contaminated, then the entire sample and the SF remaining in that stifle joint are also affected. However, if the SF sample is contaminated mostly with red blood cells (RBCs) rather than plasma then contamination may not lead to significantly altered drug levels. With treatment groups of six dogs, it is not experimentally practical to discard all SF samples that have been, or may have been, contaminated with blood and as a result, all samples were included in the analytical comparisons. Since SF sample collection is a technically difficult task, potential contamination with blood is possible. Some SF samples collected were deemed contaminated on visual inspection, however these samples were not statistical outliers, thus they remained in the statistical analysis. Therefore experimental SF meloxicam levels achieved in this study need to be considered strictly as an approximation in healthy
canines, with the understanding that they may be a slight overrepresentation in some cases.

Transdermals deliver drugs directly across the stratum corneum, through sweat ducts or through hair follicles and sebaceous glands. The drug then diffuses into the dermis and epidermis and finally into the systemic circulation where the drug travels to and concentrates in the target site (Benson, 2005; Naik et al., 2000; Ueda et al., 2009). In a previous PK study (Yuan et al., 2009), researchers demonstrated that following topical administration of meloxicam in a gel to healthy canines, plasma drug levels remained significantly lower than SF drug levels throughout all of the time points tested. These researchers concluded that the gel formulation investigated thus delivered meloxicam locally through the skin layers into the SF, rather than via absorption into the systemic circulation and subsequent concentration in the SF. If this is true, then the gel formulation used was acting as a topical drug delivery system and not a transdermal delivery system. In this study, in both the oral Metacam® and TDM treatment groups, plasma meloxicam concentrations were roughly two-fold higher in comparison to SF meloxicam levels. This suggests that the delivra™ base is likely acting as a transdermal delivery system, delivering meloxicam through the systemic circulation, rather than acting as a topical agent, delivering meloxicam only locally at the site of application. It would also be useful to analyze the stifle skin biopsy samples to determine meloxicam levels achieved in the local tissue. However, in this study plasma samples were only collected at baseline and following six treatments. Without plasma, tissue and SF drug level measurements taken at various time points following TDM administration, it is not possible to truly determine
whether meloxicam levels achieved in the SF were the result of meloxicam transported topically or transdermally by the delivra™ base.

Since systemic meloxicam levels achieved with the delivra™ base were similar to that of Metacam®, it is likely that the systemic meloxicam related ADRs, such as those affecting the kidneys or the GIT through inhibition of PG synthesis, would not be significantly decreased with this novel transdermal route of administration. However, transdermal formulations provide parenteral drug delivery, therefore the GIT should not be directly exposed to meloxicam by this route of administration. It has been reported that the weakly acidic properties of many NSAIDs, including meloxicam (Pomykalski et al., 2011), promotes the movement of the drug into epithelial cells lining the surface of the gastric mucosa, where the drug becomes ionized and trapped, and promotes release of hydrogen ions that damage mucosal cells (Schoen et al., 1989). Additionally, it is thought that NSAIDs are able to decrease gastric mucosal hydrophobicity, which would allow for direct contact of the mucosa with gastric acid and pepsin, further damaging the gastric epithelium (Wolfe et al., 1988). Therefore, avoiding direct exposure of the GIT to meloxicam through transdermal drug delivery could offer a mechanism for reducing meloxicam related ADRs in the GIT, despite there being similar systemic meloxicam levels.

While the stifle skin biopsy samples were not analyzed in this study, it is not expected that the TDM formulation would cause adverse skin reactions. The surface of the skin is slightly acidic, with a pH of about 5.4 to 5.9 (Paudel et al., 2010) and it has been reported that the ideal pH of transdermal systems should range from 5 to 9 to allow passive transdermal drug delivery while avoiding skin irritation (Naik et al., 2000). When
comparing slightly acidic (pH 4 or 6.5) formulations to slightly basic (pH 10) formulations, it was reported that the slightly acidic formulation resulted in fewer adverse skin reactions (Ananthapadmanabhan et al., 2003). The pH of the TDM formulation batch used in this study was not measured, however was measured from another batch (see Chapter 2). The pH was reported to be 4.58 in this batch. Assuming the pH is similar between the tested batch and that used in this study, the TDM formulation should be well tolerated when applied topically.

Overall it appears that the TDM formulation used in this study resulted in plasma and SF meloxicam levels comparable to those achieved with the oral Metacam® formulation. Additionally, it appears that the TDM formulation did not cause adverse systemic or local adverse reactions in healthy canines. Therefore, this TDM formulation has the potential to be a novel transdermal delivery system for meloxicam for treatment of canine OA.
Table 1. Plasma meloxicam levels (ng/mL) measured in oral and transdermal meloxicam treatment groups before and 6 hours after treatment administration in phase 1 and phase 2.

<table>
<thead>
<tr>
<th>Dog name</th>
<th>Phase 1</th>
<th></th>
<th></th>
<th>Phase 2</th>
<th></th>
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</thead>
<tbody>
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<td>Post-treatment meloxicam concentration (ng/mL)</td>
<td>Treatment</td>
<td>Side</td>
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<tr>
<td>Major</td>
<td>0</td>
<td>1383.86</td>
<td>Transdermal</td>
<td>L</td>
<td>1.73</td>
</tr>
<tr>
<td>Janey</td>
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<td>1162.11</td>
<td>Oral</td>
<td>R</td>
<td>0.61</td>
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<tr>
<td>Baron</td>
<td>0</td>
<td>808.62</td>
<td>Oral</td>
<td>L</td>
<td>0.76</td>
</tr>
</tbody>
</table>
Table 2. Synovial fluid meloxicam levels (ng/mL) measured in oral and transdermal meloxicam treatment groups before and 6 hours after treatment administration in phase 1 and phase 2.

| Dog name | Phase 1 | | Phase 2 | |
|----------|---------|---|---------|---|---|
|           | Pre-treatment meloxicam concentration (ng/mL) | Post-treatment meloxicam concentration (ng/mL) | Treatment | Side | Pre-treatment meloxicam concentration (ng/mL) | Post-treatment meloxicam concentration (ng/mL) | Treatment | Side |
| Ruby     | 0       | 963.59 | Transdermal | L | 0 | 335.74 | Oral | R |
| Hunter   | 0       | 532.41 | Oral | L | 0 | 731.55 | Transdermal | R |
| Baby     | 0       | 32.08  | Transdermal | R | 0 | 251.06 | Oral | L |
| Major    | 0       | 690.01 | Transdermal | L | 0 | 279.47 | Oral | R |
| Janey    | 0       | 743.81 | Oral | R | 0 | 341.57 | Transdermal | L |
| Baron    | 0       | 438.01 | Oral | L | 0 | 150.64 | Transdermal | R |
Table 3. Mean±SE plasma meloxicam levels (ng/mL), measured in oral and transdermal meloxicam treatment groups before and 6 hours after treatment administration in phase 1 and phase 2. The difference in means was not significant, with a P-value of 0.6360.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th></th>
<th>Post-treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (ng/mL)</td>
<td>SE</td>
<td>Mean (ng/mL)</td>
<td>SE</td>
</tr>
<tr>
<td>Oral</td>
<td>0.84</td>
<td>0.40</td>
<td>812.40</td>
<td>89.86</td>
</tr>
<tr>
<td>Transdermal</td>
<td>0.41</td>
<td>0.15</td>
<td>962.70</td>
<td>287.77</td>
</tr>
</tbody>
</table>
Table 4. Mean±SE synovial fluid meloxicam levels (ng/mL), measured in oral and transdermal meloxicam treatment groups before and 6 hours after treatment administration in phase 1 and phase 2. The difference in means was not significant, with a P-value of 0.7525.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (ng/mL)</td>
<td>SE</td>
</tr>
<tr>
<td>Oral</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Transdermal</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Figure 1. Plasma meloxicam concentrations measured before and after oral Metacam® and transdermal meloxicam in delivra™ base treatment for 6 days. No statistically significant differences (P>0.05) were detected in meloxicam levels.
Figure 2. Synovial fluid meloxicam levels measured before and after oral Metacam® and transdermal meloxicam compounded in delivra™ base treatment for 6 days. Statistically significantly different (P>0.05) meloxicam levels were not detected between treatment groups.
CHAPTER 2 – EVALUATION OF MELOXICAM STABILITY IN VITRO WHEN COMPOUNDED IN DELIVRA™
Introduction

The VDD of Health Canada is responsible for ensuring the safety, quality and effectiveness of veterinary drugs in Canada. This is done by carefully reviewing information provided in the VNDS prior to approval of the drug. In partial fulfillment of a VNDS, drug sponsors are required to complete manufacturing and quality control, animal safety, and efficacy sections. These sections involve studies of drug stability, laboratory animal toxicity and clinical efficacy, respectively (Health Canada, 2011). The VDD collaborates with various national and international organizations to develop guidelines and policies for applicants to follow when completing a VNDS, to demonstrate that their drug meets the Food and Drug Regulations. Health Canada has also adopted some guidelines provided by ICH, an international program between the EU, Japan and USA (Health Canada, 2014).

According to the ICH guidelines, stability testing should include studies of varying storage conditions, such as temperature, light and humidity. The ICH guidelines also suggest timelines for sampling and duration of stability studies. According to ICH, test storage conditions should include (1) a long term test condition for 1 year at 25°C±2°C with a relative humidity (RH) of 60%±5%, (2) an intermediate test condition for 6 months at 30°C±2°C with a RH of 65%±5% and (3) an accelerated test condition for 6 months at 40°C±2°C with a RH of 75%±5% (VICH, 2007). For drug formulations that may be stored in the refrigerator or freezer, it is also suggested that a refrigerator storage condition for 3 months at 4°C and a freeze-thaw (FT) storage condition from -10°C to ambient room temperature (RT) be included as well (TO, 2011). Light exposure studies are termed photostability studies and it is suggested that a dark control under the
same conditions is included. ICH suggests a minimum overall illumination of 1.2 million lux hours and a minimum ultraviolet (UV) energy of 200 watt hours per square meter for confirmation of photostability (VICH, 1999). Results obtained from the photostability tests can then be used to justify the final packaging of the drug product in the VNDS. ICH also provides guidelines for the duration of the study as well as for frequency of sample collection, which differ depending on if the storage is for long term, intermediate or accelerated conditions.

Due to the proprietary nature of pharmaceutical drug development, most of the information provided in a VNDS is held under strict confidentiality and, therefore, much of the data, protocols and methods obtained in previous studies are not available as scientific justification for future studies. As a result, the current stability study is designed to most closely fulfill the criteria for the manufacturing and quality control section of a VNDS, based on previous work that is accessible, as well as guidelines posted by ICH and Health Canada. For example, one study suggested that samples be stored in glass rather than plastic, for better observation and physical measurements (TO, 2011), thus glass containers were used for storage. However, due to financial restrictions and specialized equipment needs all details of the protocols used may not be the same as those used in the industry, and may not be acceptable in a VNDS for the VDD.

The objectives of this study were:

1) Assess meloxicam concentration in the delivra™ base under varying storage conditions

2) Assess the pH changes of meloxicam compounded in the delivra™ base under varying storage conditions
It is hypothesized that meloxicam compounded in the delivra™ base will be stable for up to 3 months, with minimal changes in pH and meloxicam concentration, under some storage conditions.

Materials and methods

Drug formulation

Meloxicam compounded in the delivra™ base was prepared by a compound chemist in Quebec Canada at a final meloxicam concentration of 5% (50 mg/g, LivCorp Inc., Ontario, Canada). As discussed in Chapter 1, the formulation of the delivra™ base is proprietary and therefore cannot be disclosed. However the process of compounding of meloxicam in the delivra™ base is the same as that described previously (See Chapter 1 Methods, Preparation of meloxicam treatments).

Sample storage

One large batch of 5% meloxicam compounded in the delivra™ base was stirred using a metal spatula in a clockwise motion for 1 minute to maximize homogeneity of the formulation before dividing it into test samples for stability testing. Aliquots of 10mL of the cream were taken from the initial batch and stored in 20 mL clear borosilicate glass vials and capped with black phenolic screw caps (Wheaton Industries, New Jersey, USA). The necks of the glass vials were then wrapped in Parafilm® to improve humidity control. The treatment groups used in this study were 4°C with no light (4NL), FT cycles with no light (FTNL), RT (25°C±2°C) with no light (RTNL), RT with light (RTL) and elevated temperature (40°C±2°C) with no light (40NL).
Study duration and time points

Prior to sample collection, aliquots were stirred with a metal spatula for 30 seconds in a clockwise motion. To ensure homogeneity of the TDM formulation, ten samples were taken at baseline from varying locations within the batch container. For all other time points three samples were collected from the top, middle and bottom of the containers. Samples were measured at baseline, then at 7, 14, 21, 28 days, 2 months and 3 months after storage for all of the treatment conditions except the FT condition. Samples from the FT condition were collected up to 3 weeks after storage. All samples were collected in triplicate from the top, middle and bottom layers of one vial per each treatment condition.

Temperature

Temperatures used in this study were 4°C in a standard refrigerator, -20°C in a standard freezer, 25°C on a shelf at room temperature in the presence and absence of normal light, and 40°C in a thermal incubator with no light. Samples undergoing the FT cycles were subject to either 1, 2 or 3 FT cycles. In the 1 FT cycle treatment group, samples were frozen at -20°C for 1 week and then thawed in a water bath set at ambient RT (25°C±2°C). In the 2 FT cycle treatment group, samples were frozen at -20°C for 1 week, thawed in RT water bath, stirred with a metal spatula for 30 seconds and returned to the freezer for another 1 week. Following the second week of total storage, samples were then removed from the -20°C freezer, and thawed in a RT water bath, for a total of 2 FT cycles. Samples in the 3 FT cycle treatment group underwent the same protocol as the 2 FT cycle, but were subject to 3 weeks of -20°C storage and 3 total FT cycles.
Light

Samples in the light (L) treatment group were exposed to a minimum of 1.2 million lux hours of fluorescent light and 200 W/m² of UV light. The samples in the no light (NL) treatment groups were covered on the outside of the glass vials with aluminum foil. The amount of lux exposure was determined by measuring the lux output of the fluorescent bulb using a lux meter (Sper Scientific, Arizona, USA).

pH measurement

The pH of collected samples was measured using a pH meter with a glass, flat-surface electrode (Extech, New Hampshire, USA). Prior to sample analysis at each time point, the pH meter was calibrated with pH 7.00 and 4.00 buffer solutions (Fisher Scientific, Ontario, Canada). One pH measurement was taken for the 3 sample replicates from the same vial for each treatment condition at each time point.

Meloxicam extraction and HPLC analysis

HPLC was performed by a trained technician to determine meloxicam concentration within the collected samples. Samples in glass vials were mixed with a metal spatula for 30 seconds prior to HPLC analysis. For a single sample, 3 to 5 mg of the transdermal meloxicam formulation was placed into a 2 mL tube (Eppendorf, Ontario, Canada) and the weight of the sample was recorded. Subsequently, 1 mL of methanol was pipetted into the tube. Tubes were then closed and vortexed for 10 seconds, then placed in a water bath in a sonicator for 30 minutes. Following this, samples were spun down for 10 seconds in a mini centrifuge (Mandel Scientific Company Inc., Ontario, Canada) and 200 µL of supernatant was pipetted into a new labeled 1 mL cryovial.
Samples were stored at -20°C for analysis by HPLC within 5 days of meloxicam extraction.

Standard curves for meloxicam were created prior to unknown sample analysis. Stock solutions of meloxicam (Sigma, Ontario, Canada) were made in methanol at 1 mg/mL and stored in a -80°C freezer. Stock solutions of meloxicam were diluted in the mobile phase at concentrations of 0.5, 1, 2.5, 5, and 10 µg/mL, to create standard curves for calibration. Test samples were then thawed, and 990 µL of mobile phase was added to a new tube. The mobile phase consisted of 40% milliQ water, 60% acetonitrile and 1% acetic acid by volume, with a pH of 4.5. Once test samples had thawed, 10 µL from the top of each sample was pipetted into the new tube containing the mobile phase. The new tubes were then centrifuged and a 50 µL aliquot of each sample was loaded into a Sunfire C18, 2.5µm chromatography separation column (Waters Alliance®, Massachusetts, USA) utilizing a flow rate of 1 mL/minute. The Waters Alliance® 2695 HPLC separations system connected to a personal computer was used to determine meloxicam concentrations in test samples. Eluent was monitored at a wavelength of 355.0 nm, and data collection and analysis was completed using Empower 2 software (Waters®, Ontario, Canada).

**Visual inspection**

Visual inspection was performed to determine if any evaporation, separation, color changes or odor changes occurred.

**Statistical analysis**

This study was conducted with triplicate samples being collected from the same vial, representing sub-samples. Interaction terms were initially included in the model but
shown to be insignificant, therefore, they were removed. This statistical model included adjustments for time and treatment effects. In the model, the random effect of treatment by time was removed due to the small variance. Pairwise differences in least square means was used due to the highly significant treatment and time effects in this study. Differences were adjusted by the Tukey-Kramer method due to unequal sample sizes. ANOVA assumptions in the model for pH all seemed to be satisfied based on tests for normality and analyses of residuals. There was one outlier in the HPLC model that was not removed since it was likely a true value, and removing it did not change conclusions of the overall test. Otherwise all ANOVA assumptions were met for the HPLC model.

Results

pH

Overall mean pH values for varying treatment groups and storage times are listed in Table 5 with corresponding p-values written in Tables 6 and 7. Storage under the 40NL condition resulted in statistically significantly different mean pH levels compared to the 4NL, FTNL, and RTNL storage conditions (p<0.0001, p=0.0066, p=0.0081 respectively). Storage under the 4NL condition led to a statistically significantly different pH level compared with the RTL condition (p=0.0010). There were also statistically significantly different pH levels measured between varying time points. These differences were measured between baseline and week 2, month 2 and month 3 (p=0.0382, p=0.00023, p<0.0001), as well as week 2 and month1 and 2 (p=0.0004, p=0.0043). Statistically significant differences were also demonstrated between month 3 and week 1 and 3 (p=0.0073, p=0.0396) and week 2 and week 1 and 3 (p<0.0001, p<0.0001).
HPLC analysis was used to determine meloxicam concentration (%) under each storage condition. No statistically significant overall differences in mean meloxicam concentration were detected between any of the treatment conditions (Data not shown; P>0.05). However, there were statistically significant differences in mean meloxicam concentration between the various time points. Overall mean meloxicam concentrations in % for each treatment group and storage time period are listed in Table 8 and p-values are listed in Table 9. Briefly, statistically significantly lower meloxicam concentrations were measured between baseline and month 1 and 2 (P=0.0324, P=0.0167), and statistically significantly higher meloxicam concentrations were measured between baseline and month 3 (P<0.0001). Statistically significant differences were also measured between week 1 and month 1, 2 and 3 (P=0.0264, P=0.0150, P=0.00031) as well as week 2 and month 2 and 3 (p=0.0497, p=0.0007). Statistically significantly higher meloxicam concentrations were also measured between month 3 and week 3 and month 1 and 2 (p<0.0001, p<0.0001, p<0.0001).

Visual inspection

No noticeable separation or changes in color or odor occurred in any of the treatment groups at any time points. Visually, the 40NL treatment condition appeared to have condensation on the inner side of the glass vials for all time points tested.

Discussion

Statistically significantly different pH levels were measured with varying storage conditions and time points throughout the study. However, the overall pH range was between 4.34 and 4.61, which may not be biologically significantly different. The skin’s
pH is typically acidic, with a pH in the range of 5.4 to 5.9 (Kaplan et al., 1984). It has been suggested that increasing acidity of a transdermal formulation may increase the permeability of the skin to acidic NSAIDs (Henrotin et al., 1998), and, therefore, may increase meloxicam entry into the systemic circulation. As such, a pH in the above range would facilitate meloxicam entry and may be biologically beneficial. It has also been shown that formulations with a pH close to this range, for example pH of 4 or 6.5, leads to fewer adverse skin reactions compared with more basic formulations with a pH of 10 (Willis-Goulet et al., 2003). Additionally, one study reported that a pH in the range of 5 to 7 in transdermal products does not cause skin irritation in humans (Riviere et al., 2013). While there was an overall significant effect of treatment condition and time on the measured pH of meloxicam compounded in delivra™, the range of pH values measured suggest they may not be biologically significant changes and, based on the literature, are likely to produce minimal local irritant effects. Therefore, despite the overall statistical significance of treatment conditions and time on pH changes, the meloxicam compounded in delivra™ base appears to be stable under the given test conditions for up to 3 months of storage. Lastly, it is important to note that the pH meter used in this study has a resolution of 0.01 units. As such, triplicate measurements taken at each time point were generally within 0.02 pH units resulting in a very small variance, which could cause a more enhanced sensitivity to statistical significance. If a pH meter with a higher resolution were used, the variance may have been higher and could result in fewer significant findings.

The HPLC analysis measured under varying storage conditions showed no overall significantly different meloxicam concentrations. However the meloxicam concentrations
compared at different time points led to significantly different results. The statistical analysis suggests that meloxicam compounded in the delivra™ base is stable for up to 1 month under the test conditions. As with the pH measurements, the meloxicam concentrations may have been statistically significantly different but may have differing practical significance. The average measured meloxicam concentrations throughout the study ranged from 3.03% to 5.44%. Since the meloxicam compounded in delivra™ cream was formulated at a concentration of 5% but had a true average initial concentration of 3.87%, this range correlates to -21.71% to +40.57% of the initial mean concentration. According to guidelines given by Health Canada, a change of +5% of the initial concentration of a drug product is acceptable (Paudel et al., 2010), therefore indicating a product’s stability under the test condition. Based on this guideline the meloxicam compounded in delivra™ base formulation is stable for up to 3 weeks of storage at 40NL, RTNL and 4NL, less than 1 week of storage at RTL, and less than 1 freeze-thaw cycle. However, this stability conclusion is based on the measured initial mean concentration of 3.87% meloxicam rather than the targeted concentration of 5%. Thus, final formulation changes may have to be made in order to ensure that the desired concentration is consistently obtained. While the guideline for acceptable change is seemingly very strict, it is important to ensure that the drug formulation meets this requirement to prevent under or over dosing of the patient.

ICH guidelines suggest storage durations that vary considerably, depending on test conditions, such as long-term, intermediate or accelerated. However, due to financial considerations, simplified timelines and test conditions based on some previously published stability work (Bachhav et al., 2010; Hawkins et al., 2006) have been used in
this study. One published stability study (Hawkins et al., 2006) used short-term storage conditions, testing samples once per week for 1 month. In a separate stability study (Bachhav et al., 2010) researchers used long-term storage conditions, testing samples once per month for 3 months and then again at 6 months. The current study, therefore, tested samples once per week for 1 month, followed by once per month for 3 months in order to best satisfy criteria for a VNDS. Alsante et al. conducted a study summarizing storage conditions used by various pharmaceutical companies (Alsante et al., 2003). While confidentiality of the exact protocols used by individual companies was maintained, this group was able to demonstrate that about 72% of companies included a storage condition of 41-50°C or 51-70°C, supporting the importance of this temperature extreme as a test condition. As such, for financial considerations, the intermediate storage condition was thus removed from this study and 4°C, freeze-thaw cycles, room temperature and high temperature conditions were used. To satisfy the photostability portion of the study, a UV light and separate fluorescent light were used. A lux meter was used to ensure that the 1.2 million lux hour requirement was met with the fluorescent bulb. Varying temperature and light conditions are useful for both the drug developer and VDD in determining the best storage condition and packaging of the final drug product.

Additionally, due to financial restrictions and a lack of facilities and equipment, laboratory conditions that satisfy the requirements for good manufacturing practice (GMP) have not been fully met in this study. While the following series of experiments have been conducted to most closely resemble a stability testing study, results obtained would likely not be acceptable for inclusion in a VNDS. In addition to not satisfying GMP test conditions, humidity was not measured in this study. Based on visual
inspection, it appears that condensation was present and therefore some evaporation of the TDM likely occurred, however it was not possible to quantify the level of moisture loss that occurred. Measuring humidity levels would have been useful for determining evaporation or moisture loss of the delivra™ cream, or for controlling the test environment to prevent moisture loss. However, the data may still be used to assist in designing future stability studies, as well as assisting in the determination of the shelf life or beyond use date of meloxicam compounded in delivra™. One study suggested that naturally aged samples may provide better evidence for the product’s degradation profile (Hou et al., 2001), thus repetition of the RTNL and RTL conditions may provide unique opportunities for future analysis of degradation products.
Table 5. Mean±SE pH values following storage under varying conditions at multiple time points.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean±SE pH value</th>
<th>Baseline</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Month 1</th>
<th>Month 2</th>
<th>Month 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>40NL</td>
<td>4.58±0.00</td>
<td>4.52±0.00</td>
<td>4.34±0.00</td>
<td>4.49±0.00</td>
<td>4.45±0.00</td>
<td>4.43±0.00</td>
<td>4.38±0.02</td>
<td></td>
</tr>
<tr>
<td>RTL</td>
<td>4.58±0.00</td>
<td>4.55±0.00</td>
<td>4.38±0.00</td>
<td>4.52±0.00</td>
<td>4.50±0.00</td>
<td>4.44±0.00</td>
<td>4.37±0.01</td>
<td></td>
</tr>
<tr>
<td>RTNL</td>
<td>4.58±0.00</td>
<td>4.58±0.00</td>
<td>4.40±0.00</td>
<td>4.55±0.00</td>
<td>4.54±0.00</td>
<td>4.52±0.00</td>
<td>4.52±0.01</td>
<td></td>
</tr>
<tr>
<td>4NL</td>
<td>4.58±0.00</td>
<td>4.61±0.00</td>
<td>4.42±0.01</td>
<td>4.60±0.00</td>
<td>4.58±0.00</td>
<td>4.59±0.00</td>
<td>4.59±0.01</td>
<td></td>
</tr>
<tr>
<td>FTNL</td>
<td>4.58±0.00</td>
<td>4.60±0.00</td>
<td>4.44±0.00</td>
<td>4.60±0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Statistically significantly different pH values were detected between the above treatment groups outlined in Table 5. P values listed in the table have been adjusted by the Tukey-Kramer method.

<table>
<thead>
<tr>
<th>pH comparison</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>40NL vs. 4NL</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>40NL vs. FTNL</td>
<td>0.0066</td>
</tr>
<tr>
<td>40NL vs. RTNL</td>
<td>0.0081</td>
</tr>
<tr>
<td>4NL vs. RTL</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

Table 7. P values for statistically significant results obtained from comparison of pH values between varying time points for all treatment groups. Values have been adjusted by the Tukey-Kramer method.
Table 8. Mean±SE meloxicam concentration detected by HPLC analysis following storage under varying conditions and times.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean meloxicam concentration (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Week 1</td>
<td>Week 2</td>
<td>Week 3</td>
<td>Month 1</td>
<td>Month 2</td>
</tr>
<tr>
<td>40NL</td>
<td>3.87±0.17</td>
<td>3.73±0.28</td>
<td>3.69±0.63</td>
<td>4.71±0.69</td>
<td>3.03±0.32</td>
<td>3.19±0.45</td>
</tr>
<tr>
<td>RTL</td>
<td>3.87±0.17</td>
<td>4.24±0.50</td>
<td>4.11±0.29</td>
<td>3.51±0.09</td>
<td>3.20±0.13</td>
<td>3.32±0.22</td>
</tr>
<tr>
<td>RTNL</td>
<td>3.87±0.17</td>
<td>3.94±0.12</td>
<td>3.72±0.28</td>
<td>3.50±0.09</td>
<td>3.25±0.36</td>
<td>3.19±0.05</td>
</tr>
<tr>
<td>4NL</td>
<td>3.87±0.17</td>
<td>3.84±0.20</td>
<td>3.99±0.68</td>
<td>3.27±0.19</td>
<td>3.44±0.23</td>
<td>3.05±0.73</td>
</tr>
<tr>
<td>FTNL</td>
<td>3.87±0.17</td>
<td>4.31±0.21</td>
<td>4.06±0.17</td>
<td>3.63±0.45</td>
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<td></td>
</tr>
</tbody>
</table>

Table 9. P values listed for statistically significantly different average meloxicam concentrations between various time points.

<table>
<thead>
<tr>
<th>HPLC comparison</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline vs. month 1</td>
<td>0.0324</td>
</tr>
<tr>
<td>Baseline vs. month 2</td>
<td>0.0167</td>
</tr>
<tr>
<td>Baseline vs. month 3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Week 1 vs. month 1</td>
<td>0.0264</td>
</tr>
<tr>
<td>Week 1 vs. month 2</td>
<td>0.0150</td>
</tr>
<tr>
<td>Week 1 vs. month 3</td>
<td>0.0031</td>
</tr>
<tr>
<td>Week 2 vs. month 2</td>
<td>0.0497</td>
</tr>
<tr>
<td>Week 2 vs. month 3</td>
<td>0.0007</td>
</tr>
<tr>
<td>Week 3 vs. month 3</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
CHAPTER 3 – EVALUATION OF ANTI-INFLAMMATORY AND ANALGESIC EFFECTS OF MELOXICAM IN A RAT PAW EDEMA MODEL WHEN COMPOUNDED IN DELIVRA™
**Introduction**

Canine OA is a degenerative joint disease that affects articular and periarticular joint structures and results in the destructive processes of inflammation (Garnero *et al.*, 2003). While all aspects of OA development and progression are not fully understood, it has been suggested that OA is mediated by many proinflammatory cytokines (Dean *et al.*, 1989; Dijkgraaf *et al.*, 1995), with evidence of elevated concentrations of IL-6, IL-1, and TNFα in joints affected by OA (Goldring, 2000; Venn *et al.*, 1993). TNFα and IL-1 have been shown to have catabolic effects by promoting cartilage destruction (Health Canada, 2012; Pelletier *et al.*, 1985), inducing the release of proteolytic enzymes by chondrocytes (Lefebvre *et al.*, 1990; McDevitt *et al.*, 1977; Pelletier *et al.*, 1983) and inducing the production of other proinflammatory mediators (Dingle *et al.*, 1979; Fujita *et al.*, 2006; Saklatvala, 1981; Steinberg *et al.*, 1983) and inhibiting chondrocyte induced anabolism (Saklatvala, 1986; Schäfer-Korting *et al.*, 1994). IL-6 has been suggested to promote bone resorption through increasing differentiation of bone resorbing osteoclasts (Wilkosz *et al.*, 2003). Therefore, many OA management strategies are targeted at decreasing inflammation, providing symptomatic relief and possibly disease modifying effects.

Carrageenan is a plant mucopolysaccharide that causes an acute inflammatory response when administered to various animal species (Lees, 2003). A rodent model of carrageenan-induced inflammation in the rat paw is commonly used for comparison of short-term anti-inflammatory effects of various NSAIDs (Chi *et al.*, 1990a; Winter *et al.*, 1962) such as meloxicam (Dudhgaonkar *et al.*, 2006b), diclofenac (Sumen *et al.*, 2001), and transdermal ketoprofen (Chi *et al.*, 1990a). These studies have demonstrated significant reductions in carrageenan-induced paw inflammation with drug treatment,
suggesting effective anti-inflammatory effects of these NSAIDs. Many NSAIDs including meloxicam (Engelhardt et al., 1995) and diclofenac (Metselaar et al., 2004) also have analgesic activities in addition to their anti-inflammatory properties. These analgesic effects could provide further benefits to canines suffering from OA, by enabling use of the affected limb, preventing muscular atrophy from disuse and enhancing quality of life.

As discussed in chapters 1 and 2, the transdermal delivra™ product is composed of a combination of plant based oils and waxes, making it a completely all-natural product. In addition to compounding NSAIDs in delivra™, LivCorp Inc. is interested in developing an effective all-natural anti-inflammatory cream. Avocado soybean unsaponifiables (ASU) are a combination of avocado and soybean oils that have been recently suggested to have beneficial effects in OA through encouraging repair of OA affected cartilage and preventing further cartilage degradation (Henrotin et al., 2003). These beneficial effects of ASU are suggested to occur through increasing collagen and aggrecan synthesis and inhibiting the destructive effects of IL-1 (Henrotin et al., 1998). Therefore, LivCorp Inc. is beginning to investigate ASU as a potential additive to the delivra™ base for all natural anti-inflammatory effects.

To fulfill part of the efficacy section of a VNDS, the following study was conducted to compare various drugs compounded in the delivra™ product with a current veterinary approved meloxicam formulation.
The objectives of this study were:

1) Compare the anti-inflammatory effects of meloxicam, diclofenac and ASU compounded in the delivra™ base with an injectable meloxicam (Metacam®) formulation;

2) Compare the analgesic effects of meloxicam, diclofenac and ASU compounded in the delivra™ base with an injectable meloxicam (Metacam®) formulation;

3) Compare the degree of inhibition of tissue inflammation, edema, necrosis and infiltration following treatment with meloxicam, diclofenac and ASU compounded in the delivra™ base with an injectable meloxicam (Metacam®) formulation.

The hypothesis for this study was that meloxicam, diclofenac and ASU when compounded separately in the delivra™ base will produce anti-inflammatory and analgesic effects comparable to injectable meloxicam (Metacam®) in a carrageenan-induced paw model of inflammation in the rat.

Materials and methods

Animals

The following study was conducted according to CCAC guidelines and following animal use protocol (AUP) approval by the University of Guelph Animal Care Committee (ACC). Adult male Sprague-Dawley rats (Charles-River, Quebec, Canada) weighing between 180-220 g were used in this study. Rats were acclimated for a minimum of 3 days prior to study, and housed in the Central Animal Facility at the University of Guelph. Rats were on a 12h:12h reverse light cycle and given standard rat
chow and water ad libitum. Following the final data collection point, rats were
anesthetized in a chamber using 2.5% isoflurane and subsequently euthanized via cardiac
exsanguination via needle and syringe.

Treatment groups

Rats (n=10 per treatment group) were randomly assigned to one of 6 treatment
groups: PLO gel base (Transderma Pharmaceuticals Inc., British Columbia, Canada),
diclofenac compounded in delivra™ base (TDD) (11.6 mg/g, LivCorp Inc., Ontario,
Canada), meloxicam compounded in delivra™ base (30 mg/g, LivCorp Inc., Ontario,
Canada), ASU compounded in delivra™ base (TDA) (30 mg/g, LivCorp Inc., Ontario,
Canada), delivra™ base alone (LivCorp Inc., Ontario, Canada) or injectable Metacam®
solution (5 mg/mL stock concentration, Boehringer Ingelheim, Missouri, USA). All
treatment drugs were administered at 1 mg/kg BW prior to carrageenan injection. The
PLO base treatment group served as the negative control group. The PLO base and
delivra™ base treatment groups were dosed at amounts equal to the meloxicam and ASU
groups on a gram of formulation/kg body weight basis.

A pilot study was first conducted to determine the optimal timing of treatment
administration prior to carrageenan injection. Rats (n=3 per group) were randomized to
one of 4 treatment groups: 3 hr pre-carrageenan with ASU in delivra™ base, 3 hr pre-
carrageenan with meloxicam in delivra™ base, 1 hr pre-carrageenan with ASU in
delivra™ base, 1 hr pre-carrageenan with meloxicam in delivra™ base. Hind paws (right
or left) were randomly selected for treatment application in a balanced fashion. Pre-
carrageenan treatment time for the main study was determined based on the results from
the pilot study. Additionally, data obtained from rats in the selected group (1 hr or 3 hr)
were used in the main study, therefore reducing the number of rats needed in the ASU in delivra™ and meloxicam in delivra™ treatment groups to n=7 in the main study.

Treatment application

Rat hind paws (right or left) were randomly selected in a balanced manner for each treatment group. On the day of the study, rats were weighed and the amount of test article was calculated for each rat. Selected paws were then shaved (Wahl, Connecticut, USA) on the dorsal side of the hind paw. A dot was drawn on the center of the dorsal and ventral sides of the hind paw with permanent marker to serve as landmarks for caliper measurements (see Figures 3a, b).

The transdermal cream treatments (PLO base, delivra™ base, TDD, TDM and TDA) were each stirred with a metal spatula for 30 seconds in a clockwise motion and weighed out on individual weigh boats. Once all drugs had been weighed, the treatments were applied with a gloved hand to the dorsal side of the selected and shaved hind paw. Transdermal creams were rubbed in a clockwise motion with the index finger for 30 seconds and gloves were changed between each treatment application. Treatment groups were blinded to the evaluator for all rats throughout the duration of the study.

The injectable Metacam® formulation was diluted to a concentration of 1 mg/mL in sterile injectable water (Hospira, Quebec, Canada) to provide a more convenient dose volume. The calculated amount of drug needed was aspirated into a 1 mL syringe with a 27 gauge needle and allowed to come to room temperature. Meloxicam was injected into the SC tissue overlying the hip of the selected side of the rat. All creams were absorbed within the application period so no noticeable signs of treatment groups were discernable during sample and data collection.
Carrageenan-induced paw inflammation

A volume of 0.1 mL of 1% lambda carrageenan (Sigma Aldrich, Ontario, Canada) in 0.9% sterile saline (Hospira, Quebec, Canada) was injected into the subplantar region of the hind paw of each rat. Prior to the study day, the lambda carrageenan powder was dissolved in sterile saline at 90°C and stirred for 45 minutes with a metal stir bar. The prepared carrageenan solution was then autoclaved to ensure sterility and 0.1 mL of solution was loaded into 1 mL syringes with 27 gauge needles. The prepared carrageenan solution was kept for no more than 5 days at 4°C and was wrapped in aluminum foil.

On the day of the study, syringes containing carrageenan were allowed to come to room temperature. One hour (determined from pilot work) after drug treatment, selected rat hind paws were injected into the subplantar region, just dorsal to the footpad, between the 3rd and 4th digit (see Figure 4).

Analgesic efficacy

Analgesic efficacy of drug treatments was determined using an infrared (IR) plantar test instrument (Ugo Basile, Varese, Italy). An IR intensity of 60 was used in the main study. This intensity was chosen based on the data obtained from standardization of the thermal apparatus using normal rats. For the standardization procedure, IR intensities of 20, 30, 40, 50 and 60 were tested on healthy rats and the intensity with the lowest variability was used for the main study.

The thermal plantar test apparatus consisted of a mobile IR beam placed under a glass surface divided into 6 chambers. Each chamber was separated from other chambers by an opaque plastic wall. Outer walls of the chamber were clear to enable visualization of the rat hind paws. An infrared beam was focused on the center of the subplantar region
of the rat hind paw and acted as a heat source to provide thermal pain. An automatic timer began when the beam was turned on and automatically stopped once the rat withdrew its paw from the glass surface. The time to withdrawal was termed the latency time and was measured in seconds. A maximum latency time of 60 seconds was used to prevent permanent injury to the rat paw. A longer latency time represented a greater analgesic effect (Hargreaves et al., 1988).

All rats were acclimated to the analgesiometer once daily for a minimum of 3 days prior to the study. On the day of the study, the time to withdrawal for the injected paw was measured at baseline (prior to treatment application), 0.5 hr, 1 hr, 3 hr and 6 hr post-carrageenan injection.

**Anti-inflammatory effects**

The amount of edema was determined in the injected hind paw as changes in paw thickness using a digital caliper (General® Tools, New York, USA). The data was reported in mm and was used as an indication of the inflammatory response of the injected paw (Morris, 2003). The landmarks drawn previously on the dorsal and ventral sides of the paw were used as landmarks to ensure consistency of measurements. Thickness measurements were made at baseline (prior to treatment application), immediately after carrageenan injection, 0.5 hr, 1 hr, 3 hr and 6 hr post-carrageenan injection.

**Histopathological assessment of paw tissues**

After the 6 hr time point measurements were recorded, rats were anesthetized with isoflurane and sacrificed via cardiac exsanguination. Injected paws were then removed at the level of the ankle joint with bone shears and sectioned with a scalpel (No.
11 blade, No. 4 handle) along the parasagittal plane dividing the injected footpad equally (see Figure 4). The medial sides of the collected hind paws were used for biomarker gene analysis described below. The lateral sides of the collected hind paws were used for histopathology sections and were immediately placed in jars containing 10% formalin and stored for a minimum of 2 days. Paws for histopathology were then transferred to a decalcifying solution for 1 day after which point they were trimmed to ensure the section was level, and returned to the 10% formalin solution. Prepared hind paws were sent to the animal health laboratory (AHL) at the University of Guelph where they were embedded in paraffin, sliced, mounted onto glass slides and stained with hematoxylin and eosin (H&E).

A boarded pathologist who remained blinded to treatment group allocations provided histopathology scores for all sectioned paws. Paws were scored for four separate features: edema, hemorrhage, inflammatory cell infiltrate and necrosis. Each feature was scored semi-quantitatively from 0 to 4 (0=normal, 1= minimal [<5% of section affected], 2=mild [>5% but <10% affected], 3=moderate [>10% but <25% affected] and 4=marked [>25% of section affected]), and summated to achieve an overall score (maximum section score=16).

Analysis of biomarkers: tissue dissection

The following procedures were conducted in the National Research Council (NRC) at the University of Prince Edward Island (UPEI) (Delivra Inc., UPEI, Canada). Following collection, the medial sides of the hind paws were immediately flash frozen in 2 mL self-standing polypropylene cryovials (Corning Inc., New York, USA) in liquid nitrogen. On the day of biomarker gene extraction, all surfaces were wiped with 95%
isopropyl alcohol followed by RNAse ZAP™ (Life Technologies, Ontario, Canada). Frozen paws were placed on a glass petri dish over ice and a 5 mm X 5 mm X 3 mm section of tissue was dissected as follows: (1) an incision was made from the distal to proximal end of the hind paw, ventral to the metatarsal bones isolating the subplantar tissue, (2) a second incision was made from the medial to lateral side of the hind paw, proximal to the footpad at the level of the 1st digit, and (3) a third incision was made from the proximal end to the distal end, between the 2nd and 3rd digits (see Figure 8).

Analysis of biomarkers: RNA isolation

The following procedures were applied to all frozen paw tissue samples. Once the frozen tissue sample was dissected, it was placed in 1.0 mL TRIzol® reagent (Life Technologies, Ontario, Canada) at 4°C and kept over ice. The sample was homogenized with a homogenizer (Pro Scientific, Connecticut, USA) equipped with a 7 mm X 95 mm saw tooth generator (Pro Scientific, Connecticut, USA) in the TRIzol® reagent for 1 minute, rested for 1 minute, and homogenized for an additional 30 seconds. The homogenizer was washed between samples for 30 seconds with RNAse ZAP™, 30 seconds with diethylpyrocarbonate (DEPC) treated water (Life Technologies, Ontario, Canada), 30 seconds with 95% ethanol and 30 seconds with another aliquot of DEPC treated water. The homogenate was kept at RT for a minimum of 5 minutes during which time other samples were homogenized. Following this incubation period, 200 µL of chloroform (Caledon Laboratories Ltd., Ontario, Canada) was added to the homogenate and vortexed (Fisher Scientific, Ontario, Canada) for 15 seconds on level 10. The sample was rested for 2 to 3 minutes at RT, then centrifuged at 12,000 X g for 15 minutes at 4°C. Following centrifugation the sample was divided into an upper aqueous phase,
middle interphase and organic phenol-chloroform phase. The upper aqueous phase was removed 200 µL at a time and placed into a new tube into which 500 µL of 100% isopropanol (Caledon Laboratories Ltd., Ontario, Canada) was added. The tube was inverted 10 times at left to stand at RT for 10 minutes. The tube containing the remaining interphase and organic layer was stored at 4°C. Following the rest period, the sample was then centrifuged at 12, 000 X g for 10 minutes at 4°C. The supernatant was discarded and the sample was washed with 1 mL of 75% ethanol and gently inverted 10 times. The sample was centrifuged at 7, 500 X g for 5 minutes at 4°C and the ethanol wash was subsequently discarded. The sample was dried at RT for 5 to 10 minutes, resuspended in 100 µL of RNase free water (Life Technologies, Ontario, Canada) and heated on a thermal block at 54°C for 10 to 15 minutes. The sample was then centrifuged in a mini centrifuge (Mandel Scientific Company Ltd., Ontario, Canada) for 10 seconds and placed back on ice. A NanoDrop™ spectrophotometer (Thermo Scientific, Delaware, USA) was used to determine the purity of the RNA sample based on the absorption values at 230 nm, 260 nm and 280 nm (A_{230}, A_{260}, A_{280} respectively) as well as the concentration of RNA in ng/µL. For samples with A_{260}:A_{280} ratios lower than 1.8 or A_{260}:A_{230} ratios lower than 2, RNA purification steps were performed as described below. Samples that were sufficiently pure were stored at -80°C.

Analysis of biomarkers: RNA purification

Samples with poor absorption ratios were purified with the Qiaex II Gel Extraction Kit (Qiagen, Ontario, Canada). Buffer QX1 was inverted 25 times and 600 µL per RNA sample was aliquoted into labeled tubes. Samples of RNA were thawed and 10 µg of RNA per sample was mixed with buffer QX1 by pipetting up and down 10 times.
Qiaex II silica solution was vortexed (Fisher Scientific, Ontario, Canada) for 30 seconds and 20 μL was added to each sample tube. Tubes were gently inverted 30 times and incubated at RT for 5 minutes. Samples were then centrifuged at 5,000 X g for 30 seconds at RT and supernatant was discarded. 500 μL of buffer QX1 was then added to the sample tube, inverted 15 times and centrifuged at 5,000 X g for 30 seconds at RT. Supernatant was discarded and 500 μL of buffer PE was added to resuspend the pellet. The tubes were flicked gently 15 times with the index finger and centrifuged at 5,000 X g for 30 seconds at RT. The supernatant was discarded, 500 μL of buffer PE was added to the sample tube and the tube was inverted 15 times. The sample was centrifuged at 5,000 X g for 30 seconds at RT and the supernatant was discarded. Samples were dried for 30 minutes and 50 μL of RNase free water was added to resuspend the pellet. The sample was heated on a thermal block at 50°C for 5 minutes and centrifuged for 2 minutes in a mini centrifuge (Mandel Scientific Company Ltd., Ontario, Canada) at high speed. The solution was placed in a new tube and measured for absorption ratios and RNA concentration.

**Analysis of biomarkers: RNA gel**

RNA gels were performed on a few randomly selected samples that underwent purification to determine if any RNA degradation occurred. The 1% RNA gel solution was composed of 50 mL of 1X Tris acetate ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich, Ontario, Canada) (TAE) running buffer (RB) mixed with 0.5 g of low melt agarose (Sigma Aldrich, Ontario, Canada) which was microwaved for 30 seconds on high power, swirled for 10 seconds in a clockwise motion then microwaved for another 10 seconds. The mixture was allowed to cool slightly and 1 μL of 10 mg/mL ethidium
bromide (Sigma Aldrich, Ontario, Canada) was pipetted into the solution and swirled. The solution was poured into a gel mold fitted with an 8 well comb and allowed to set for about 1 hour. While the gel was setting, 2 µg of total RNA from the selected sample was mixed with RNAse free water and 5X loading buffer (LB) (Bio-Rad, Ontario, Canada) diluted down to a concentration of 1X in a final sample volume of 15 µL. Once the gel was firm, the comb was carefully removed and placed in a gel electrophoresis chamber filled with 1X TAE RB. RNA samples mixed with LB were loaded into the wells beginning at the second well from the left. A 2 µL aliquot of a 500 base pair (bp) molecular ruler was mixed with 4 µL of 5X LB and 14 µL of RNAse free water and loaded into the first well on the left side of the gel (see Figure 9). The gel electrophoresis was conducted at 120 V for roughly 40 minutes, until the dye traveled two thirds of the way across the gel. Once the RNA fragments had migrated a sufficient distance, a picture of the gel was taken with the BioSpectrum Imaging System (UVP, California, USA, see Figure 9).

Analysis of biomarkers: Protein isolation

Any remaining aqueous phase was removed from samples containing the interphase and organic phenol-chloroform phase. 0.3 mL of 95% ethanol was then added to each sample and inverted 10 times. Samples were incubated at RT for 2 to 3 minutes and centrifuged at 2,000 X g for 5 minutes at 4°C. Supernatant containing protein was then aliquoted into a new tube and stored at -80°C.

Analysis of biomarkers: RT-qPCR

Samples containing isolated RNA were analyzed for reference and inflammatory biomarker gene expression by RT-qPCR. Primers for detection of IL-1β, TNFα, actinβ
and HPRT1 (Qiagen, Ontario, Canada) were reconstituted separately in 1.1 mL of Tris-EDTA (TE) buffer at a pH of 8 and aliquots were stored at -20°C. On the day of RT-qPCR analysis, RNA samples were thawed and a diluted 25 µL aliquot of 25 ng/µL was made for each sample and stored on ice. A master mix was prepared for each primer containing 12.5 µL of 2X QuantiTect SYBR Green RT-CR Master Mix, 2.5 µL of 10X QuantiTect Primer Assay, 0.25 µL of QuantiTect reverse transcriptase (RT) Mix and 8.75 µL of RNAse free water for a total volume of 24 µL per reaction. A no RT (NRT) master mix and no template control (NTC) master mix were also made for the actinβ primer. The NRT master mix was made in a similar fashion to the main master mix described above for each primer, however no QuantiTect RT Mix was added and instead RNAse free water was included. The NTC master mix was identical to the main master mix described above, however no template RNA was added to the reaction well.

A volume of 24 µL of the main master mixes was loaded into each well of a 96 well PCR plate (Bio-Rad, Ontario, Canada) over a 96 well PCR cooler rack (Eppendorf, New York, USA). A volume of 24 µL of the NRT and NTC master mixes was also loaded into the PCR plate (see Figure 10). Once all of the master mixes were loaded into the plate, 1 µL of diluted template RNA was added to each well. For each RNA sample, triplicate reactions were conducted for all 4 primers. Aliquots of 1 µL of template RNA was also added to the NRT wells and aliquots of 1 µL of RNAse free water were added to the NTC wells.

Once loading was complete, the PCR plate was covered with a Microseal® Adhesive Seal (Bio-Rad, Ontario, Canada) and was loaded into a PCR machine. A one-step PCR reaction was used for this study with the following specifications: a reverse
transcription step at 50°C for 30 minutes, a PCR initial activation step at 95°C for 15 minutes, a 3-step cycling step repeated 40 times involving denaturation for 15 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 30 seconds at 72°C, followed by a 1 minute 95°C and 5 second 55°C step.

Plasma and paw tissue drug level determination

In order to evaluate drug levels attained in serum and in hind paw tissues, and due to limited paw tissues available from the main study, a separate drug level determination experiment was conducted. Samples for analysis of serum (n=10 per group) and hind paw tissue (n=6 per group) drug levels were collected for drug treatment groups. For paw tissue drug level determination, 24 rats were randomly allocated for treatment with one of: ASU compounded in delivra™ base, injectable Metacam®, meloxicam compounded in delivra™ base or TDD. Rats were given drug treatments and inflammation was induced by the same protocol as described above. Anti-inflammatory and analgesic effects were not assessed in these rats. At 6 hrs post-carrageenan injection rats were sacrificed according to the above protocol. Carrageenan-injected paw tissue was collected from each rat and the lateral sides of the paws were flash frozen in liquid nitrogen for drug level analysis.

Samples for serum drug level analysis were collected from all of the main study rats (n=10 per group, 6 treatment groups total). Blood collected from cardiac exsanguination at the time of sacrifice was immediately placed into 3 mL BD Vacutainer® blood collection tubes with no additive (BD Diagnostics, New Jersey, USA). Tubes were stored at RT for a minimum of 30 minutes, then centrifuged at 1, 100
X g for 20 minutes at RT. Serum was pipetted into a 1.2 mL cryovial (Corning Inc., New York, USA) and stored at -80°C.

**Statistical analysis: anti-inflammatory and analgesic activity**

The statistical model was a completely randomized split plot design with repeated measures for paw thickness and sub-sampling. The repeated measures were collected from the injected side of the rats. The sub-sampling was handled by pre-averaging the measures prior to analysis. In addition, the model allowed for unequal variance over time and the data was log transformed. Residual analyses suggested that all other ANOVA assumptions were adequately met by the data. All interactions, variables and quadratics for continuous variables were initially included in the model and non-significant (P<0.05) terms were removed.

The model used for the thermal plantar latency data was the same as described above. The model initially included all variables and then non-significant (P<0.05) terms were removed, leaving a random effect in the model. A log transformation was performed on the latency time data, but otherwise all other ANOVA assumptions seemed to be met based on the analyses of residuals.

**Statistical analysis: biomarker gene expression**

The statistical approach taken for data obtained for biomarker gene expression was a 2-factor factorial completely randomized design with sub-sampling. A generalized, linear mixed model was performed to analyze the biomarker gene expression using Proc MIXED (SAS 9.2). Models initially included all variables, interactions and quadratics of continuous variables, and non-significant (P<0.05) variables were removed. For the actinβ data set, a transformation was not required and all ANOVA assumptions seemed
to be met based on residual analyses. For the HPRT1 data set, a log transform was performed but otherwise all other ANOVA assumptions seemed to be met. For analysis of the TNFα data set, an analysis of covariance approach was used and the data was log transformed, otherwise all other ANOVA assumptions seemed to be met. A log transformation was also performed on the IL1β data set, with all over ANOVA assumptions being met. The IL1β model initially included all variables and any non-significant (P<0.05) variables were removed. The data showed no significant changes when adjusting for actin levels, therefore the actin variable was removed from this model.

**Statistical analysis: histopathological analysis**

Data for histopathological analysis was reported as subjective scaled scores made up of scores for 4 different features. This study was a standard 1 factor completely randomized design (CRD) that used the non-parametric Kruskal Wallis test.

**Results**

**Analgesic efficacy**

Latency time to paw withdrawal was used as an indication of the level of analgesia achieved with each drug treatment, with an increase in latency time representing a greater level of analgesic efficacy of the drug treatment. Statistical analysis showed no significant differences in overall level of analgesia achieved with the different drug treatments (P=0.2136). However, there were statistically significant differences in level of analgesia between varying time points (P<0.0001) as well as between drug treatments at specific time points (P=0.0216) (Table 10). Significantly greater paw thickness was measured in the PLO treatment group after 0.5, 3, and 6 hrs (P<0.0001, P<0.0001, P<0.0001), in the injectable Metacam® treatment group after 0.5, 1, 3 and 6
hrs (P=0.0035, P=0.0237, P=0.0004, P<0.0001), in the TDM treatment group after 0.5, 1, 3 and 6 hrs (P<0.0001, P<0.0001, P<0.0001, P<0.0001), in the TDD treatment group after 0.5, 1, 3 and 6 hrs (P=0.0051, P=0.0099, P=0.0017, P=0.0032), in the TDA treatment group after 0.5, 1, 3 and 6 hrs (P<0.0001, P<0.0001, P<0.0001, P<0.0001) and in the delivra™ base treatment group after 0.5, 1, 3 and 6 hrs (P=0.0016, P=0.0042, P<0.0001, P<0.0001) compared with baseline.

Anti-inflammatory effects

Inflammation, and therefore anti-inflammatory activity of treatments, was indicated by the presence and amount of paw edema measured as paw thickness for each drug treatment group; with decreasing paw thickness representing increasing anti-inflammatory activity. Statistically significant increases were detected between injected and non-injected mean paw thicknesses (P<0.0001). Statistically significant increases were also found in mean paw thicknesses between 0.5, 3 and 6 hrs and immediately after carrageenan injection (P=0.0019, P<0.0001, P<0.0001) (Table 11). No significant differences in mean paw thicknesses were detected between any of the treatment groups (Table 11).

Histopathology analysis

Overall histopathological score was based on scores for 4 different features: level of edema, level of hemorrhage or fibrin, level of infiltrate, and level of necrosis (see Table 12). Statistical analysis revealed no significant differences in overall histopathological score of inflammation between treatment groups (P=0.7099, 99% confidence interval). Marked edema (expansion of tissues, as represented by section pallor and thickening) and hemorrhage (RBCs free within the tissue sections) were noted
Marked inflammatory cell infiltration (consisting predominantly of an influx of neutrophils, lymphocytes, and mast cells) was noted in perivascular and subcutaneous regions (Figure 6 B, C) as well as intramuscular regions (Figure 7). Finally, necrosis (tissue and cell lysis, usually associated with hemorrhage and inflammation) was noted in some areas (Figure 6 A).

**Inflammatory biomarker gene expression**

The RT-qPCR data for all biomarkers investigated (actinβ, HPRT1, TNFα and IL1β) were reported as quantification cycle (Cq) values. Due to sponsor financial and time constraints, a total of 20 samples only were analyzed, with a focus on the meloxicam treatment groups as follows: samples from the PLO treatment group (n=6), meloxicam compounded in delivra™ base treatment group (n=7), and injectable Metacam® treatment group (n=7). The meloxicam treatment groups were of particular interest, and therefore chosen for analysis, given meloxicam’s current applications in veterinary medicine. The Cq values for actinβ and HPRT1 in rat paw tissue samples were used as reference genes and the target genes of interest were IL1β and TNFα and are reported in Table 13. Analysis of actinβ and HPRT1 levels between different treatment groups and time points revealed no statistically significantly different Cq values (P>0.05), suggesting that they were both appropriate reference genes for the study. The target gene Cq values were normalized to the reference gene Cq values and compared relative to the Cq values obtained from the negative control (PLO treatment) group, giving delta delta Cq values (ΔΔCq). Analysis of the IL1β and HPRT1 ΔΔCq values demonstrated no significant changes between PLO, injectable Metacam® or meloxicam compounded in delivra™ base treatment groups (Table 13).
Drug level analysis

Serum and paw tissues were collected and stored for drug levels determination at -80°C. They are currently not being analyzed for drug levels at the request of the study sponsor (Dr. Joseph Gabriele, LivCorp Inc.). It is anticipated that they will be analyzed at a future date at the discretion of the sponsor.

Discussion

The carrageenan-induced rat paw edema model has been used previously to assess anti-inflammatory and analgesic activities of various drugs (Willoughby et al., 2000; Wunder et al., 2003). NSAIDs such as meloxicam and diclofenac have been shown to have effective anti-inflammatory and analgesic effects (Metselaar et al., 2003; Sandersoln et al., 2009) and are commonly used in the treatment of OA (Monteiro-Steagall et al., 2013). Therefore, evaluation of the anti-inflammatory and analgesic effects of meloxicam and diclofenac when compounded in a novel drug delivery system, delivra™ base was performed. ASU has also been recently suggested to have anti-inflammatory effects in vitro in human cells (Henrotin et al., 1998; Henrotin et al., 2003) so investigation was warranted. The injectable meloxicam (Metacam®) treatment group was used as a positive control as meloxicam is approved for use in Canada as an analgesic drug in veterinary patients.

There were statistically significantly different levels of analgesia between the PLO treatment group and TDA, TDD, TDM, injectable meloxicam (Metacam®) and delivra™ base treatment groups at specific time points. However, there were no significant differences in overall level of analgesia achieved between varying drug treatments and the negative control group in this study, suggesting no overall analgesic
efficacy of any of the drugs at the dosage regimens tested. The analgesic activities of
tested drugs were determined using an IR analgesiometer (Ugo Basile, Varese, Italy), set
at an intensity of 60 and a maximum latency time of 60 seconds to prevent permanent
damage to the rat paws. Many analgesic studies have reported the temperature used with
similar equipment in degrees Celsius. However, the newer analgesiometer used in this
study (Ugo Basile, Varese, Italy) displayed the thermal stimulus as an IR intensity rather
than degrees Celsius. Therefore, standardization of the equipment in order to determine
the optimal IR intensity was performed. An IR intensity of 60 units was found to have the
smallest variance between normal rats, thus this was chosen for use in the main study. A
previous study (Hargreaves et al., 1988) has reported similar variances in measured
latency times as this current study. Hargreaves et al. were able to detect significant
differences in latency times with their treatment groups, thus it is unlikely that the high
variances in this study have contributed to the lack of significance detected between
treatment groups.

The positive control group treated with injectable Metacam®, which is currently
approved for analgesic use in veterinary patients and has documented analgesic efficacy,
did not result in significant analgesic effects suggesting that the inflammatory model may
have been too severe, or alternatively that the dose of meloxicam was not high enough in
the rat compared to other species. A previous study that used a similar experimental
protocol demonstrated that an oral meloxicam dose of 1 mg/kg was also not sufficient to
inhibit carrageenan induced paw edema in the rat (Dudhgaonkar et al., 2006a). However,
this dose was chosen based on recommendations found in previous literature for this
species (Allen et al., 2005; Engelhardt, 1996). In addition, the study that demonstrated a
lack of efficacy of the 1 mg/kg dose of meloxicam administered the drug orally to rats (Dudhgaonkar et al., 2006a), and it has been suggested that oral bioavailability of meloxicam is less than that of injected meloxicam (Fleischmann et al., 2002). Therefore, a dose of 1 mg/kg of injected meloxicam was thought to be sufficient for use in this study. Few studies have been conducted investigating ASU and its effects on carrageenan-induced paw inflammation. One study that used an avocado extract demonstrated that it had anti-inflammatory activities in a carrageenan-induced model of inflammation (Ding et al., 2007), supporting its potential use as a natural additive to the delivra™ base. Another study conducted investigated diclofenac administered via intraperitoneal (IP) injection at 10 and 20 mg/kg and demonstrated that these doses effectively inhibited carrageenan-induced inflammation (Sumen et al., 2001). In the current study, a dose of 1 mg/kg was used for all treatment drugs to standardize the dosage regimens across treatment groups to the positive control (SC Metacam®) group. Based on the above findings, higher meloxicam and diclofenac dosages were likely necessary to demonstrate significant clinical effects with this model of inflammation. Should future work be done with these treatment groups and the rat paw edema model, a range of dosages (dosage justification study) would be recommended. Certainly, a dosage regimen of 1 mg/kg in the current rat paw edema model study was a reasonable choice based on results obtained in dogs in Chapter 1 of my thesis. Inter-species differences with respect to dosage regimens necessary to produce analgesic and anti-inflammatory effects also need to be considered.

To evaluate the amount of inflammation achieved in each group, a caliper (General® Tools, New York, USA) was used to measure changes in paw thickness which
has been shown to correlate well with the degree of inflammation in other studies (Morris, 2003). Other studies investigating carrageenan-induced paw inflammation have also employed displacement of fluid when the paw is immersed, i.e. changes in paw volume as an indication of inflammatory-induced tissue edema. Unfortunately, the necessary equipment for measuring paw volume was not available. However, previous work has shown very good correlation with measures of paw thickness compared to paw volume, as well as changes in overall paw circumference as indicators of changes in paw inflammation (Morris, 2003). While no significant differences were found between treatment groups in median paw thickness, there were significant increases in paw thickness at 0.5, 3 and 6 hrs after carrageenan injection for all groups when compared to baseline. These results suggest that the carrageenan-induced model of inflammation is a robust model, however, they also may suggest that the degree of inflammation produced was too severe for the dosage regimens of the treatment groups studied to yield significant and detectable clinical effects. The concentration and dosage of lambda carrageenan, an extract of red seaweeds, used in this study were based on previously published work in rats showing that a 0.1 mL subplantar injection of 1% (10 mg/mL) carrageenan produces a predictable acute inflammatory response that peaks at about 4 to 5 hours post injection (Chi et al., 1990b; Jadot et al., 1986; Morris, 2003; Winter et al., 1962). Several studies (Chou, 2003; Loram et al., 2007; Yamamoto et al., 1993) have employed larger dosages than that used in my study.

The inflammatory response evoked by carrageenan injection has been reported to produce specific cellular effects in paw tissue (Vinegar et al., 1987). The associated edema is suggested to be restricted to vasodilation, exudate and neutrophil and monocyte
migration (Jadot et al., 1986). This edema has been reported in the plantar muscle and to a greater extent in the subplantar tissue of the rat hind paw (Boschi et al., 2008) with primarily neutrophilic migration occurring (Aaron et al., 1987; Santer et al., 1983; Vinegar et al., 1987). The inflammation is described as biphasic in nature, with the 1st phase involving the release of mainly of histamine, bradykinin and 5-hydroxytryptophan (5-HT) in the 1st hour after carrageenan injection, followed by a 2nd phase with the release of PGs detected in paw tissues (Di Rosa et al., 1971; Guay et al., 2004; Jadot et al., 1986). This timing of phases seems to correlate well with findings that COX-2 expression is at its maximum approximately one hour after carrageenan injection (Nantel et al., 1999). Therefore, NSAIDs may not have an effect until after the first hour of carrageenan-induced inflammation. The current study found no significant differences in histopathological scores with any drug treatment. However, there were extensive local effects with carrageenan injection that seem to agree with findings from previous studies (see Figures 5-7). As discussed previously, histopathological score was based on four features; edema, hemorrhage, infiltrate and necrosis and were detected in many samples (see Table 12). Additionally, vasodilation was demonstrated in histological sections, as can be seen in Figure 12. Cellular infiltration was mostly neutrophilic, with mast cells also present (see Figure 13). These inflammatory characteristics seem to correlate well with previously described carrageenan-induced inflammatory responses.

It has been reported that the inflammatory response to carrageenan injection involves the release of many proinflammatory factors including PGs and ILs, which can further enhance the inflammatory reaction (Guay et al., 2004). Detectable levels of TNFα in the serum and TNFα and IL1β in paw exudate of carrageenan-injected rats have been
well documented (Amdekar et al., 2012; Chou, 2003), with levels being detected after 1.5 and 4 hrs following injection (Chou, 2003). Additionally, increased levels of PGE$_2$ have been reported in paw exudates of injected rats (Chou, 2003). The overall sequence of events has been suggested to begin with the release of TNF$\alpha$, which induces the expression of ILs such as IL1$\beta$, IL6 and IL8. These ILs then promote the production of COX downstream products (Nakamura et al., 1987). Therefore, the effects of varying drug treatments on TNF$\alpha$ and IL1$\beta$ levels during carrageenan-induced inflammation were investigated. Due to financial restrictions, 20 samples from the meloxicam compounded in delivra™ base (n=7), injectable Metacam® (n=7) and PLO (n=6) groups were analyzed only from the main study. Similar to previous work, levels of TNF$\alpha$ and IL1$\beta$ were detected, however, there were no significant differences between measured TNF$\alpha$ and IL1$\beta$ levels in these drug treatment groups compared to negative control (PLO).

Potential explanations for the lack of significant differences between treatments and negative control in my study may include the high variability of latency times found in the thermal plantar test, potentially low treatment drug concentrations and/or too severe an inflammatory model. Another explanation could be poor or no drug delivery to target tissues. While this may be possible with the transdermal preparations given the exploratory nature of these products, it is anticipated that the injectable meloxicam should achieve adequate absorption and penetration of the target tissue. Samples for measurement of drug levels in serum and paw tissue were collected but are not currently analyzed. Analysis of these samples would allow for confirmation of the presence of drugs at the biophase or target site. In conclusion, under the test conditions employed in
my study, the transdermal treatments and the injectable formulation of meloxicam did not provide significant analgesia or anti-inflammatory effects in a rat paw edema model. Further dose justification and additional proof of concept studies are still warranted.
Table 10. Changes in median latency times (%) measured between varying time points for each treatment group and between treatment groups and the PLO group at varying time points. 95% confidence intervals are displayed in parentheses. Significant (P<0.05) differences are indicated by an asterisk (*).
Table 11. Median±SE paw thickness values (in mm) measured for each treatment group. P-values reported are for comparisons between treatment groups and the PLO negative control group. No significant (P>0.05) differences were detected.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median paw thickness (mm, ±SE)</th>
<th>p-value when compared to PLO</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLO</td>
<td>5.25±0.72</td>
<td></td>
</tr>
<tr>
<td>Injectable meloxicam</td>
<td>5.24±0.62</td>
<td>0.83</td>
</tr>
<tr>
<td>Meloxicam + delivra™</td>
<td>5.24±0.86</td>
<td>0.91</td>
</tr>
<tr>
<td>Diclofenac + delivra™</td>
<td>5.20±0.50</td>
<td>0.55</td>
</tr>
<tr>
<td>ASU + delivra™</td>
<td>5.22±0.78</td>
<td>0.68</td>
</tr>
<tr>
<td>Delivra™ base</td>
<td>5.18±0.73</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Table 12. Mean±SE overall histopathology scores for each treatment group. No significant (P>0.05) differences were detected between any treatment groups, when compared with the PLO negative control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean±SE histopathology score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>edema</td>
</tr>
<tr>
<td>PLO</td>
<td>2.4±0.31</td>
</tr>
<tr>
<td>Injectable meloxicam</td>
<td>2.5±0.17</td>
</tr>
<tr>
<td>Meloxicam + delivra™</td>
<td>2.2±0.13</td>
</tr>
<tr>
<td>Diclofenac + delivra™</td>
<td>2.3±0.15</td>
</tr>
<tr>
<td>ASU + delivra™</td>
<td>2.3±0.33</td>
</tr>
<tr>
<td>Delivra™ base</td>
<td>2.5±0.22</td>
</tr>
</tbody>
</table>
Table 13. Mean±SE replication cycles required for fluorescence detection for the PLO, injectable meloxicam (Metacam®), and meloxicam compounded in delivra™ base treatment groups. Values for the TNFα biomarker are normalized to actinβ and HPRT1. No significant (P>0.05) differences in mean cycle numbers were detected between treatment groups for any of the biomarkers measured.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Actinβ</th>
<th>HPRT1</th>
<th>IL1β</th>
<th>TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLO (n=6)</td>
<td>15.32±0.16</td>
<td>3.09±0.01</td>
<td>3.09±0.02</td>
<td>3.37±0.03</td>
</tr>
<tr>
<td>Injectable meloxicam (n=7)</td>
<td>15.21±0.14</td>
<td>3.07±0.01</td>
<td>3.05±0.02</td>
<td>3.35±0.03</td>
</tr>
<tr>
<td>meloxicam + delivra™ (n=7)</td>
<td>15.16±0.14</td>
<td>3.08±0.01</td>
<td>3.08±0.02</td>
<td>3.34±0.03</td>
</tr>
</tbody>
</table>
Figure 3. Ventral (A) and dorsal (B) view of a rat hind paw. Black filled circle indicates location marked with permanent marker for caliper measurement.
Figure 4. Ventral view of a rat hind paw. Filled circle (A) indicates location of lambda carrageenan injection. Line (B) indicates location of the parasagittal incision separating the injected footpad for histology and biomarker gene analysis. The lateral sides of the hind paws were used for histopathological analysis and the medial sides were used for biomarker gene analysis. Line (C) indicates location where hind paw was removed for sample collection.
Figure 5. Sagittal section of the subplantar tissue of a rat hind paw. The dorsal side of the paw tissue is located at the top of the image. (A) represents an area with severe edema, as can be seen by the light purple H&E staining. (B) represents an area with severe hemorrhage, with RBC staining dark red.
Figure 6. Sagittal section of a rat hind paw with the dorsal side at the top of the image.

Necrosis can be seen in the area to the right of (A), where there is degradation of the tissue and concentration of cells on the collagen fibers. Cell infiltration was present in both perivascular (B) and subcutaneous (C) regions of the hind paw.
Figure 7. Sagittal section of a rat hind paw showing long dark pink muscle fibers.

Muscular cell infiltrate can be seen in the muscle fiber containing several dark purple cells spanning the length of the fiber.
Figure 8. Image of a rat hind paw. (A) shows incisions made separating proximal and distal ends as well as medial and lateral sides of the subplantar tissue. (B) shows incision separating dorsal and ventral aspects of paw tissue. The grey box in both images shows the section of subplantar tissue used for biomarker gene analysis.
Figure 9. Image of an RNA gel. The gene ladder is loaded in the far left well and samples are subsequently loaded in the wells to the right.

Figure 10. Plate set up for the RT-qPCR analysis. Triplicates were used for each biomarker and each sample across rows. 4 different biomarkers were tested for each sample and were loaded down columns.
Statistically significant increases in paw thickness were detected 1/2 hr, 3 hr, and 6 hr after carrageenan injection (p<0.05) when compared to immediately after injection.
Figure 12. A sagittal section of a rat hind paw with the dorsal side of the paw at the top of the image. A dilated blood vessel containing dark pink staining RBCs can be seen below (A).
Figure 13. Sagittal section of a rat hind paw with the dorsal side at the top of the image. Large, purple staining mast cells (A) can be seen infiltrating the tissue. Smaller neutrophils (B) can be seen with a segmented nucleus.
GENERAL DISCUSSION

The delivra™ cream base is currently sold in a line of LivRelief™ creams for use in human medicine. The LivRelief™ and delivra™ creams are composed of all natural ingredients, with no pharmaceuticals, and therefore fall under the category of natural health products (NHPs) in Canada. Approval of NHPs in Canada is still regulated by Health Canada, however the approval process is less stringent than that of veterinary drugs containing pharmaceutical ingredients such as meloxicam. The contents of the product, potency, quality, safety and efficacy are assessed and if approved, the product is given a natural product number (NPN) or a homeopathic medicine number (DIN-HM). Unlike pharmaceutical products, NHPs that have not been fully evaluated but that have passed the initial safety, quality and efficacy standards can be sold in Canada. These products are given exemption numbers (EN). In order to pass the full evaluation by Health Canada, NHP applications are required to provide information on safety and efficacy, which can be based on published literature or data from clinical trials. GMPs are also expected in the application, providing information such as quality assurance and stability of the NHP. NHP license holders are required to monitor any adverse reactions that occur, even after the product has been approved (Health Canada, 2012). Since the LivRelief™ cream containing delivra™ base holds an NPN, it has been approved by Health Canada and has therefore met all of the above requirements. However, LivCorp Inc. is now hoping to compound meloxicam into the delivra™ base for veterinary use, thus a VNDS needs to be completed.

The veterinary drug approval process for a VNDS involves investigation of many aspects of the drug product including manufacturing and quality control, animal safety
and efficacy. In order to evaluate a role for meloxicam formulated in the delivra™ base for use in veterinary patients, a pilot PK canine study, a drug stability study and rat paw edema proof of concept study were performed. The pilot PK canine study was conducted to investigate the transdermal delivery of meloxicam by the delivra™ base and compare it to the systemic delivery of meloxicam by the oral Metacam® formulation in healthy synovial joints of the intended target species. This study provided basic information regarding proof of concept and safety that would fall under the preclinical section of a VNDS. The drug stability study was performed to investigate the stability of the TDM formulation under varying storage conditions and time points, in order to meet some of the requirements of the manufacturing and quality control section of a VNDS. However, in the current thesis work, this study was not performed under GMP and, therefore, the data would not be used in a VNDS. The rat paw inflammation study was conducted to investigate the anti-inflammatory and analgesic proof of concept for the TDM, which would also be found in the preclinical section of a VNDS.

In the pilot canine study it was demonstrated that using the delivra™ base as a delivery vehicle it is possible to transport meloxicam into the SF and plasma of healthy canines at comparable levels to the oral Metacam® formulation. This provided support for the probable efficacy of a novel formulation of meloxicam compounded in delivra™ base. Additionally, no clinical signs of meloxicam intolerance, ie. ADRs, were noted during each phase in either of the treatment groups. These findings provide preliminary support for the safety of meloxicam compounded in the delivra™ base in the target species. Based on this pilot study, future PK and PD studies may be used to provide a better drug profile for meloxicam in the canine when transported in the delivra™ base.
including transdermal movement of meloxicam through the skin. Future studies may be designed to elucidate whether the delivra™ base is acting as a transdermal delivery system, delivering meloxicam into the systemic circulation where it recirculates and concentrates in the target tissue, and/or if it is acting as a topical delivery system, delivering meloxicam directly through the skin to the target tissue (synovial tissues) with minimal systemic exposure.

In this current study, meloxicam levels were measured in the plasma and SF of healthy canines. Articular cartilage does not have a direct blood, nerve or lymph supply (Mobasheri et al., 2010; Schindler, 2011), and the SF is responsible for providing this cartilage with nutrients as well as removing waste products (Garvican et al., 2010). However, it is important for therapeutic levels of meloxicam to be delivered to the synovial membrane as well, since much of the inflammation in naturally occurring and experimental models of canine OA occurs in the synovial membrane (Fujita et al., 2006; Hegemann et al., 2002; McDevitt et al., 1977; Pelletier et al., 1983). Additionally, studies have shown that factors produced by inflamed synovial tissues can stimulate the breakdown of the cartilage matrix, perpetuating the effects of OA (Dingle et al., 1979; Saklatvala, 1981; Steinberg et al., 1983). Therefore, a future study investigating the meloxicam levels achieved in inflamed synovial tissues following TDM administration is warranted.

In order to be granted approval by the VDD, the drug product needs to meet the manufacturing and quality control requirements set by Health Canada. Studies demonstrating evidence of drug stability and consistency in composition and manufacturing need to be conducted to fulfill part of these requirements. A drug stability
study was performed to provide evidence for some of these criteria. The VDD requires specific tests to be conducted for semi-solid drug products. These tests are for pH, specific gravity, antimicrobial preservative content, antioxidant content, viscosity, sterility, particle size, content uniformity, dose delivery and weight variation, syringe ability and separation (Health Canada, 2011). An emulsion such as the delivra™ cream base needs to undergo stability testing in order to demonstrate that the formulation will remain unchanged for the duration of the shelf life. Separation can occur with emulsions by various mechanisms such as: creaming, flocculation, and disproportionation. Creaming refers to the separation of the emulsion into two phases based on density. Flocculation refers to the process of large molecules within the emulsion colliding with and trapping smaller molecules. Disproportionation occurs when smaller molecules within the emulsion dissolve and are integrated into larger molecules (TO, 2011). All of these processes can occur at different times, or in combination, ultimately leading to separation of the emulsion into oil and water components. Evaporation may also occur, which can result in concentration of active drug within the remaining emulsion that, when applied at recommended label doses, may result in overdosing of the drug. Overdosing or inadequate dosing of the drug can also occur if the transdermal product is not uniform. If biologically significant changes in pH occurred before the shelf life was reached, then repeated exposure of the skin to the transdermal product, as is necessary for chronic conditions such as OA, may lead to significant local skin irritation.

In this drug stability we tested pH and meloxicam content at each time point. Samples for meloxicam content analysis were taken from three distinct locations within each vial to demonstrate content uniformity within each vial. It was found that under the
RT with no light exposure, 40°C with no light exposure and 4°C with no light exposure conditions, the TDM formulation was stable for up to 3 weeks of storage. Additionally, the TDM formulation did not appear to have any separation or color changes, as determined by visual observations that were made during study time points. However, this study was not conducted under GMP, thus information obtained would likely not be acceptable to satisfy part of the manufacturing and quality control section of a VNDS. Despite this fact, conclusions from this study can be used to optimize the final formulation of meloxicam in the delivra™ base, and may aid in the design of future drug stability studies investigating meloxicam stability and shelf life when compounded in the delivra™ base. Since the above test conditions led to the longest acceptable storage duration, it may be beneficial to conduct another drug stability study using these select storage conditions under true GMP settings to determine the true drug stability and degradation profile of this formulation.

The results of the pilot canine and drug stability studies supports the continued development of the TDM formulation for veterinary application. Clinical trials evaluating the efficacy of the TDM formulation in the management of naturally-occurring canine OA, as well as safety and other preclinical studies such as PK studies, would be required for approval of a VNDS. However, before any clinical efficacy studies in dogs would be considered or conducted, scientific support (proof of concept) for the analgesic and anti-inflammatory effects of TDM are strongly recommended. Thus, a carrageenan-induced inflammatory model in the rat paw was employed to evaluate the analgesic and anti-inflammatory effects of the TDM formulation.
Conclusions made from the rat paw inflammation study suggest a lack of anti-inflammatory and analgesic efficacy of meloxicam, ASU and diclofenac when delivered in the delivra™ base. NSAIDs including meloxicam and diclofenac are commonly used in veterinary medicine for their analgesic and anti-inflammatory effects (Monteiro-Steagall et al., 2013). It has been reported that these drugs exhibit analgesic effects only when inflammation is present (Botting, 2003), suggesting that their anti-inflammatory activities are related to their ability to reduce pain. As such, the NSAIDs meloxicam and diclofenac were expected to exhibit both analgesic and anti-inflammatory effects in this rat model. However, this study also suggested a lack of anti-inflammatory and analgesic efficacy of the injectable meloxicam (Metacam®) formulation, which is used commonly in rodents at the dosage (1 mg/kg BW) used in the current study. It is postulated that the lack of efficacy of all treatment groups may be due to substantial inflammation produced in the carrageenan model rendering detectable clinical effects not possible. Alternatively, it is also possible that meloxicam, and the other active agents (diclofenac, ASU) did not achieve adequate levels in the target tissue. The results obtained in research dogs in chapter 1 refute the latter explanation. Analysis of paw tissue and blood drug levels from collected samples would provide definitive evidence of drug penetration to the target site. It is anticipated that these samples will be run by the sponsor in the near future. Conclusions obtained from this study may be used to optimize future study design with this model in order to confirm proof of concept and dose justification for drug development.

The ideal transdermal drug delivery system is suggested to have a shelf life of up to 2 years (Sharma et al., 2012). Transdermal drug delivery systems can be applied as
reservoir or matrix patches, or as creams, ointments, or gels, that are topically applied to the skin surface. The reservoir patch typically consists of an impermeable backing to prevent exposure of the drug product to the external environment, a reservoir containing the active drug, a membrane that controls the rate of drug release, an adhesive that keeps the patch on the skin surface and a release liner that is removed just prior to patch application. The matrix style transdermal patch has a similar design but does not contain a membrane to control drug release rates, and typically the active drug is compounded in the adhesive layer. The components within the matrix control the rate of drug release in this system (Wilkosz et al., 2003). Transdermal gels, creams, ointments, etc. that are applied topically work by driving the drug into the stratum corneum which then acts as a reservoir for long-term (hours) release of the drug product (Prausnitz et al., 2008).

Transdermal drug delivery offers many advantages such as ease of administration, sustained release of drug (Berba et al., 1991; Naik et al., 2000) and avoidance of the hepatic first pass effect (Prausnitz et al., 2008). Some dogs may be difficult to orally medicate and for chronic illnesses, this can lead to reduced owner compliance. Additionally, injections are typically not feasible for the average pet owner, especially on a long-term basis, as is required with chronic illnesses such as OA. Since the transdermal route of administration is usually non-invasive and pain free, it has generally been associated with good patient compliance (Guy et al., 1987; Prausnitz et al., 2008; Willis-Goulet et al., 2003) and has therefore been investigated as an alternative route of drug delivery. As discussed previously, topical drug delivery systems are designed to transport the drug through the skin where they are applied and concentrate the drug in the target tissue locally, with minimal drug reaching the systemic circulation. However
transdermals are designed to transport the drug through the systemic circulation where
the drug then concentrates in more distant target tissues. As such, transdermals offer the
advantage of having the potential to be applied to an area with a large surface area, or an
area that has potentially higher drug permeability. Skin surface permeability varies with
body region, and it has been reported that the most permeable regions are mucous
membranes, scrotal skin and eyelids, the least permeable regions are palmar and plantar
surfaces and nails, and regions with intermediate permeability are the face, chest and
back, buttocks, abdomen and upper arms and legs (Wilksz et al., 2003). Therefore,
transdermal formulations may be applied to areas with intermediate or high permeability
to increase drug penetration into the body. In veterinary medicine, transdermal
formulations offer an advantage over topical formulations because they may be applied to
regions with greater permeability but that are less accessible to the animal, such as the
back, thereby reducing potential oral dosing resulting from ingestion of the patch or
emulsion. There are currently transdermal veterinary formulations being used in
veterinary medicine with demonstrated efficacy and safety (Egger et al., 2007; Likar,
2006; Pieper et al., 2011; Savides et al., 2012), such as fentanyl or buprenorphine
patches, however these formulations have not been approved by the VDD and are
therefore considered to be used off-label.

Despite the advantages offered with a transdermal drug delivery system in
veterinary patients, there are many challenges in developing an effective transdermal
delivery system, in particular the optimizing of the formulation. The transdermal delivery
system needs to be able to transport therapeutic levels of drug across the skin and into the
target tissues, with minimal ADRs including local skin irritation. Many mechanisms for
enhanced drug delivery have been suggested including incorporating active pharmaceutical ingredients in liposomes, altering the pH of the system, and using sorption enhancers. The pH of the skin typically falls between 5.4 to 5.9 (Paudel et al., 2010) and creams that are topically applied to the skin that change the pH significantly from this range can cause adverse skin reactions. However it has also been shown that slightly more acidic compounds can increase skin permeability, allowing for enhanced delivery of the drug. Minimal skin irritation has been reported with the use of transdermals in the pH range of 4 to 6.5 (Ananthapadmanabhan et al., 2003). Thus, it has been suggested that an optimal transdermal drug delivery system will have a pH in the range of 5 to 9 (Sharma et al., 2012). The delivra™ cream base that was used in my thesis work had a pH of approximately 4.58, which is close to the ideal range. Additionally, since the delivra™ cream base is slightly acidic, it may allow for greater penetration of meloxicam, and other drugs, through the stratum corneum. The PLO gel base used in the rat paw edema study has been used as a transdermal delivery system for various drugs. However, LivCorp Inc. performed an in vitro skin permeability study and demonstrated that the delivra™ base was able to transport significantly higher levels of naproxen across the skin when compared with the commonly used PLO gel, further supporting the potential of the delivra™ base as a transdermal delivery system.

Physical and chemical enhancers such as microneedles and surfactants respectively, are useful in increasing the permeability of the stratum corneum, however they are more likely to cause skin irritation since they work by physically or chemically disrupting this barrier. Contrary to these enhancers, liposomes have been shown to enhance drug delivery without causing significant skin irritation (Schäfer-Korting et al.,
Liposomes form a lipid bilayer vesicle into which aqueous drugs may be entrapped in the center, and lipophilic drugs incorporated directly into the liposome bilayer (Kogan et al., 2006; Toutou et al., 2010). The delivra™ cream base used in my thesis work is composed of water in oil and wax emulsion that form liposomes. While the liposomes can increase drug penetration through the skin, they have also been shown to increase drug concentrations in local target tissues. Utilizing two experimentally induced inflamed synovial joint models in mice, it was found that an IV formulation of prednisolone encapsulated in liposomes lead to significantly higher clinical efficacy compared to administration of prednisolone intravenously at the same dose (Metselaar et al., 2004; Metselaar et al., 2003). These findings support development of transdermal formulations, such as the delivra™ cream base, that may provide increased target tissue drug concentrations with minimal adverse local skin reactions.

The delivra™ base delivered meloxicam to plasma and SF levels at levels comparable with orally administered meloxicam (Metacam®) in healthy dogs. Ideally, a transdermal drug delivery system applied near the target tissue will achieve higher levels in the target tissue compared to systemic concentrations. This would imply that drug is being delivered directly to the target site in addition to drug reaching the target site following absorption into the systemic circulation and redistribution to the target site. NSAIDs have been reported to accumulate in regions with inflammatory exudate subsequent to carrageenan injection (Lees, 2003). Additionally, a study investigating SF meloxicam levels following oral meloxicam (Metacam®) administration to healthy dogs with experimentally induced synovitis demonstrated significantly higher levels of SF meloxicam in the inflamed compared to the control joint (Johnston et al., 2012).
Therefore, the presence of inflammation at the target site, as occurs with OA, is anticipated to increase distribution of NSAIDs (unbound and protein bound) to the site, whether the drug is administered as a transdermal or via the enteral or parenteral routes. The increased drug levels potentially achieved at the joint when administered transdermally versus systemic levels achieved when compared to other routes of administration provides a clear advantage regarding potential for reduced ADRs.

Since albumin has been shown to concentrate in joints that are inflamed (Wunder et al., 2003), and most NSAIDs are greater than 95-99% bound to albumin, it has been suggested that drug conjugation with larger molecules such as albumin may allow for increased drug levels in inflamed joints (Garrood et al., 2006).

Based on the combined results from the three studies conducted during my MSc, I conclude that the delivra™ base has the potential to deliver therapeutic levels of meloxicam in the dog for use in canine OA management. Clearly, substantial studies are still required in order for this product to obtain veterinary approval in Canada. Included in the drug development plan would be future studies to further modify the drug formulation process to enhance product shelf life and optimize both pharmacokinetics and pharmacodynamics with the final formulation in the treatment of OA in the dog.
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