ABSTRACT

KuKanich, Stanley. Pharmacokinetics and its application to dosage design in veterinary medicine. (Pharmacokinetics and pharmacodynamics of opiates in dogs). (Under the direction of Mark G. Papich).

The use of analgesics in veterinary medicine has increased in recent years. Despite their increased use, little objective data is available on the efficacy of current dosages. The purpose of this dissertation was to assess the pharmacokinetics of opioids in dogs and objectively measure their pharmacodynamics by using a non-invasive, non-lethal, mechanical threshold device (von Frey device).

The pharmacokinetics of morphine were evaluated following oral and intravenous administration. Morphine was rapidly eliminated, poorly and erratically absorbed orally, and the active metabolite (morphine-6-glucuronide) was not detected.

The von Frey (vF) device was well tolerated, caused no apparent tissue damage, and showed no evidence of tolerance or aversion when repeated hourly for 8 hours. Following morphine sulfate administration,1 mg/kg, as an intravenous injection, the effective plasma concentration to elicit a 50% maximal response (EC_{50}) was 13.9 ± 2.4 ng/mL, and vF thresholds were significantly elevated above baseline values for 4 hours.

Following an intravenous infusion of morphine, the EC_{50} was 29.5 ± 5.4 ng/mL and the lowest mean plasma concentration in which vF thresholds were significantly elevated compared to saline was 31.3 ± 6.0 ng/mL. Plasma morphine concentrations were variable at higher infusion rates. Following multiple intravenous morphine sulfate doses (0.5 mg/kg every 2 hours), the EC_{50} was 27.3 ± 7.4 ng/mL, and there were no significant pharmacokinetic differences between the first and last dose. There were no significant

differences in a saline infusion group vf thresholds compared to the previous untreated group.

The pharmacokinetics of methadone following intravenous and oral administration, and oral administration with omeprazole or ketoconazole were examined. Methadone exhibited a rapid clearance, short elimination half-life, and was not detected in plasma following oral administration. Methadone was not detected following oral administration with omeprazole and only detectable in one dog following oral administration with ketoconazole.

The vf device objectively correlated morphine plasma concentrations with its antinociceptive effects in dogs. Methadone and morphine did not reach therapeutic concentrations following oral administration to dogs.

PHARMACOKINETICS AND ITS APPLICATION TO DOSAGE DESIGN IN VETERINARY MEDICINE

(PHARMACOKINETICS AND PHARMACODYNAMICS OF OPIATES IN DOGS)

by

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DEDICATION

I am dedicating this dissertation to my parents, Stanley Gregory and Patricia Evans KuKanich. My parents have been a positive influence for as long as I can remember. They have provided an excellent example of hard work, dedication, family values, respect for yourself as well as others, and love for their children and grandchildren. Thank you.

BIOGRAPHY

Dr. Stanley “Butch” KuKanich was born November 1971 in Winchester, VA. He completed the Doctor of Veterinary Medicine program at the Virginia-Maryland Regional College of Veterinary Medicine in 1997 at Virginia Tech. He spent four years working as a veterinarian in emergency medicine prior to applying and accepting a position as a Clinical Pharmacology resident and graduate student at North Carolina State University College of Veterinary Medicine. Dr. KuKanich earned Diplomate status in the American College of Veterinary Clinical Pharmacology in 2004.

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Symbol	Units	Definition
λ_z	1/hr	First-order rate constant
$t_{½}\ \lambda_z$	hr	Half-life of the terminal portion of the curve
MRT	hr	Mean residence time
Cl_T	mL/min/kg	Total body clearance
Vd_{ss}	L/kg	Volume of distribution at steady state
Vd_{area}	L/kg	Volume of distribution of the area during the elimination
$AUC_{0-\infty}$	hr*ng/mL	Area under the curve from 0 to infinity
$AUMC_{0-\infty}$	hr*hr*ng/mL	Area under the first moment curve from 0 to infinity
C_0	ng/mL	Concentration at time 0
C_{max}	ng/mL	Maximum concentration
T_{max}	hr	Time to maximum concentration
$t_{½\alpha}$	hr	Distribution half-life
$t_{½\beta}$	hr	Elimination half-life
α	1/hr	Rate constant associated with distribution
β	1/hr	Rate constant associated with elimination
A	ng/mL	Intercept for the distribution phase
B	ng/mL	Intercept for the elimination phase
K01	1/hr	Absorption rate
K10	1/hr	Elimination rate from compartment 1
K12	1/hr	Rate of movement from compartment 1 to compartment 2
K21	1/hr	Rate of movement from compartment 2 to compartment 1
K01 $t_{½}$	hr	Half-life of the absorption phase
K10 $t_{½}$	hr	Half-life of the elimination phase
V1	L/kg	Volume of compartment 1
V2	L/kg	Volume of compartment 2
%F	%	Bioavailability
MEC	ng/mL	Minimum effective concentration
EC_{50}	ng/mL	Concentration to achieve 50% maximal effect
E_{MAX}	%	Maximal effect

EQUATIONS

One Compartment Model

$$Cp = Cp_0 e^{Kel*t}$$

$$T_{1/2} = \frac{0.693}{K_{el}}$$

$$K_{el} = \frac{0.693}{T_{1/2}}$$

Total Body $T_{1/2} = \frac{0.693 * Vd}{Cl}$

$$Vd = \frac{Dose}{Cp_0}$$

$$Cl = \frac{Dose}{AUC}$$

$$AUC = \frac{Cp_0}{Kel}$$

Two Compartment Model

$$Cp = Ae^{\alpha*t} + Be^{-\beta*t}$$

$$T_{1/2} = \frac{0.693}{\beta}$$

$$T_{1/2\alpha} = \frac{0.693}{\alpha}$$

$$T_{1/2} = \frac{0.693 * Vd}{Cl}$$

$$Vd_{ss} = V_{1*}(\frac{k_{12} + k_{21}}{k_{21}})$$

$$Cl = \frac{Dose}{AUC}$$

$$AUC = \frac{A}{\alpha} + \frac{B}{\beta}$$

$$Vd_{area} = \frac{Dose}{AUC * \beta}$$

$$k_{10} = \frac{(\alpha * \beta)}{k_{21}}$$

$$k_{12} = \alpha + \beta - k_{21} - k_{21}$$

$$k_{21} = \frac{(A * \beta) + (B * \alpha)}{A + B}$$

Noncompartmental

$$MRT = \frac{AUMC}{AUC}$$

$$Cl = \frac{Dose}{AUC}$$

$$T_{1/2} = \frac{0.693}{\lambda_Z}$$

$$Vd_{ss} = Cl * MRT$$

$$Vd_{area} = \frac{Dose}{AUC * \lambda_Z}$$

1 INTRODUCTION

The use of analgesics in veterinary medicine has increased markedly over the last two decades. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been the most extensively studied and used analgesics in veterinary medicine. There are currently 7 NSAIDs registered for use in dogs in the USA. However, the effectiveness of NSAIDs is limited to mild to moderately painful conditions and adverse effects preclude their use in certain patients. Only three drugs, other than NSAIDs, are labeled for analgesic use in dogs and are only available as injectable solutions (xylazine, medetomidine, and butorphanol). Xylazine and medetomidine are α-2 adrenergic receptor agonists, which possess very good sedative and mild to moderate analgesic effects having a 0.5 - 1.5 hour duration of effect (Tyner, et al 1997; Hamlin, et al, 1988; Paddelford, et al, 1999). Butorphanol is an opiate which acts as a partial μ and full κ agonist and possesses mild to moderate analgesic and sedative properties having a 0.5 - 1 hour duration of activity (Sawyer, et al, 1991).

There are no veterinary medications labeled for moderate to severe pain control in dogs. Morphine, oxymorphone, fentanyl, hydromorphone, and meperidine are μ opiate agonists that have been recommended for controlling moderate to severe pain in dogs. Only meperidine has been objectively assessed in dogs; however this study did not correlate drug concentration to effect (Lascelles, et al, 1997). In some studies, subjective scoring systems have been an inaccurate method of assessing pain in animals (Greisneaux, et al, 1999; Reese, et al, 2000; Lemke, et al, 2002). Blinded, placebo controlled trials using subjective scoring systems failed to distinguish the placebo treated

from NSAID and oxymorphone treated dogs (Greisneaux, et al, 1999; Reese, et al, 2000; Lemke, et al, 2002).

The use of analgesics in dogs for moderate to severe pain has been based on subjective scales or extrapolation from human studies. An objective measurement of time - effect or concentration - effect of these analgesics has not been successfully determined. The purpose of this dissertation was to assess the pharmacokinetics of analgesics to screen drugs for potential use in dogs. If pharmacokinetic parameters were favorable, then assess the pharmacodynamics with an appropriate model. Chapter 2 will give an overview of the pathways associated with nociceptive stimuli and important mediators of the stimulation. Chapter 3 will discuss different objective measurements commonly used, mostly in laboratory rodents, with their respective advantages and disadvantages. The pharmacokinetics of morphine following oral and i.v. administration are examined in chapter 4. Chapter 5 reports the results of pharmacodynamic modeling of morphine with the von Frey device. The pharmacokinetics and pharmacodynamics of morphine are assessed in chapter 6. Chapter 7 examines methadone as an alternative for an orally administered opioid in dogs as morphine exhibited poor oral bioavailability.

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2. NOCICEPTION: PHYSIOLOGY AND PHARMACOLOGY

2.1 PHYSIOLOGY OF NOCICEPTION

A nociceptive stimulus is one that produces an unpleasant sensation. Pain is a coupling of the unpleasant sensation with conscious perception and an emotional response. There is debate on whether animals can feel pain, but there is no debate that nociception is present in animals. Nociceptive receptors are free nerve endings distributed throughout the body, and detect a nociceptive stimulus. Nociceptive stimuli are transmitted from peripheral nerves to the brain via the spinal cord.

Peripheral nerves have three different types of axons; sensory afferents, motor efferents, and autonomic. Peripheral nerve fibers have also been classified according to their size. A fibers are large fibers, which are myelinated rapidly conducting, and transmit tactile as well as nociceptive stimuli (Björkman, 1995; Markenson, 1996; Wood & Docherty, 1997; Julius & Basbaum, 2001). A fibers are further subcategorized into α, β, γ, and δ fibers. Aα and Aβ fibers are associated with proprioceptive and tactile stimuli, whereas Aδ fibers are the fibers associated with a nociceptive stimulus (Markenson, 1996; Julius & Basbaum, 2001). Aδ fibers rapidly transmit mechanical and thermal nociceptive stimuli, are thinly myelinated, and associated with the first pain sensation (Björkman, 1995; Markenson, 1996; Julius & Basbaum, 2001). B fibers are myelinated preganglionic autonomic fibers and not associated with nociceptive stimuli (Markenson, 1996). C fibers are unmyelinated, slow conducting fibers activated by mechanical, thermal, and chemical nociceptive stimuli and are associated with the second pain sensation (Björkman, 1995; Markenson, 1996; Wood & Docherty, 1997; Julius & Basbaum, 2001). Aδ fibers produce stimuli (first pain sensation) that are well localized

and occur immediately, whereas c fibers carry diffuse pain sensations (second pain sensation), which are poorly localized and persistent (Björkman, 1995; Markenson, 1996; Wood & Docherty, 1997; Julius & Basbaum, 2001).

Sensitization is the phenomenon by which a stimulus that is ordinarily innocuous becomes a nociceptive stimulus. Sensitization can be either centrally mediated (occurring in the central nervous system) or peripherally mediated (occurring at the nociceptor). Central sensitization is primarily a result of n-methyl-d-aspartate (NMDA) receptor activation, resulting in an increase in the magnitude and response, reduction in the activation threshold, and response from stimuli that would not ordinarily elicit a response of nociceptive receptors (Markenson, 1996; Cousins & Power, 1999). Additionally, in patients with chronic pain decreased concentrations endogenous opioids are present with a concomitant desensitization of opiate receptors (Bruehl,et al, 1999). Peripheral sensitization is a result of nociceptor depolarization occurring at lower thresholds. Peripheral sensitization has been associated with decreased tissue pH, and generation of protons, cytokines, bradykinin, serotonin, histamine, ATP, prostaglandins, and leukotrienes (Markenson, 1996; Julius & Basbaum, 2001; Riedel & Neeck, 2001).

Peripheral nerve fibers originate in the dorsal root ganglion, which also contains projections into the dorsal horn of the spinal cord to perpetuate the nociceptive signal (Markenson, 1996; Julius & Basbaum, 2001; Riedel & Neeck, 2001). Glutamate, and to a lesser degree, aspartate are the primary neurotransmitters in the dorsal horn, however vasoactive intestinal peptide (VIP), substance P (SP), cholecystokinin (CCK), and neurotensin have all been identified as neurotransmitters (Markenson, 1996; Julius & Basbaum, 2001; Riedel & Neeck, 2001). The predominant receptor type in the dorsal

horn is the α - amino - 3 - hydroxy - 5 - methyl - 4- isoxazole propionic acid (AMPA) receptor, with glutamate as its endogenous ligand, as well as SP receptors as a less predominant type (Markenson, 1996; Doubell, et al, 1999; Julius & Basbaum, 2001; Riedel & Neeck, 2001).

The second order neuron's cell body is located in the dorsal horn and primarily projects its axons through the spinothalamic tract to terminate in the thalamus (Markenson, 1996; Riedel & Neeck, 2001). However, projections also extend to the medulla and brainstem, the spinoreticular and spinomesencephalic tract, respectively, and spinobulbar tract collectively (Craig & Dostrovsky, 1999). The spinobulbar tract is responsible for homeostatic processes controlled by the brainstem (Craig & Dostrovsky, 1999). Direct projections from the dorsal horn also terminate in the hypothalamus and ventral forebrain, the spinohypothalamic tract, with resultant effects on autonomic, neuroendocrine, and emotional aspects of pain (Craig & Dostrovsky, 1999).

The nociceptive stimuli is propagated through the spinothalamic tract through glutamate, and to a lesser extent SP and CCK (Craig & Dostrovsky, 1999; Riedel & Neeck, 2001). γ–amino butyric acid (GABA) and glycine receptors are also present in the spinothalamic tract and provide inhibitory signals to nociceptive transmission (Craig & Dostrovsky, 1999; Riedel & Neeck, 2001). Prostaglandin E_2 (PGE_2) and activation of NMDA receptors decrease the depolarization threshold of the nociceptor resulting in propagation of the nociceptive stimuli (Markenson, 1996; Cousins & Power, 1999; Julius & Basbaum, 2001; Hinz & Brune, 2004). The thalamus is the crucial relay for processing nociceptive signals to the cortex and for the sensation of nociceptive stimuli (Riedel & Neeck, 2001).

Thalamocortical projections propagate the nociceptive signal from the thalamus into the cerebral cortex (Riedel & Neeck, 2001). Numerous areas in the cortex have been identified in the nociceptive pathways including: the ventral posterior nuclei, the posterior portion of the ventral medial nuclei, the ventral lateral nuclei, the central nuclei, parafascicular nuclei, and the ventral caudal portion of the medial dorsal nuclei (Craig & Dostrovsky, 1999). Species specific differences are present in the distribution of the thalamocortical projections, but with the exception of a few species, have not been well characterized (Craig & Dostrovsky, 1999).

Descending inhibitory pathways from the central nervous system have been identified in the corticodiencephalic, diencephalic, mesencephalic, periventricular grey, medulla, spinal and medullary dorsal horns, and locus ceruleus via norepinephrine pathways (Markenson, 1996; Riedel & Neeck, 2001). The spinobulbar tract also communicates with descending antinociceptive pathways. Catecholamine cell groups, the periaqueductal grey, and reticular formation have also demonstrated descending nociceptive inhibitory pathways by opiate, GABA, glutamate, norepinephrine, and serotonin receptors (Craig & Dostrovsky, 1999; Riedel & Neeck, 2001). The spinohypothalamic tract also exhibits inhibitory responses at all levels of the nociceptive pathway through the release of corticotropin-releasing hormone (CRH), which stimulates opiate and non-opiate receptor pathways (Riedel & Neeck, 2001). In addition to release of β-endorphin, CRH directly stimulates cortical, thalamic, hypothalamic, and spinal inhibition of nociceptive pathways (Lariviere & Melzack 2000; Riedel & Neeck, 2001).

2.2 PHARMACOLOGY

2.2.1 Opiate Receptors

Opiates are considered the prototypical analgesics by which all others are compared. Opiates were originally derived from the pods of the poppy seed, which contains numerous opiate alkaloids. Three classes of opiate receptors have been identified: μ, κ, and δ. The endogenous opioid peptide with the highest affinity for μ receptors is β-endorphin, which is derived from proopiomelanocortin. Leucine- and methinone-enkephalin are the endogenous ligands for the δ receptor and are derived from proenkephalin. Dynorphin A is derived from prodynorphin and is the endogenous ligand for the κ receptor. However the role of dynorphins remains controversial because they induce sensitization of nociceptive transmission through activation of NMDA receptors (Wollemann & Benyhe, 2004).

An endogenous opioid peptide with similar homology to dynorphin, has recently been described, termed orphanin FQ or nociceptin (Meunier, et al, 1995; Reinschied, et al, 1995). Orphanin precursors have been identified in the hippocampus, cortex, and numerous sensory sites (Neal, et al, 1999). However the effects of orphanin have produced conflicting results and its significance is currently unknown (Pan et al, 2000).

Opiates are classified by the receptor type with which they interact (μ, κ, and δ) and by the effect elicited upon binding. Opiates may be full agonists, partial agonists (sub-maximal response), antagonists, and combinations thereof. In addition to analgesia, opiates can also produce euphoria, sedation, respiratory depression, cough suppression, miosis or mydriasis depending on the species, nausea and vomiting, bradycardia,

constipation, biliary stasis, decreased uterine contractions, release of anti-diuretic hormone, pruritus, and increased urethral sphincter tone (Schumacher, et al, 2004).

Opiates exert their analgesic effect by binding to spinal and supraspinal receptors, which exhibit synergistic effects when simultaneously activated (Yeung & Rudy, 1980; Roerig & Fujimoto, 1988). Presynaptic spinal receptors are present for μ, κ, and δ in the dorsal horn (Schumacher, et al, 2004). Presynaptic opiate receptors decrease neurotransmitter release (glutamate and SP) by decreasing the rate of calcium influx (Schumacher, et al, 2004). Postsynaptic μ receptors hyperpolarize the neuron by increasing potassium channel conductance and decreasing response from nociceptive stimuli (Schumacher, et al, 2004).

Numerous supraspinal areas have been identified as having opiate receptors including the rostral ventral medulla, locus ceruleus, and periaqueductal gray area as well as the thalamus and hypothalamus (Markenson, 1996; Riedel & Neeck, 2001; Ko et al 2003; Schumacher, et al, 2004). Functional opiate receptors have also been identified in peripheral tissues including the knee, gastrointestinal tract, and leukocytes (Keates, et al, 1999; Sheehy, et al 2001; Beck, et al, 2002; Elvenes, et al, 2003; Sun & Loh, 2003, Suzuki, et al, 2003; Schumacher, et al, 2004; Patierno, et al, 2004).

2.2.2 Norepinephrine (NE) Receptors

Norepinephrine is a monoamine neurotransmitter present at the cortical and spinal level. Noradrenergic bundles send input to the rostral ventral medulla and descending axons to the dorsal lateral funniculus to inhibit nociceptive pathways (Markenson, 1996; Riedel & Neeck, 2001). Presynaptic α-2 receptors present in the spinal cord decrease the

ascending nociceptive stimulus by decreasing neurotransmitter release (Doubell, et al, 1999; Markenson, 1996; Riedel & Neeck, 2001).

Stimulation of α-2 receptors leads to inhibition of adenylyl cyclase, decreasing intracellular levels of cyclic adenosine monophosphate (cAMP) in addition to regulation of ion (potassium) channels leading to hyperpolarization and alteration enzyme activity (Nicoll, 2004; Potter & Hollister, 2004). Tricyclic antidepressants (TCAs) and serotonin norepinephrine reuptake inhibitors (SNRIs) decrease the reuptake of NE and serotonin, leading to increased levels of NE in the synapse and increased receptor stimulation (Potter & Hollister, 2004). α-2 receptor agonists, such as xylazine and clonidine, directly stimulate α-2 receptors to produce a sympathomimetic effect (Hoffman, 2004).

2.2.3 Serotonin (5-HT) Receptors

Serotonin, also known as 5-hydroxytryptamine (5-HT), decreases nociceptive stimuli at the level of the spinal cord by decreasing neurotransmitter release and inhibiting postsynaptic transmission (Markenson, 1996; Doubell, et al, 1999; Riedel & Neeck, 2001). More than 12 5-HT receptor subtypes have been identified (Nicoll, 2004). With the exception of 5-HT3, 5-HT exerts an inhibitory action in the CNS through hyperpolarization caused by an increased potassium conductance (Nicoll, 2004). Direct intrathecal injection of serotonin produced analgesia in the rat tail flick test (Reiman, et al, 1999). Selective serotonin reuptake inhibitors (SSRIs), SNRIs, and TCAs are therapeutic classes of drugs, which can increase CNS serotonin levels (Potter & Hollister, 2004).

2.2.4 γ-aminobutyric acid (GABA) Receptors

GABA is an inhibitory NT, which decreases nociceptive stimuli through spinal and supraspinal sites of action (Riedel & Neeck, 2001). Two subtypes of GABA receptors have been well documented, $GABA_A$ and $GABA_B$. $GABA_A$ receptors increase chloride channel conductance, are antagonized by picrotoxin and bicuculline, and mediate the fast component of the inhibitory post synaptic action potential (Nicoll, 2004). $GABA_B$ receptors are coupled to G proteins that either inhibit calcium channels or activate potassium channels (shared by 5-HT) and mediate the slow component of the inhibitory post synaptic potential (Nicoll, 2004). GABA acts at pre- and postsynaptic sites in the dorsal horn to decrease NT release and inhibit postsynaptic transmission which decreases nociceptive transmission through the spinothalamic tract (Doubell, et al, 1999; Riedel & Neeck, 2001). GABA acts at supraspinal sites in the periaqueductal grey to activate the descending antinociceptive pathways (Riedel & Neeck, 2001).

Barbiturates bind to allosteric binding sites on $GABA_A$ receptors to enhance GABA activity by prolonging chloride channel opening (Porter & Meldrum, 2004). Additionally, barbiturates block glutamate activation of excitatory AMPA receptors, suppress high frequency repetitive firing by altering sodium conductance, and block some L - type and N – type calcium channels (Porter & Meldrum, 2004). Benzodiazepines bind to allosteric benzodiazepine binding sites on $GABA_A$ receptors to increase the opening frequency of chloride channels (Trevor & Way, 2004). Baclofen selectively activates $GABA_B$ receptors. The mechanism of action for gabapentin is poorly understood, but acts as a GABA agonist (Nicoll, 2004; Porter & Meldrum, 2004). Baclofen, gabapentin, barbiturates and benzodiazepines have shown analgesic activity (Boivie, J, 1999;

Doubell, et al, 1999; Riedel & Neeck, 2001). Gabapentin (Neurontin) is registered by FDA for treatment of neuropathic pain in people.

2.2.5 Glutamate (AMPA & NMDA) Receptors

Glutamate is the major excitatory NT in the brain and spinal cord, which stimulates AMPA, NMDA, and kainate (KA) receptors (Petrenko, et al, 2003; Nicoll, 2004). AMPA and KA receptors are permeable to sodium, potassium, and some types of calcium, whereas the NMDA receptors are highly permeable to sodium, potassium, and calcium (Nicoll, 2004). AMPA receptors are the major receptor in the afferent nociceptive pathway, including the dorsal horn of the spinal cord, spinobulbar tract, spinothalamic tract, and spinocortical tract (Yaksh, 1999).

NMDA receptors are present in the dorsal horn of the spinal cord, spinothalamic tract, and spinocortical tract (Yaksh, 1999). NMDA receptors are essential for normal CNS functions and chronic blockade causes memory impairment and psychotomimetic effects (Petrenko, et al, 2003). NMDA receptors are the primary regulators associated with central sensitization, but do not primarily transmit nociceptive stimuli (Markenson, 1996; Cousins & Power, 1999; Doubell, et al, 1999; Petrenko, et al, 2003). NMDA receptor ion channels are physically blocked by magnesium, when nociceptors are at resting membrane potentials. Therefore, upon NMDA receptor activation no response is seen because ions cannot pass through the channel due to the magnesium blockade (Markenson, 1996; Cousins & Power, 1999; Doubell, et al, 1999; Petrenko, et al, 2003). However upon nociceptor depolarization (through AMPA), the magnesium ion is removed from the NMDA ion channel resulting in increased ion flow resulting in

propagation of the nociceptive signal as seen in central sensitization (Markenson, 1996; Cousins & Power, 1999; Doubell, et al, 1999; Petrenko, et al, 2003). Additionally, metabotropic glutamate receptors act via G - protein activation of protein kinase C (PKC) to phosphorylate the serine / threonine residues to decrease the magnesium block potentiating depolarization (Doubell, et al, 1999; Petrenko, et al, 2003). Tyrosine kinase phosphorylation of tyrosine residues on the NMDA receptor following initial depolarization results in prolonged opening of the channel and increased burst clusters contributing to central sensitization (Doubell, et al, 1999; Petrenko, et al, 2003). Therefore, direct effects of AMPA receptor depolarization and post - translational modification (phosphorylation) of the NMDA receptor results in increased ion permeability, resulting in increased frequency and rate of depolarization and decreased nociceptive excitatory thresholds, due to concurrent activation of AMPA and NMDA receptors.

NMDA receptors have also been isolated from peripheral tissues on myelinated and unmyelinated axons (Petrenko, et al, 2003). Administration of glutamate results in nociceptive stimulation that can be subsequently blocked by an NMDA antagonist (Petrenko, et al, 2003). Therefore NMDA receptors may also play a role in peripheral sensitization.

Ketamine and tiletamine are noncompetitive NMDA receptor antagonists, which are commonly used as sedatives and anesthetics, but are associated with adverse effects (Petrenko, et al 2003). Dextromethorphan, memantine, and amantadine are noncompetitive NMDA antagonists available as oral products, which decrease opiate

consumption when administered concurrently and are currently being investigated as analgesic adjuncts (Petrenko, et al 2003; Nitu, et al, 2003; Snijdelaar, et al, 2004).

2.2.6 Phospholipase, Cyclooxygenase (COX), & Lipoxygenase (LOX)

Tissue injury results in release of phospholipids from cell membranes. Phospholipase A_2 catalyzes the reaction forming arachidonic acid from phospholipids, which is in turn catalyzed to leukotrienes (LTs) by lipoxygenase (LOX) and prostaglandins (PGs), prostacyclin (PGI_2) and thromboxane (TX) by cyclooxygenase (COX). LOX catalyzes the formation of LTB_4, LTC_4, LTD_4, and LTE_4, which mediate inflammation, vasoconstriction, gastric acid secretion, bronchoconstriction, and increase the recruitment, activation, adhesion, and migration of inflammatory cells (Brune, 2004). LTB_4 also increases the production of tumor necrosis factor-α (TNF-α) and interleukin1-β (IL-1β), inflammatory cytokines (Brune, 2004).

Two isoforms of COX have been identified, COX-1 and COX-2. COX-1 is constitutively expressed in tissues, catalyzing the formation of PGD_2, PGE_2, PGF_2, and thromboxane A_2 (Hinz & Brune, 2004). COX-1 products are associated with physiologic responses such as platelet aggregation, vasodilation, and mucous secretion, and inhibition of acid secretion in the gastrointestinal tract (Hinz & Brune, 2004). COX-2 catalyzes the formation of PGI_2 (prostacyclin) and PGE_2, and is primarily associated with inflammatory processes (Hinz & Brune, 2004). COX-2 expression has also been demonstrated in the dorsal horn and becomes upregulated following tissue injury (Hinz & Brune, 2004). COX-2 catalyzed formation of PGE_2 in the dorsal horn lowers the depolarization thresholds of the nociceptors (Hinz & Brune, 2004). Additionally, a COX-

1 variant termed COX-3 has also been identified in central nervous tissue, however the significance and activity of COX-3 is still unknown (Hinz & Brune, 2004).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the agents most commonly used to inhibit the cyclooxygenase enzyme. NSAIDs can selectively inhibit COX-2 or COX-1 with others showing nonselective inhibition of both isoforms of COX. Zileuton is a selective inhibitor of LOX. Tepoxalin inhibits COX-1, COX-2, and its active metabolite inhibits LOX. Efficacy studies evaluating the effects of tepoxalin compared to a COX only inhibitor (carprofen) resulted in a higher percentage improvement for tepoxalin, although the improvement was not statistically different (Zubrin Technical Monograph, Schering-Plough Animal Health).

2.3 References

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3. NOCICEPTION: ANIMAL MODELS

3.1 INTRODUCTION

As stated in chapter 2, nociception is a complex combination of electrophysiological, neurochemical, and behavioral responses to a noxious stimulus. Nociception is hypothesized to have three functions: 1) a warning of tissue injury; 2) a warning of impending tissue damage; 3) a warning of danger to a social group (Le Bars, et al, 2001). However, not all types of nociception fit discretely into one of these categories. In people, many assessments of nociception are made in postoperative patients, with the degree of nociception subjectively quantified by the patient. Experimental testing in people, regardless of the stimulus, is also subjectively assessed by the subject. However this is not possible with animals. Subjective assessment of postoperative nociception in animals, by people, has yielded variable results and appears inadequate to distinguish treatment from placebo in some trials (Greisneaux, et al, 1999; Reese, et al, 2000; Lemke, et al, 2002).

The goal of nociceptive modeling in animals is to construct a model that allows an objective assessment (quantifiable response) of the antinociceptive effects of a treatment, is noninvasive, and is repeatable. Four basic types of nociceptive stimuli have been used in animal modeling: 1) mechanical; 2) thermal; 3) chemical; and 4) electrical. An extensive review of the theory behind each of the stimuli has been published (Le Bars, et al, 2001). A summary review of the animal models is included followed by representative models used in dogs.

3.2 Mechanical Stimulation

3.2.1 Theory

Mechanical stimulation is the application of a stimulus, which elicits a nociceptive response by applying pressure to an area. The stimulus can be applied as a constant pressure, coarsely applied, or as an increasing pressure with time. The stimulation is applied until a predetermined response is obtained. Mechanical models provide a quantifiable stimulus, are noninvasive, and repeatable.

Mechanical stimulation, in addition to activating nociceptors, activates mechanoreceptors, resulting in a nonspecific stimulation (Le Bars, et al, 2001). A withdrawal response to application of the stimulus can occur despite the lack of nociception. Both Aδ and c fiber nociceptors can be stimulated by a mechanical device. Aδ fibers are nerves that transmit acute sharp nociceptive stimuli, whereas c fibers transmit dull aching stimuli and are the most likely source of nociceptive stimuli in patients. Application of a mechanical stimulus can result in tissue damage that will increase the response from repeated application because of sensitization. Conversely, repeated stimuli may diminish the response owing to habituation. Restraint, which is typically needed during the use of a mechanical stimulus, can alter the response seen. Application of a mechanical stimulus also results in an asynchronous depolarization of peripheral nerve fibers, which does not accurately reflect the neurophysiology of nociception. Finally, differentiation of the response to the stimulus can be difficult if application of increasing pressure may elicit passive movement. A simple withdrawal reflex is a spinal reflex and is not indicative of a nociceptive response, and also must be distinguished from the nociceptive response in a reliable model (Le Bars, et al, 2001).

The pressure exerted by a mechanical stimulus is related to the surface area, weight applied, and rate of application. As the surface area is decreased, the pressure is increased if the weight and the rate of application are held constant. As the rate of application is increased the pressure is increased if the surface area and weight are held constant. An ideal mechanical device is one that maintains a constant surface area and rate of application.

3.2.2 Canine Models

A pneumatic mechanical device that delivered a constant rate of pressure, from a rigid tip applied to the proximal portion of the tibia, which was immobilized in a cast, failed to demonstrate the antinociceptive effects of morphine. The model described in the Barnhart, et al study, increased the force until signs of discomfort (unstated) were seen. The investigators acknowledged that the response may have been misinterpreted during the study because withdrawal responses were typically seen at initial contact of the tip (activation of mechanoreceptors), not necessarily due to nociception. Additionally, the application of a cast to immobilize the limb may have caused distress to the animals, which altered the responses and served as a source of mechanical stimulation in itself (Barnhart, et al, 2000). The quantifiable measurement of the antinociceptive effect recorded was the pressure applied and changes in response were compared to baseline (control) measurements (Barnhart, et al, 2000).

A custom built device, which measured the pressure applied to a toe pinch, quantified the antinociceptive effects of xylazine and medetomidine in dogs. The device resembled a large set of pliers with a set of interchangeable cylinders that applied

pressure to a toe, and a gauge that quantified the pressure applied (Hamlin, et al 1988; Tyner, et al 1997). Significant elevations in thresholds were present following intravenous xylazine administration (1.1 mg/kg) for 15 minutes (Hamlin, et al 1988). No significant differences in threshold measurements were present between the two α-2 agonists in the Tyner study, which evaluated xylazine (1.1 mg/kg IV and 2.2 mg/kg IM) and medetomidine (750 μg/m^2 IV and 1000 μg/m^2 IM) (Tyner, et al 1997). A similar mechanical device evaluated the response of saline and 4 dosages of butorphanol in conscious dogs. Increases in thresholds were observed, but no statistical analysis was used to evaluate the results, making the interpretation of the results difficult (Quandt, et al, 1994). The operator controlled the rate of application in all of the toe pinch models, which can alter the actual pressure applied. This may have affected the thresholds, decreasing the sensitivity of the model (Hamlin, et al 1988; Quandt, et al, 1994; Tyner, et al 1997).

A palpation pressure device was tested which utilized an 18 mm pad with a pressure transducer that was applied to a surgical site with the tip of the operator's finger (Slingsby & Waterman-Pearson, 2000; Caulkett, et al 2003). The device was initially used in cats, but was subsequently applied adjacent to the surgical site in post-operative dogs. No significant differences in responses between butorphanol and meloxicam were noted for a period of 24 hours. Butorphanol has demonstrated antinociceptive effects lasting less than 1 hour in dogs, therefore the palpation pressure device either was not effective in determining antinociceptive effects or neither drug exhibited antinociceptive properties. The device did not control the rate of application, the study was not placebo (or positive) controlled, and the large surface area of the pad resulted in uneven pressure

application. Additionally, the baseline values were taken from a different body parts as compared to the tested area, which would result in variability and inaccuracy. Finally, normal tissues were compared to tissues in which sensitization was present due to the surgical site.

A mechanical distension model, which distended the large bowel with a balloon, was assessed to quantify the antinociceptive effects of butorphanol. The balloon was distended until a response was noted, at which time the distension was discontinued and the pressure recorded. Significant changes (% change from baseline) in the pressures were present for a period of 0.5 - 1 hour following butorphanol administration (Sawyer, et al, 1991).

A variant of the mechanical stimulation model involves surgical models, which destabilize a joint with arthritis as a consequence. Surgical transection of the cranial cruciate ligament is the primary model used, however a surgical model of the coxofemoral joint has also been described (McDevitt & Muir, 1976; McDevitt, et al, 1977; Schiavinato, et al, 1989; Abatangelo, et al, 1989; Visco, et al, 1996; Pelletier, et al, 2000; Renberg, et al, 1999; Renberg, et al, 2000 Budsberg, 2001). Surgical models are invasive, require anesthesia, result in conditions that are not identical to naturally occurring arthritis, and produce variable results (Liu, et al, 2003).

A combination of mechanical and chemical testing has been described in laboratory rodents, but not in dogs. In this model, a chemical stimulus is injected into the animal, then a mechanical stimulus is applied to the area and pressures are recorded with time. Increased sensitivity is achieved by using a mechanical / chemical combination, however specificity is decreased as described in more detail in section 3.4 (Le Bars, et al,

2001). Additionally, studies have utilized hemostats or forceps to apply pressure to body parts, but since this measure is not consistent or accurately quantifiable these methods produce unreliable data.

3.2.3 Von Frey Device

The von Frey (vF) device has been described as an instrument for experimental use in animals and people. The vF device acts as a mechanical stimulus by applying increasing pressure until a response is noted. Early models consisted of fine hairs that bent at a predetermined pressure and were applied in increasing sizes until a reaction occurred without the hair bending. Models with digital load cells and rigid tips are now available for increased accuracy and precision.

Despite the advent of newer models of nociception, vF devices are still commonly used in research. A study examining the effects of serotonin and bradykinin utilized a vF device to determine significant differences in muscular hyperalgesia compared to saline controls in people (Babenko, et al, 2000). The vF device also has been utilized as a comparison to visual analogue scales in people examining the hyperalgesic effects of serotonin and bradykinin in muscular hyperalgesia (Babenko, et al, 1999). Decreased wound hypersensitivity following hysterectomy was demonstrated in women pre-treated with morphine as compared to post-operative treatment with a vF device (Richmond, et al, 1993). The peripheral effects of fentanyl following local administration into a surgical wound were evaluated in people with a vF device (Tverskoy, et al, 1998).

The vF device has been used extensively in laboratory rodents, in particular rats. A study assessing hyperalgesia secondary from surgical incision yielded significant

changes in vF threshold measurements in the rat and selective neurectomy abolished the changes in vF thresholds (Brennan, et al 1996). A study assessing the effects of non-NMDA excitatory amino acid (EAA) antagonists on surgical incision vF thresholds demonstrated receptor systems for EAA other than NMDA are involved in acute nociception in rats (Zahn, et al, 1998). The antinociceptive effects of gabapentin in a peripheral neuropathy model in rats were demonstrated using vF thresholds (Back, et al, 2004). Pharmacokinetic - pharmacodynamic modeling of the anti-hyperalgesic effects of 5'-deoxy-N6-cyclopentyl-adenosine in neuropathic pain were demonstrated in rats by use of vF threshold measurements (Schaddelee, et al, 2004).

Instruments based on the von Frey device have successfully determined the antinociceptive effects of drugs in dogs. A portable device consisting of a rigid fixed diameter tip applied at a constant rate to various locations has demonstrated significant responses to meperidine (an opioid) and in a separate study carprofen (an NSAID) in post-operative patients (Lascelles, et al, 1997; Slingsby & Waterman-Pearson, 2001). The tip was attached to a load cell, which quantified the pressure applied (Lascelles, et al, 1997). The rate of application was controlled by the operator, but verified as constant by use of a measuring device (Lascelles, et al, 1997).

3.3 THERMAL STIMULATION

3.3.1 Theory

Application of heat or cold stimulates thermosensitive nerve fibers, and if the intensity is high enough, nociceptors will also be activated. Therefore, similar to mechanical stimulation, thermal stimulation tends to be nonspecific. Additionally, the weak caloric power of most thermal stimulation models (radiant heat, contact thermodes) cause an asynchronous nerve depolarization, similar to mechanical threshold devices. The initial skin temperature is also a variable, which can affect the response to a heat stimulus (Le Bars, et al, 2001).

Conventional radiant heat sources have the additional problem of emitting radiation in the visible spectrum, which the skin is a good reflector and poor absorber. Blackening the skin with India ink can minimize this effect. The area of radiant heat application is also variable and difficult to control. Radiant heat also disrupts the normal blood flow present in the capillary bed, which can alter the response (Le Bars, et al, 2001).

Thermodes are solid elements, which are placed in direct contact with the skin and heat rapidly. Thermodes are not affected by the color of the skin, but their fixed, rigid, and flat nature can be difficult to maintain consistent contact with the skin. Additionally, thermodes can activate nociceptor inhibitory pathways due to asynchronous depolarization of the nerves. Thermodes also present a source of mechanical stimulation (Le Bars, et al, 2001). A water bath can be used in a similar manner to the thermodes as a contact thermal device, but the rate of heating is slower than with thermodes, and its use in dogs has not been described.

Carbon dioxide lasers produce an almost instantaneous rise in skin temperature, have no direct contact with the test subject, are not effected by pigmentation, and are concentrated to a fixed area (and depth), to produce an almost synchronous nerve depolarization limited to nociceptors. Additionally, lasers do not require direct contact with the skin reducing variability due to concurrent mechanical stimulation (Le Bars, et al, 2001). However, lasers are expensive to purchase and maintain, and present a potential hazard for personnel using them.

3.3.2 Canine Models

A radiant heat source examined the effects of 4 opiates in dogs, all of which yielded an increase in thresholds (Andrews & Workman, 1941). However, only 2 dogs were examined and statistical analysis was not performed. A heat source evaluated the antinociceptive effect of morphine following intravenous and intramuscular administration, but failed to demonstrate a significant effect (Barnhart, et al, 2000). The heat source consisted of an incandescent light bulb housed in a metal cylinder. The cylinder made direct contact with the skin and was immobilized in a fiberglass cast, therefore transferred heat by conductance, similar to a thermode. The device had a slow rate of heating, was dependant on contact with the skin, provided a source of mechanical stimulation, and the cast provided a source of mechanical stimulation, all of which could have affected results reported.

3.4 CHEMICAL STIMULATION

3.4.1 Theory

Chemical agents result in a progressive nociceptive stimulation, which is of longer duration and inescapable nature once administered. Chemical irritants elicit a biphasic pain response and most closely imitate clinical pain. However, inflammation elicited from chemical stimuli adds variability as anti-inflammatory properties of compounds may alter the nociceptive response; therefore both anti-inflammatory and antinociceptive effects are assessed (Le Bars, et al, 2001). Additionally, chemical models are more invasive, and less humane because they require an injection of an algogenic substance.

3.4.2 Canine Models

Numerous models utilizing chemical stimulation have assessed the antinociceptive and anti-inflammatory properties of drugs in dogs. The majority of models use algogenic substances (urate crystals, carageenan, bradykinin, formalin) injected into an extremity, usually a joint, with gradation of lameness assessed as the outcome (McCarty, et al, 1966; Phelps & McCarthy, 1967; Chang, 1972; Dorwart, et al 1974; Niemegeers & Janssen, 1975; Sancilio, et al, 1987;Toutain, et al, 2001; Millis, et al, 2002; Hazewinkel, et al, 2003; McCann, et al 2004). Due to the widespread use and the invasive nature of this model an extensive review of the results is not included.

3.5 ELECTRICAL STIMULATION

3.5.1 Theory

The use of electrical current as a nociceptive stimulus provides a quantifiable, reproducible, and noninvasive model, which produces a synchronous depolarization of nerve fibers. However electrical stimulation is not a natural stimulation and it causes depolarization of all types of nerve fibers (including inhibitory fibers), not just nociceptors. Additionally, electrical stimuli completely short circuit peripheral receptors preventing assessment of nerve transduction. Finally, variation of impedance occurs with different tissue locations or electrode placement, but impedance can be assessed by monitoring the voltage and current applied (Le Bars, et al, 2001).

Application of electrical stimuli results in a double pain phenomenon. The first pain results from activation of Aδ fibers and typically results in a well localized stinging sensation in human volunteers. The second pain is slower in onset and typically consists of a difficult to localize burning sensation in human volunteers and results from activation of c fibers (Le Bars, et al, 2001). The separation of the first and second pain sensations is a result of the differing nerve conducting velocities as described in chapter 2.

3.5.2 Canine Models

The use of electrical currents to elicit nociceptive stimulation of the tooth pulp has been used by one group in conscious dogs. Electrodes were surgically implanted prior to the study and the effects of opiates and noradrenergic drugs were assessed. The model demonstrated statistical differences for the noradrenergic drugs, however statistical

analysis was not performed for the opiate studies (Skingle & Tyers, 1980; Hayes, et al, 1986).

A similar study utilizing electrical stimulation of dental pulp has been conducted on anesthetized dogs. The study required anesthesia, but was not as invasive as the previous dental pulp studies were. An alligator clip applied to the exposed dentin of the canine tooth provided the electrical stimulus, while a needle placed in subcutaneous tissues of the masseter muscle served as the ground. The jaw opening reflex was the parameter quantitatively assessed by electrodes placed in the digastricus muscle and the subcutaneous tissues of the zygomatic arch. Statistically significant results, compared to saline, were obtained following intravenous and intrathecal administration of morphine (Brown, et al, 2002). However anesthesia was a confounding factor to the antinociceptive effects of morphine.

Direct electrical stimulation of peripheral nerves (radial and tibial) as well as limbs, oral mucosa, and periosteum have evaluated the antinociceptive effects of drugs in dogs (Niv, et al, 1983; Hamlin, et al, 1988; Wang, et al, 1994; Quandt, et al, 1994, Valverde, et al, 2003). Statistically significant differences in treatment groups were noted in dogs treated with clonidine and midazolam under anesthesia, and xylazine in unanesthetized dogs (Niv, et al, 1983; Hamlin, et al, 1988; Wang, et al, 1994). Threshold elevations were present, but not statistically assessed following administration of butorphanol, and the minimum alveolar concentrations of halothane and isoflurane were calculated using electrical stimulation in a separate study (Quandt, et al, 1994; Valverde, et al, 2003).

3.6 CONCLUSIONS

Numerous models have been developed to quantitatively assess the antinociceptive effects of drugs administered to dogs. Each model has advantages and disadvantages with none of them being ideal for use in dogs. The choice of the appropriate model depends on the cost, the mechanism of action of the test drug, the confounding effects of anesthesia if needed, the degree of invasiveness, duration of the study, ability to repeat measurements over time, sensitivity, humane treatment of the animal, and specificity required.

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PHARMACOKINETICS OF MORPHINE AND PLASMA CONCENTRATIONS OF MORPHINE-6-GLUCURONIDE FOLLOWING MORPHINE ADMINISTRATION TO DOGS.

RH: Pharmacokinetics of morphine in dogs.

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4.1 ABSTRACT

The purpose of this study was to evaluate the pharmacokinetics of morphine and morphine-6-glucuronide (M-6-G) following morphine administered intravenously and orally to dogs in a randomized crossover design. Six healthy 3-4 year old Beagle dogs were administered morphine sulfate (0.5 mg/kg) as an i.v. bolus and extended release tablets were administered orally as whole tablets (1.6 ± 0.1 mg/kg) in a randomized crossover design. Plasma concentrations of morphine and M-6-G were determined using high-pressure liquid chromatography (HPLC) and electrochemical coulometric detection. Following i.v. administration all dogs exhibited dysphoria and sedation, and 4/6 dogs vomited. Mean ± SE values for half-life, apparent volume of distribution, and clearance after i.v. administration were 1.16 ± 0.15 hours, 4.55 ± 0.17 L/kg, and 62.46 ± 10.44 ml/min/kg, respectively. One dog vomited following oral administration and was excluded from the oral analysis. Oral bioavailability was 5% as determined from naïve pooled analysis. M-6-G was not detected in any plasma samples following oral or i.v. administration of morphine at a (25 ng/mL LOQ). Computer simulations concluded morphine sulfate administered 0.5 mg/kg intravenously every 2 hours would maintain morphine plasma concentrations consistent with analgesic plasma levels in people. Oral morphine is poorly and erratically absorbed in dogs.

4.2 INTRODUCTION

Morphine is the prototypical opiate analgesic. Experimental studies have shown that morphine interacts with μ and κ opiate receptors to exert its analgesic effect (Gutstein & Akil, 2001). Morphine-6-glucuronide (M-6-G) has been shown to exhibit analgesic activity in people following M-6-G administration, and the levels produced following morphine administration almost equal that of the parent drug (Penson, et al, 2000; Murthy, et al, 2002; Skarke, et al, 2003). M-6-G has been shown to be produced as a metabolite in isolated canine hepatocytes in low concentrations (Milne, et al, 1996; King, et al, 2000). The analgesic effects of M-6-G in people following morphine administration have been variable ranging from less than 1% to 66% of the total analgesic effect from parenterally administered morphine (Penson, et al, 2000; Murthy, et al, 2002; Skarke, et al, 2003).

There are no reports of pharmacokinetic – pharmacodynamic modeling in dogs to more accurately calculate an MEC. Additionally, no reports evaluated the plasma concentrations of M-6-G in dogs following i.v. and oral administration of morphine. In people the reported MEC of morphine has ranged from 9.1 to 40 ng/mL and as high as 364 ng/mL with chronic administration (Neumann, et al, 1982; Dahlstrom, et al, 1982; Graves, et al, 1985; Gourlay, et al, 1986; Eisenach, et al, 1989; Sarton, et al, 2000; Skarke, et al, 2003). In people, a measure of the MEC for morphine is confounded by the contribution of M-6-G, which may also provide analgesic effects.

The purpose of the study was to evaluate the pharmacokinetics of morphine and M-6-G following intravenous and oral administration of morphine to dogs. A second purpose of this study was to calculate dosing strategies based on the calculated

pharmacokinetic parameters to maintain a targeted plasma concentration of 20 ng/mL that could be used as a basis for further pharmacodynamic studies.

4.3 MATERIALS AND METHODS

Six healthy beagle dogs (3 male, 3 female), ranging in weight from 7.3 – 13.0 kg, and age 3 - 4 years were used in this study. Food was withheld 12 hours prior to the study. The North Carolina State University Institutional Animal Care and Use Committee approved the study.

Plasma was analyzed using HPLC with electrochemical coulometric detection. The HPLC system consisted of a quaternary pump, degasser, and auto injector (Agilent 1100 series, Agilent technologies, Wilmington, DE). Plasma was extracted using solid phase extraction cartridges (Varian C-8, Varian Inc. Palo Alto, CA). Briefly, 1 mL of plasma was mixed with 1 mL 0.2 M borate buffer (pH 9.0), vortexed, followed by addition of 0.4 mL 0.1 M 1-pentanesulfonic acid, and vortexed again. The cartridges were conditioned with 1 mL methanol, 1 mL distilled water, followed by loading the plasma - buffer mixture (2.4 mL), washing with 1mL distilled water, and elution with 1 mL methanol. The eluent was evaporated under nitrogen gas, reconstituted with 0.2 mL mobile phase, and 0.05 mL injected. The mobile phase consisted of 95% 0.01 M acetate buffer with 0.1% triethylamine, and 5% acetonitrile with the pH adjusted to 4.5 with glacial acetic acid. A mobile phase gradient was programmed in the following manner: 0 - 8 minutes, 100% mobile phase, 8 – 11 minutes ramped to 85% mobile phase and 15% acetonitrile, 11 – 14 minutes ramped to 100% mobile phase, then 14 – 20 minutes 100 % mobile phase to re-equilibrate the column. Separation was achieved with a 4.6 x 150 mm, 5 - micron, phenyl column (Zorbax SB-Phenyl, Agilent technologies, Wilmington, DE) maintained at 40° C. Settings for the electrochemical coulometric detector (ESA, Coulochem II, Bedford, MA) included: guard cell +750 mV; cell 1 +300 mV; cell 2 +450

mV, with cell 2 being quantified. The retention times for morphine and M-6-G were 7.5 and 5.5 minutes respectively. Calibration curves were made by analyzing fortified canine plasma with morphine HCl (Lipomed, Inc., Cambridge, MA) and M-6-G (Lipomed, Inc., Cambridge, MA), and analyzing the detector response vs. drug concentration with linear regression. The calibration curve for each day's run was accepted if the coefficient of determination (r^2) was at least 0.99, and the calculated values for each point were within 15% of the expected values, except the lowest and highest points on the calibration curve, which were within 20% of the expected values (Shah, et al, 1992).

Dogs were randomly assigned to receive either 0.5 mg/kg morphine sulfate (0.38 mg/kg base) (Baxter Healthcare, Deerfield, IL) i.v. or 15 mg morphine sulfate (11.28 mg/kg base) extended release tablets (Endo Pharmaceuticals, Chad's Ford, PA) orally in a randomized crossover design with at least a 7 day washout period between treatments. Morphine tablets were administered as whole tablets. Intravenous morphine was administered through an aseptically placed 20 gauge cephalic catheter (Angiocath, Becton Dickinson, Sandy, Utah) and flushed with 10 mL of 0.9% saline solution following injection. Tablets were administered per os followed by 20 mL of tap water to ensure swallowing. Blood samples were collected from aseptically placed 19 gauge jugular catheters (Intracath, Becton Dickinson, Sandy, Utah), placed prior to drug administration. Blood, 7 mL per time point, was collected into evacuated glass tubes containing lithium heparin as an anticoagulant (BD Vacutainer, Franklin Lakes, NJ). Blood samples were placed on ice, centrifuged for 10 minutes at 1000g, plasma was separated, and stored frozen at –80° C prior to analysis. Blood samples were collected prior to and at 10, 20, 30, and 45 minutes and at 1, 1.5, 2, 3, and 4 hours after intravenous

morphine administration. Blood samples were collected prior to, and at 15, 30, and 45 minutes and at 1, 1.5 2, 3, 4, 5, and 6 hours following oral administration.

Pharmacokinetic analysis and dose simulations were performed with a computer program (WinNonlin, 4.0, Pharsight Corporation, Mountain View, CA). Compartmental and noncompartmental analyses were performed in a standard two-stage design. Pharmacokinetic variables were calculated from equations published elsewhere (Gibaldi & Perrier, 1982). The area under the curve was calculated using the linear trapezoidal rule. A $1/y^2$ weighting factor, where y is the plasma concentration, was applied to the noncompartmental analysis. The compartmental analysis was calculated with a uniform weighting factor of 1. Oral bioavailability (%F) was calculated from the area under the curve (AUC) from the naïve pooled plasma profiles using the equation:

$$\%F = \frac{100\% \bullet Dose_{i.v.} \bullet AUC_{p.o.}}{Dose_{p.o.} \bullet AUC_{i.v.}}$$

The computer program was also used to simulate dosing regimens that would be required to maintain plasma levels greater than or equal to 20 ng/mL using intravenous or oral administration.

4.4 RESULTS

The limit of quantification (LOQ) for morphine and M-6-G analysis in plasma was 5 ng/mL and 25 ng/mL respectively and is defined as the lowest point on the standard curve, with an r^2 at least 0.99 and predicted values within 20% of the actual concentration. The mean ± SE values for accuracy (deviation from actual concentration) and precision (coefficient of variation) of the morphine assay were 8% ± 2% and 3% ± 1% and were calculated from equations published elsewhere (Shah, et al, 1992)

Following i.v. administration of morphine, all dogs exhibited sedation and dysphoria, and four of the dogs vomited. The plasma profile for i.v. morphine was characterized by a rapid distribution phase, followed by a slower elimination phase and best fit a two compartment model (figure 4.1). The elimination half-life was 1.16 ± 0.15 hrs, the total body clearance 62.46 ± 10.44 mL/min/kg, and the apparent volume of distribution at steady state 4.55 ± 0.17 L/kg (table 4.1).

The actual dose of morphine sulfate administered orally was 1.59 ± 0.14 mg/kg. One of the dogs vomited following oral administration and was excluded from the oral analysis. Morphine was poorly and erratically absorbed following oral administration (figure 4.2). The oral bioavailability was 5.31%. Morphine - 6 - glucuronide was not detectable in any plasma sample following either route of administration.

Computer simulations, using the calculated pharmacokinetic variables, predicted a morphine dose of 0.5 mg/kg i.v. every 2 hours to achieve peaks of 163 ng/mL and troughs of 22 ng/mL at steady state. Computer simulations on naïve pooled oral pharmacokinetic variables calculated a morphine dose of 10 mg/kg every 2.5 hours would achieve 48 ng/mL peaks and 25 ng/mL troughs.

4.5 DISCUSSION

This study found the pharmacokinetic parameters for intravenous morphine to be similar to parameters from previous published studies (table 2). When morphine extended release tablets were administered, oral bioavailability was found to be poor, with plasma levels detected infrequently. This is in contrast to previous studies in dogs. Morphine-6-glucuronide, which may contribute to the analgesic effect of morphine in people, was not detectable in dogs following either intravenous or oral dosing.

When administered orally to dogs in this study, morphine was poorly and erratically absorbed. Variable absorption has been seen in previous studies of oral administration of morphine in dogs (Dohoo, 1997). Oral morphine can result in gastrointestinal adverse effects including vomiting, inappetance, and constipation, despite the lack of systemic analgesic effects (Manara & Bianchetti, 1985). Pilot studies conducted with immediate release morphine tablets (0.66 mg/kg) resulted in no detectable levels in 1 dog. A second dog that received 1.26 mg/kg of the immediate release tablet vomited; therefore studies with immediate release tablets were not pursued. Following dosing of the sustained release tablets, 1 dog vomited (not the same dog as the immediate release tablets). Morphine administered orally in this study did not reach plasma concentrations that are considered therapeutic in people, which suggests that published recommendations for oral administration to dogs for pain may need to be re-evaluated (Wagner, 2002; Dohoo, et al, 1994; Dohoo & Tasker, 1997; Doohoo, 1997). In this study, plasma levels were low following oral administration of morphine, and not detectable 4 hours following dosing. Dosing regimens calculated for oral morphine sustained release tablets resulted in impractical recommendations.

Previous studies on sustained release tablets administered to dogs reported higher (but very variable) plasma levels, but a different formulation was used (Dohoo, et al, 1994; Dohoo & Tasker, 1997; Doohoo, 1997). The design and composition of sustained-release tablets can have profound effects on the drug's absorption (Sabinis, 1999). Previous studies comparing differing formulations of sustained release theophylline products in dogs yielded different pharmacokinetic parameters and subsequently different dosing recommendations for each formulation (Bach, et al, 2004). Additionally, the previous studies on oral morphine, analyzed plasma samples with a radioimmunoassay, which could cross-react with unidentified metabolites, overestimating the actual morphine concentration (Dohoo, et al, 1994; Dohoo & Tasker, 1997; Doohoo, 1997). Unidentified metabolites of morphine produced by dogs may contribute to the analgesic effects, although this has never been demonstrated.

Morphine - 6 - glucuronide was not detected in any plasma sample at a level of sensitivity of 25 ng/mL, and therefore is unlikely to contribute to the analgesic effects of morphine in dogs. In human studies, peak concentrations of M-6-G following therapeutic doses of morphine are in the range of 60 – 300 ng/mL, which if produced in similar levels in the current study would have been detected (Westerling, et al, 1995; Meineke, et al, 2002; Skarke, et al, 2003; Whittinigton & Karasch, 2003). The very low levels of M-6-G may increase the plasma concentrations of morphine required to control pain in dogs compared to people. This however assumes that dogs and people demonstrate similar levels of antinociception to similar plasma concentrations of morphine and M-6-G; this has not been evaluated.

Morphine sulfate is commonly used as an analgesic agent in dogs with dosage recommendations ranging from 0.05 – 2 mg/kg intravenously, intramuscularly, or subcutaneously every 2-6 hours (Carroll, 1999; Thurmon, et al, 1996; Wagner, 2002). These dosages are based on clinical impressions, subjective visual scoring systems, or dogma. No dose titration studies have been reported in dogs. Clinical impressions and subjective visual assessment of analgesia and pain in dogs appear to be inaccurate (Grisneaux, 1999; Reese, et al, 2000). Intravenous doses of 0.5 mg/kg or intramuscular doses of 1 mg/kg in another study did not produce an increase in threshold response to applications of noxious mechanical and thermal stimuli (Barnhart, et al, 2000). However, the lack of response may have been related to the model, as stated by the authors (Barnhart, et al, 2000).

Studies in people have related the plasma concentration of morphine to its analgesic effect (Neumann, et al, 1982; Dahlstrom, et al, 1982; Graves, et al, 1985; Gourlay, et al, 1986; Eisenach, et al, 1989; Sarton, et al, 2000; Skarke, et al, 2003). Since the effective plasma concentration of morphine in dogs is currently unknown, we calculated dosages to maintain a minimum targeted plasma concentration of 20 ng/mL, based on comparison with effective levels in people. We recognize that the accuracy of this estimate for dogs will have to be determined through pharmacodynamic studies, but this approach has been used in the past to estimate dosage regimens for other opioids (Kyles, et al, 1996). Plasma concentration requirements may be higher for dogs compared to people because there appears to be no M-6-G contributing to analgesia in dogs. Nevertheless, using a 20 ng/mL plasma concentration as a target, and performing computerized plasma concentration simulations of morphine from the pharmacokinetic

parameters determined in this study, we calculated a dose of 0.5 mg/kg every 2 hours, i.v. Analgesic studies employing this dosage should be designed to recognize the sedative and other behavioral effects that could confound subjective evaluation overestimating the length and degree of analgesia.

Different routes of intermittent parenteral administration are unlikely to effect the dosage recommendation, as previous studies have shown that plasma profiles of intravenous and intramuscular morphine administration overlap within 15 minutes (Dohoo, et al, 1994; Barnhart, et al, 2000). This is due to rapid absorption and almost complete bioavailability of morphine administered by a non-intravenous parenteral route.

Morphine is rapidly cleared from dogs following i.v. administration. Morphine - 6 – Glucuronide was not detected in any plasma sample. A morphine sulfate dosage of 0.5 mg/kg i.v. every 2 hours is predicted to maintain plasma concentrations consistent with analgesia in people. Morphine is poorly and erratically absorbed when administered orally, therefore is not a recommended route in dogs.

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TABLE 4.1. Calculated pharmacokinetic parameters (mean ± SE) following i.v. (0.5 mg/kg) and p.o. (1.59 ± 0.14 mg/kg) administration of morphine sulfate.

Variable	Value
Noncompartmental Parameters	
λ_z (1/hr)	0.68 ± 0.12
$t_{½}$ λ_z (hr)	1.16 ± 0.15
MRT (hr)	1.36 ± 0.19
Cl_T (ml/min/kg)	62.46 ± 10.44
Vd_{ss}(L/kg)	4.55 ± 0.17
Vd_{area}(L/kg)	5.62 ± 0.32
$AUC_{0-\infty}$ (hr*ng)/ml	112.84 ± 15.86
$AUC_{0-\infty}$ % extrapolated	12.60 ± 0.80
$AUMC_{0-\infty}$ (hr*hr*ng)/ml	168.24 ± 42.04
C_0 (ng/ml)	135.18 ± 6.71
Compartmental Parameters	
$t_{½\alpha}$ (hr)	0.14 ± 0.05
$t_{½\beta}$ (hr)	1.16 ± 0.27
α (1/hr)	10.68 ± 3.89
β (1/hr)	0.74 ± 0.13
A (ng/ml)	563.37 ± 409.40
B (ng/ml)	60.61 ± 10.00
K10 (1/hr)	4.22 ± 2.38
K12 (1/hr)	4.79 ± 1.81
K21 (1/hr)	2.41 ± 0.55
K10 $t_{½}$ (hr)	0.39 ± 0.11
V1 (L/kg)	1.82 ± 0.44
V2 (L/kg)	2.11 ± 0.33
% F	5.31

λ_z = first-order rate constant. $t_{½}$ λ_z = half-life of the terminal portion of the curve. MRT = mean residence time. Cl_T = total body clearance. Vd_{ss} = volume of distribution at steady state. Vd_{area} = volume of distribution of the area during the elimination phase. $AUC_{0-\infty}$ = area under the curve from 0 to infinity. $AUC_{0-\infty}$ % extrapolated = % of the area under the curve from 0 to infinity extrapolated from the last time point. $AUMC_{0-\infty}$ = area under the first moment curve from 0 to infinity. C_0 = Concentration at time 0. $t_{½\alpha}$ = distribution half-life. $t_{½\beta}$ = elimination half-life. α = rate constant associated with distribution. β = rate constant associated with elimination. A = intercept for the distribution phase. B = intercept for the elimination phase. K10 = elimination rate from compartment 1. K12 = rate of movement from compartment 1 to compartment 2. K21 = rate of movement from compartment 2 to compartment 1. K10 $t_{½}$ = half-life of the elimination phase. V1 = volume of compartment 1. V2 = volume of compartment 2. %F = pooled oral bioavailability.

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TABLE 4.2. Comparative pharmacokinetic values following morphine administration (i.v. bolus) to dogs.

Parameter	**KuKanich, *et. al.***	Hug, *et. al*., 1981	Jacqz, *et. al*., 1986	Dohoo, *et. al*., 1994	Dohoo, *et. al*., 1997	Barnhart, *et. al*., 2000
$T_{½}$	**1.16**	1.2	1.1	1.1	0.87	1.6
Vd_{ss}	**4.6**	6.1	1.5	4.1	3.6	7.2
Cl	**63**	57	51.5	41	57	85.2
$T_{½}$ = elimination half-life (hrs.). Vd_{ss} = volume of distribution at steady state (L/kg). Cl = total body clearance (mL/min/kg)						

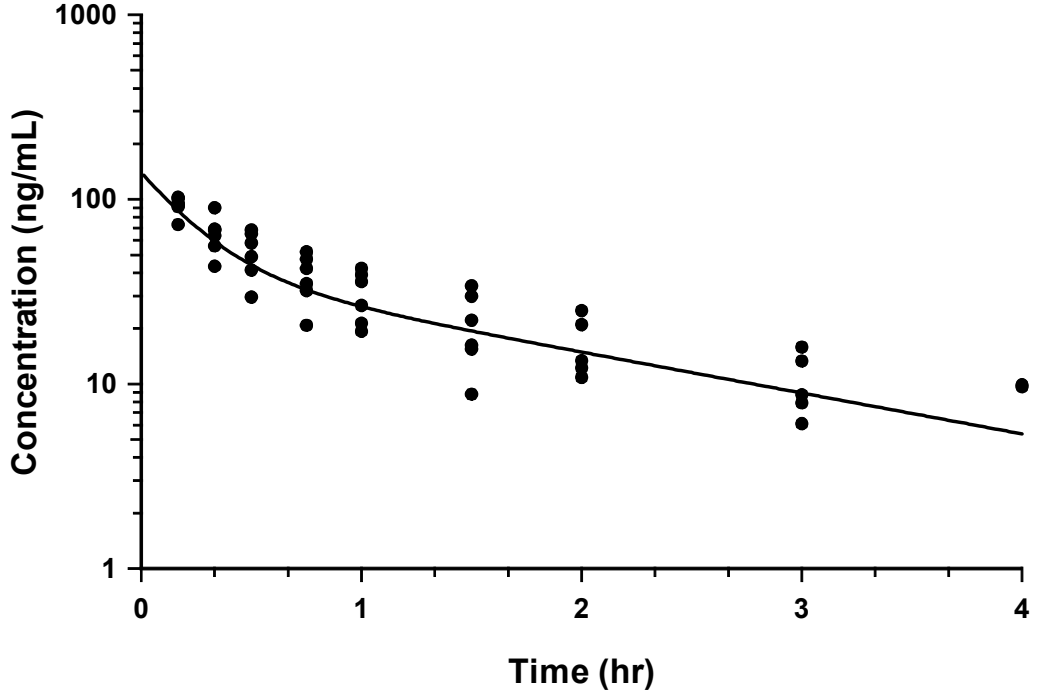

FIGURE 4.1. Plasma profile of morphine sulfate (0.5 mg/kg) administered as an i.v. bolus to 6 healthy dogs. Notice only 2/6 dogs were at or above the targeted concentration (20 ng/mL) at 2 hours. (•) Actual plasma concentrations measured for each dog. (—) Mean predicted plasma profile.

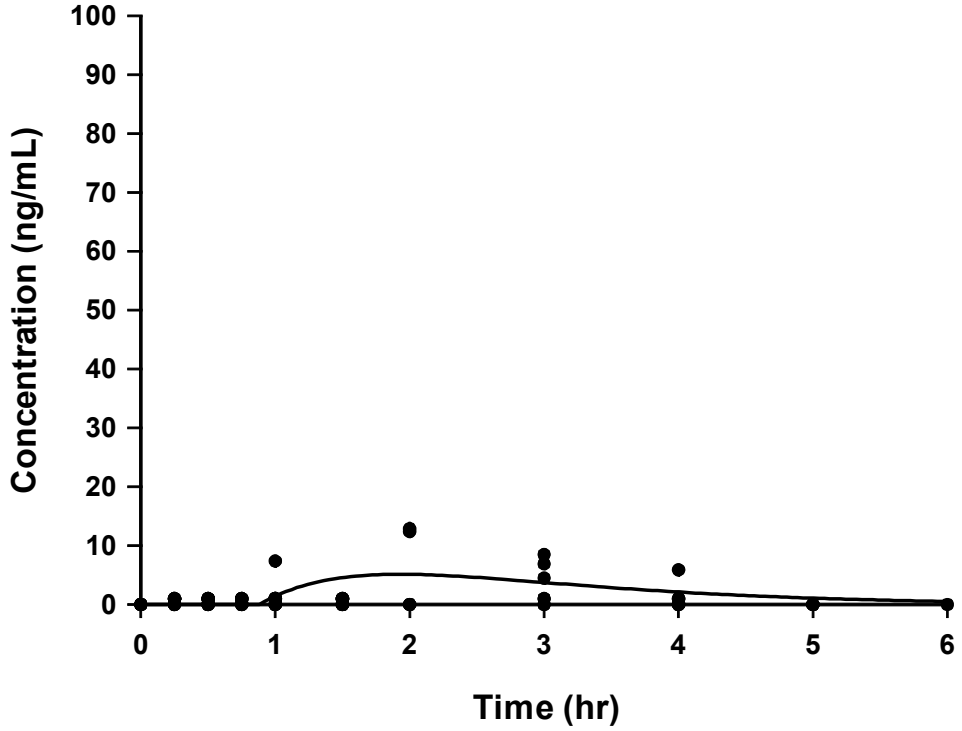

FIGURE 4.2. Plasma profile (n=5) of extended release morphine sulfate tablets (adjusted to 1.5 mg/kg) administered orally to healthy dogs. Note the y-axis is on a linear scale. (•) Actual plasma concentrations. (—) Mean predicted plasma profile.

EVALUATION OF A VON FREY DEVICE FOR PHARMACODYNAMIC MODELING OF MORPHINE IN DOGS.

RH: Pharmacodynamic Modeling of Morphine in Dogs.

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5.1 ABSTRACT

Objective - The purpose of this study was to evaluate a von Frey (vF) device as a mechanical nociceptive stimulus in dogs for pharmacokinetic and pharmacodynamic modeling of morphine. The vF device consisted of a rigid tip (0.5 mm diameter) and an electronic load cell.

Animals - Six healthy Beagle dogs were utilized in this study.

Procedure – Von Frey thresholds were measured in a control group (no treatment) and following intravenous morphine sulfate administration (treatment group) at 1 mg/kg. The operator was blinded to all measurements.

Results - The vF device was simple for the operator to apply, well tolerated by all dogs, and caused no apparent tissue damage. No significant changes ($p>0.05$) in thresholds were present in the control group for up to 8 hours, indicating a lack of acquired tolerance, learned aversion, or local hyperalgesia. The treatment group maintained significantly ($p<0.05$) elevated thresholds for 4 hours following morphine administration when assessed as a group. When assessed individually, the time for thresholds to decrease to values not significantly different from baseline was (mean ± SE) 2.8 ± 0.6 h. The maximal effect (E_{MAX}) was 213 ± 43 %, change from baseline values, and the plasma concentration to achieve 50% maximal effect (EC_{50}) was 13.92 ± 2.39 ng/mL.

Conclusions and clinical relevance - The vF device successfully evaluated the antinociceptive effect of morphine in dogs. The antinociceptive effects of morphine sulfate (1 mg/kg i.v.) last approximately 3-4 hours.

5.2 INTRODUCTION

Morphine is the prototypical opiate analgesic, which interacts with μ and κ opiate receptors to exert its analgesic effect. Despite numerous studies of morphine in dogs, the efficacy of morphine has not been objectively evaluated [1-13].

A number of studies have evaluated pharmacokinetic parameters following a single i.v. bolus administration of morphine to dogs and the results appear to be relatively consistent with morphine exhibiting a short elimination half-life (0.9 - 1.6 hrs), rapid clearance (41 - 85 ml/min/kg), and a large volume of distribution (3.6 - 7.2 L/kg) [1, 3-12]. Morphine-6-glucuronide (M6G) is a metabolite of morphine, which has been shown to have varying degrees of analgesic activity in people [14-16]. Previous studies have shown that dogs produce very low levels of M6G from the metabolism of morphine; therefore it is unlikely to contribute to analgesic effects following morphine administration [8,13].

There has not been a pharmacodynamic model to describe the relationship of morphine or M6G plasma concentrations to antinociceptive effects in dogs. The lack of dose titration data or pharmacokinetic-pharmacodynamic (PK-PD) evaluation of opiates in dogs is probably attributable to a lack of reliable, consistent, and humane outcome measures for evaluating antinociception or analgesia. Von Frey (vF) devices have been used to assess antinociceptive effects of drugs in the laboratory, generally in rodents, and also to assess the analgesic effects of drugs in human clinical patients [17-19]. The only reports of the use of a von Frey-like device in dogs has been from use of purpose-built devices, which were successfully used in studies of pre-emptive analgesia in dogs [20-22]. A device producing a mechanical stimulus, such as the vF device, stimulates both Aδ and c fiber nociceptors, which are also responsible for encoding clinical pain [23]. Opiates

primarily act on c fibers, with only very high doses acting on Aδ fiber input to the spinal cord to produce clinical analgesia [23]. A vF device consists of a tip that is applied to the skin surface with gradually increasing force to create a noxious stimulus. Fine hairs are used in some vF devices that bend at a maximum force, and larger hairs are used to re-test, until the subject reacts prior to the hair bending. In electronic devices, a rigid tip is used and the force that is applied is measured using an integrated load cell. In people, the endpoint of the reaction is determined from a verbal communication that the stimulus is perceived as noxious. The endpoint is reached in animals when the subject reacts or withdraws.

The purpose of this study was to assess a custom built vF mechanical pressure device to evaluate opiate antinociceptive effects in dogs. Our hypotheses were: 1. repeated use of the vF device would not produce changes in thresholds over time in a control group, 2. vF thresholds would increase following a bolus of morphine, and 3. vF thresholds could be used to develop a pharmacodynamic model to relate plasma morphine concentrations to changes in nociceptive thresholds.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Six healthy Beagle dogs (3 male and 3 female) between the ages of 4 and 5 years were used for this study. No previous studies utilizing a nociceptive stimulus were conducted with these dogs. Dogs were housed in runs prior to the study and fed a commercial dog food [a]. The study was conducted on groups of two animals per day in a crossover design with at least a week separation between each phase of the study. Experimental measurements were performed in an isolated, noise-free, and temperature-controlled room, with artificial lighting to minimize distractions and day-to-day variability. The North Carolina State University Institutional Animal Care and Use Committee approved the study.

5.3.2 Von Frey device

The von Frey device [b] was built to requested specifications and consisted of a load cell, handle, recording device, and tip (figure 5.1). The tip was custom built from a 0.5 mm diameter polypropylene tip [b], which was filled solid with an epoxy putty [c]. The 0.5 mm tip was then placed inside the proximal part of a 1000 μL, pipette tip [d], and filled with epoxy putty to increase the rigidity. The diameter of the tip and surface area of pressure applied was consistent throughout each study phase. The load cell was calibrated from 100 to 1000 grams. The recording device recorded the maximum weight applied to the tip. The operator controlled the rate of application and was trained in constant rate of application.

5.3.3 Von Frey threshold testing

Von Frey threshold measurements were made by the same operator (BK), who was blinded to the actual readings obtained (another operator recorded the thresholds). All measurements were made in standing dogs with minimal restraint. Pressure was exerted until a withdrawal / escape movement was made, vocalization occurred, or the maximum weight (1000 g) was applied. If maximum weight was applied the operator was notified to stop, in order to reduce the risk of tissue injury and the weight recorded as 1000 g. A simple withdrawal reflex upon first touch of the tip to the footpad was not accepted as an endpoint.

Initially, various parts of the body were assessed for ability to produce consistent results. Areas assessed included the tibial tuberosity, dorsal spinal processes, tail base, pinna, metatarsal bones, olecranon, and the spine of the scapula. Additionally, a 3 mm diameter tip was assessed, but weights applied to untreated dogs exceeded 1000 g. The most consistent results were obtained by testing the carpal pad (see results), and all subsequent experiments were performed using the carpal pads and a 0.5 mm tip.

Visual examination for tissue damage caused by pressure from the vF device was performed during each phase of the study. The carpal pads were examined for redness, swelling, pain upon palpation, bleeding, and exudate during the testing and for 24 hours after the testing was complete. Additionally, the dogs' gait was assessed for signs of lameness at the conclusion of the testing period and 24 hours after the completion of each crossover.

At each time point, three readings each were taken from the left and right leg. The vF thresholds were expressed as a percent change from baseline. Baseline values for the

control group were established as the time 0 measurements. All subsequent values were expressed as a percent change from time 0 measurements. The 1 mg/kg i.v. morphine sulfate dose group had baseline values taken prior to drug administration (time 0), and hourly measurements were expressed as percent change from baseline. All percent change values were calculated for the individual dog, and then pooled for group values.

5.3.4 Study design

A two-way crossover study consisted of a control and a treatment group. The control group received no medication (or injections) and consisted of hourly vF threshold measurements, 3 per leg (left and right) conducted on the carpal footpad, repeated hourly for eight hours. The treatment group consisted of a 1 mg/kg i.v. bolus of morphine sulfate [e] with hourly vF threshold measurements beginning at time 0 (prior to drug administration), and hourly for 4 hours, obtained in an identical manner as the control group. All dogs were subjectively assessed for sedation.

Morphine sulfate, 1 mg/kg (0.75 mg/kg base), was administered as an i.v. injection into the cephalic vein, as a bolus over 15 seconds. Blood samples (7 mL) were collected via jugular venipuncture prior to morphine administration and hourly, following the vF threshold measurement. Blood samples were collected into evacuated glass tubes containing lithium heparin [f], placed on ice, centrifuged at 1000 g for 10 min, and plasma was separated and stored frozen at -70° C. All plasma samples were analyzed within 30 days of collection.

5.3.5 Plasma drug concentrations

Plasma samples were analyzed for morphine and M6G via high-pressure liquid chromatography (HPLC) method with electrochemical coulometric detection. The HPLC system consisted of a pump [g], degasser [g], autosampler [g], and electrochemical coulometric detector [h]. A 4.6 x 150 mm, 5 - micron, phenyl column [i] maintained at 40° C was used to achieve separation. The mobile phase consisted of 95% 0.01 M acetate buffer [j] with 0.1% triethylamine [j], and 5% acetonitrile [j] with the pH adjusted to 4.5 with glacial acetic acid [j]. A gradient was programmed in the following manner: 0 - 8 minutes, 100% mobile phase, 8 – 11 minutes ramped to 85% mobile phase and 15% acetonitrile, 11 – 14 minutes ramped to 100% mobile phase, then 14 – 20 minutes 100 % mobile phase. Retention times for M6G and morphine were 5.5 and 7.5 minutes, respectively. The detector settings included: guard cell +750 mV, cell 1 +300 mV, cell 2 +450 mV, with cell 2 being quantified by computer software [k].

Plasma (1 mL) was treated with 1 mL 0.2 M borate buffer [j], pH 9.0, vortexed, and then treated with 0.4 mL 0.1 M 1-pentanesulfonic acid [j], and vortexed again. Solid phase extraction cartridges [l] were conditioned with 1 mL HPLC grade water and 1 mL 100% methanol [j], the plasma mixture (2.4mL) was loaded onto the extraction cartridges, washed with 1 mL HPLC grade water, and eluted with 1 mL 100% methanol. The eluate was evaporated under a nitrogen stream at 40° C and reconstituted with 0.2 mL mobile phase. The injection volume was 0.05 mL.

Calibration curves were made daily by fortifying pooled canine plasma with known amounts of morphine HCL [m] and M6G [m], handled in the same manner as the incurred samples. Calibration curves were accepted if the coefficient of determination (r^2)

was greater than 0.99 and the measured values were within 15% of the actual values, except the highest and lowest points, which were within 20% [24]. Controls consisting of fortified canine plasma were assessed at the beginning of the day, intermittently during the day, and the end of the day, with that day's run accepted if the controls were within 15% of the actual value.

5.3.6 Statistical analysis and pharmacokinetic & pharmacodynamic modeling

Statistical analysis was conducted using a computer software program [n] with a significance level $p < 0.05$. The Mann-Whitney Ranks sum test was used to evaluate differences between baseline thresholds in the control group and the treatment group. The Kruskal - Wallis analysis of variance was used to evaluate differences across time points within each group, as the variances were not equal. If differences were found, the Dunn's test for multiple comparisons to control (time 0) was used to discriminate the time points that were significantly different. Pharmacokinetic and pharmacodynamic modeling was conducted with a computer program [o]. Modeling was conducted in a standard two-stage design. Pharmacodynamic modeling calculated a concentration - effect relationship expressed by the equation:

$$E = \frac{E_{MAX} \bullet C}{C + EC_{50}}$$

where E is the effect (% change in von Frey threshold), E_{MAX} is the maximum effect, C is the concentration of morphine (ng/mL), and EC_{50} is the concentration of morphine which elicits a 50% maximal response.

5.4 RESULTS

The use of the vF pressure device was well tolerated by all dogs. No evidence of a learned behavior or aversion to its use was noted. Thresholds measured at the tibial tuberosity, dorsal spinal processes, tail base, pinna, metatarsal bones, olecranon, and the spine of the scapula were either variable or exceeded the maximum pressures with no treatment. For example, the baseline vF threshold (mean ± SE) at the metatarsals was 442.1 ± 34.3 g. The most consistent results were obtained at the carpal pad. The baseline vF thresholds at the carpal pad were 185.0 ± 4.1 g and 209.0 ± 12.9 g for the control group and treatment group, respectively. These values were not significantly different. There was no significant difference between the left and right footpad, therefore they were pooled for analysis. Von Frey thresholds did not vary significantly over time in the control group, either individually or as a group (figure 5.3).

The most common response to application of the vF device to the carpal pad was active withdrawal of the leg, however one dog would occasionally vocalize. The maximum weight applied (1000 g) was reached in three of the dogs in the morphine treatment group at the 1 hour time point. One of the dogs in the morphine treatment group also reached the maximum weight at 2 hours. None of the dogs reached the maximum weight at 3 or 4 hours. There was no evidence of tissue injury or lameness at any time for any of the dogs. Transient dimples were present in the footpads after the measurements, but disappeared prior to the next hourly measurement.

Following i.v. administration of morphine, all of the dogs vocalized briefly, approximately 30 seconds after the injection was completed. None of the dogs vomited, but all were sedated for 7 to 12 hours following morphine administration. The vF

thresholds were increased significantly from baseline during the testing period in the morphine treatment group, when assessed as a group (figure 5.4). However, when examined individually, the time for thresholds to decrease to values not significantly different from baseline was 2.8 ± 0.6 h. One of the dogs had vF thresholds that were not statistically different from baseline values at the 3 hr time point and was not measured at 4 hr due to a timing error. None of the dogs' vF thresholds, when assessed individually, were significantly elevated above baseline at 4 hours.

The limit of quantification (LOQ) for the M6G and morphine assay was 25 and 5 ng/mL, respectively, as defined by the lowest point on the calibration curve within 20% of the actual value. The accuracy (deviation from actual value) and coefficient of variation were 8% ± 2% and 3% ± 1% for the morphine assay and were calculated from equations published elsewhere [24].

Morphine-6-glucuronide was not detected in any sample. The mean ± SE morphine plasma elimination half-life was 0.88 ± 0.13 hr. (figure 5.5). The calculated mean ± SE for E_{MAX} and EC_{50} were 213 ± 43 % and 13.92 ± 2.39 ng/mL, respectively (figure 5.6).

5.5 DISCUSSION

Despite the common clinical and experimental administration of morphine to dogs, the dose response or the relationship between plasma concentrations and effect has never been objectively assessed. Morphine (0.5 mg/kg, unstated route of administration) administered to two dogs showed a 10% or greater increase over baselines in radiant heat thermal thresholds over a one hour period [25]. However the sample size was small, statistical analysis was not performed, and baseline variability was not stated. The responses to two doses of morphine (0.25 and 0.5mg/kg intravenously) were determined by measuring changes in hind limb reflexes in dogs [26]. Significant depression of the hind limb reflex was found over the 5 hour experimental period, and it was greater for the higher dose of morphine, however the experiments were performed in dogs with surgically severed spinal cords, and the relationship to antinociception is unclear.

Morphine at two doses (0.5 mg/kg i.v. and 1 mg/kg i.m.) did not result in significantly increased mechanical (pneumatic pressure) or thermal (incandescent bulb) thresholds, suggesting that either the dose / route combinations did not alter nociception, or the model does not predict the antinociceptive effects of morphine [1]. The authors suggested that the lack of response might be attributed to the model and study design, which precluded any pharmacokinetic-pharmacodynamic modeling [1]. Following i.v. morphine administration (0.1 mg/kg) to dogs under anesthesia (thiopental, 4 mg/kg to effect and isoflurane 1.3%), a significantly decreased response to electrical stimulation of the tooth pulp (dental dolorimetry) was exhibited for a 60 minute test period compared to saline controls [2]. However only one dose was evaluated, plasma concentrations of

morphine were not measured, and anesthetic influences were likely contributing to the effects of morphine.

Morphine was assessed as a constant rate infusion in postoperative patients by use of the University of Melbourne Pain Scale (UMPS) and compared to intramuscular morphine sulfate at 1 mg/kg i.m. [11]. No differences between the groups were found. However 9 of the 10 categories evaluated by the UMPS are affected by the pharmacology of morphine (pupil diameter, heart rate, respiratory rate, rectal temperature, salivation, sedation, mental status, posture, and vocalization) that can be independent of the antinociceptive effects. Additionally, no control (untreated) animals were used due to humane reasons. In the study reported here, the dogs had small pupil diameters (<66% of total diameter), low heart rates (<100 beats per minute), low respiratory rates (<20 breaths per minute), salivated, and appeared sedated and depressed for 7 – 12 hours following administration of morphine. Postures varied from apparently resting comfortably to sitting hunched in the back corner of the kennel, and all vocalized following the morphine injection. The incorporation of such parameters into 'pain' assessment scales used in studies to assess various doses of morphine by various routes, make it difficult to determine what dose of morphine is effective in the dog [27-32].

One reason for the lack of reliable data on the antinociceptive activity of morphine in dogs is the lack of a reliable outcome measure of antinociception. The vF device used for this study was custom built for dogs and is commercially available [b]. The carpal footpads provided the most consistent results for the control group. The metatarsals, as well as other locations, were assessed as a control group, but resulted in higher baseline values as compared to the carpal footpads. The expected increase in vF

thresholds was 150 to 200% which would have exceeded the load cell of the vF device for the metatarsals. Additionally, the standard error was higher for the metatarsals; therefore the carpal footpads were assessed for all phases of the study. Tissue damage was not observed in any of the dogs during or after either crossover. There was no evidence of hyperaesthesia or tolerance in the control group. None of the dogs displayed signs of lameness at any point prior to, during, or after the study was completed.

The vF device was technically simple to use, portable, and caused no apparent tissue damage, which are traits desirable for assessment of antinociceptive effects in a laboratory or clinical setting. In the control group, there was no evidence over an 8 hour period of changes in thresholds due to tolerance, learned avoidance, or local hyperaesthesia. Furthermore, the increased thresholds following morphine administration indicate that the vF device was able to discriminate the antinociceptive effects of morphine. Additionally, sedation (lasting up to 12 hours in some dogs), which interferes with subjective assessment scales, outlasted the measured antinociceptive effects of morphine, suggesting the vF device is able to discriminate the antinociceptive effect from the sedative effect of morphine. The treatment group was not carried out for 8 hours, as for the control group, since all dogs had returned to baseline by 4 hours. The vF device used in this study fulfils the criteria laid down by Beecher for an ideal method of producing painful stimuli [33]. As vF devices activate the same nerves transmitting clinical pain, increased vF thresholds may be indicative of clinical analgesia [23]. However, the level of increased thresholds that relate to clinically useful analgesia is not known.

The plasma profile and elimination half-life, 0.88 ± 0.13 hrs, of morphine following a 1 mg/kg dose (0.75 mg/kg base) was similar to previous studies in dogs

[1,3,4,6,9,10]. The clearance and volume of distribution were not calculated because they would have been overestimated since early time points representing the distribution phase were not collected. Morphine-6-glucuronide was not detected in any plasma sample at any time point following morphine administration (LOQ 25 ng/mL). In people, M6G is a major metabolite and may contribute up to 66% of the antinociceptive effect following parenteral administration of morphine [14-16]. It is unlikely M6G contributes to the antinociceptive effects of morphine in dogs due to the low (undetectable) concentrations produced in this study, which is consistent with previous studies [8,13]. The lack of M6G contributing to the antinociceptive effects of morphine in dogs may result in higher concentrations of morphine needed to produce similar effects as compared to people.

In people, morphine plasma concentrations have been correlated to the analgesic effect. The reported effective concentrations range from 9.1 to 40 ng/mL, in post-operative patients assessed by subjective scoring systems [16, 34-38]. In this study, the calculated value for the EC_{50} of morphine, 13.92 ± 2.39 ng/mL, is similar to plasma concentrations associated with analgesia in people.

In dogs, there is a lag phase for morphine to diffuse into the cerebrospinal fluid (CSF) following an i.v. bolus dose [9]. These investigators also showed a longer elimination half-life (mean ± SE) from the CSF (121.0 ± 5.6 min) as compared to the plasma (65.4 ± 23.4 min) [9]. Although CSF drug concentrations do not represent brain or spinal cord concentrations, the results may suggest a slower elimination from the central nervous system, compared to plasma, indicating that plasma concentrations of morphine should be used only as a guide to effective levels unless steady state is reached [39]. Studies in which morphine is administered as a constant rate infusion to steady state to

assess the pharmacodynamic effects during equilibrium between the brain and plasma drug concentrations are warranted, and the vF device may allow such studies to be performed in dogs.

The von Frey device was well tolerated and can be utilized to assess the antinociceptive effects of morphine given intravenously. Using this device, pharmacodynamic modeling of a single dose of morphine was possible, and it was found that morphine when given as a single i.v. bolus dose yields an EC_{50} of 13.92 ± 2.39 ng/mL. Further studies assessing the cumulative effects of morphine administered as a constant rate infusion or multiple dosing of morphine are warranted.

5.6 FOOTNOTES

[a] Hill's d/d or Hill's maintenance, Hill's Pet Nutrition, Inc., Topeka, KS

[b] Model 2290-4 (modified), IITC Life Science, Woodland Hills, CA

[c] Fast Steel, Polymeric Systems, Inc., Phoenixville, PA

[d] MLA, VistaLab Technologies, Mt. Kisco, NY

[e] Morphine sulfate, Baxter Healthcare, Deerfield, IL

[f] BD Vacutainer, Franklin Lakes, NJ

[g] Agilent 1100 series, Agilent technologies, Wilmington, DE

[h] ESA, Coulochem II, Bedford, MA

[i] Zorbax SB-phenyl, Agilent technologies, Wilmington, DE

[j] Fischer Scientific, Fair Lawn, NJ

[k] HP ChemStation Rev A.06.03, Agilent technologies, Wilmington, DE

[l] Varian C-8, Varian Inc. Palo Alto, CA

[m] Lipomed, Inc., Cambridge, MA

[n] Sigma Stat 3.0, Systat Software, Inc., Point Richmond, CA

[o] WinNonlin, 4.0, Pharsight Corporation, Mountain View, CA

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FIGURE 5.1. Von Frey device model 2290-4 shown with the load cell built into the handle and the actual tip used in the study.

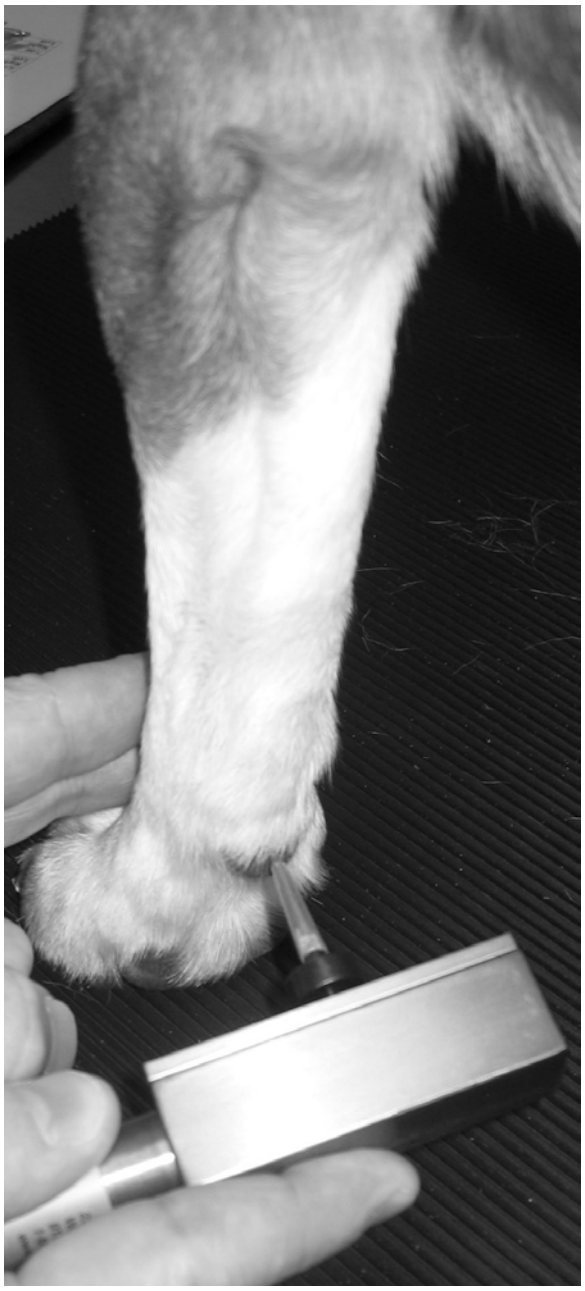

FIGURE 5.2. Demonstration of the location and method of von Frey application.

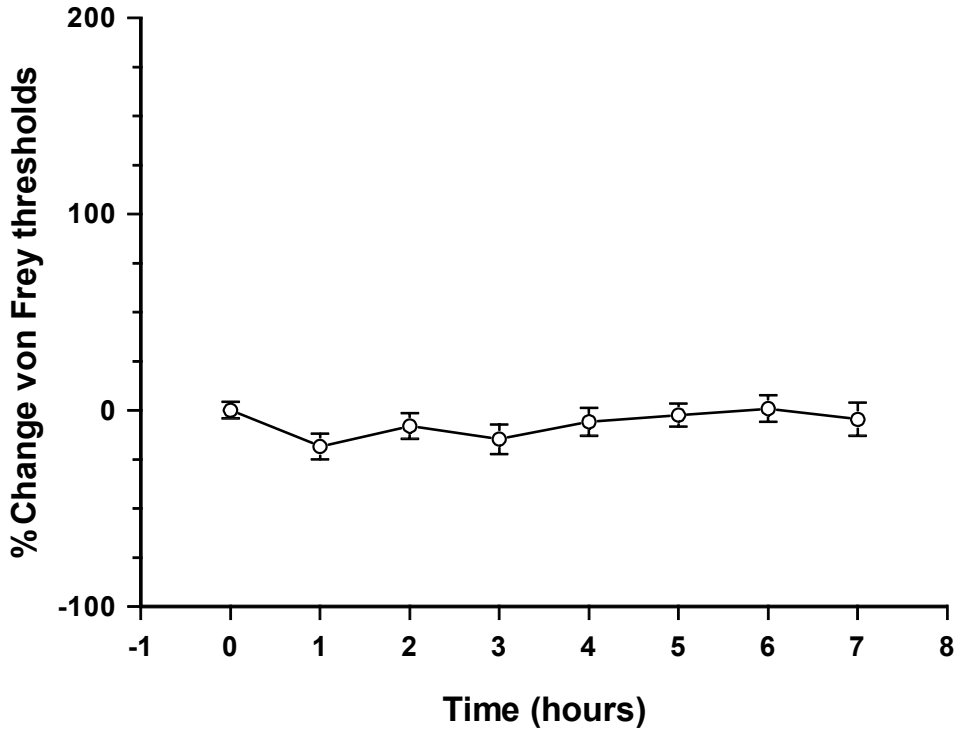

FIGURE 5.3. Carpal pad von Frey thresholds in control animals, percent change from time 0, in 6 dogs expressed as mean ± SE. There was no significant difference at any time point.

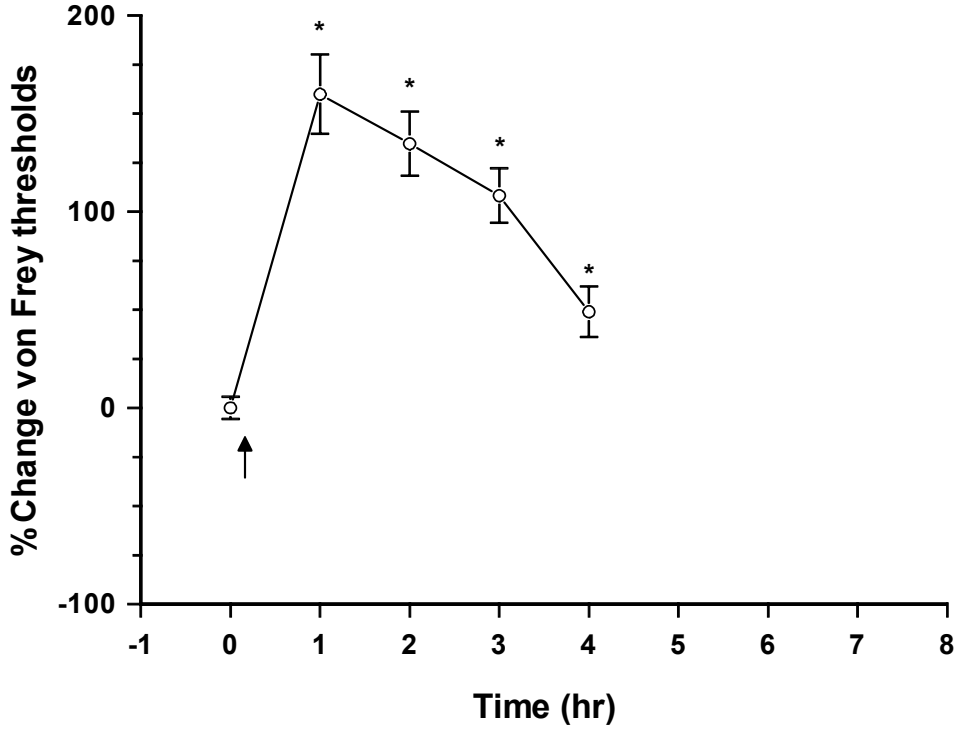

FIGURE 5.4. Von Frey thresholds, percent change from baseline (time 0), in 6 dogs expressed as mean ± SE, dosed with morphine sulfate 1 mg/kg i.v. ↑ morphine administration. * $p < 0.05$ compared to baseline.

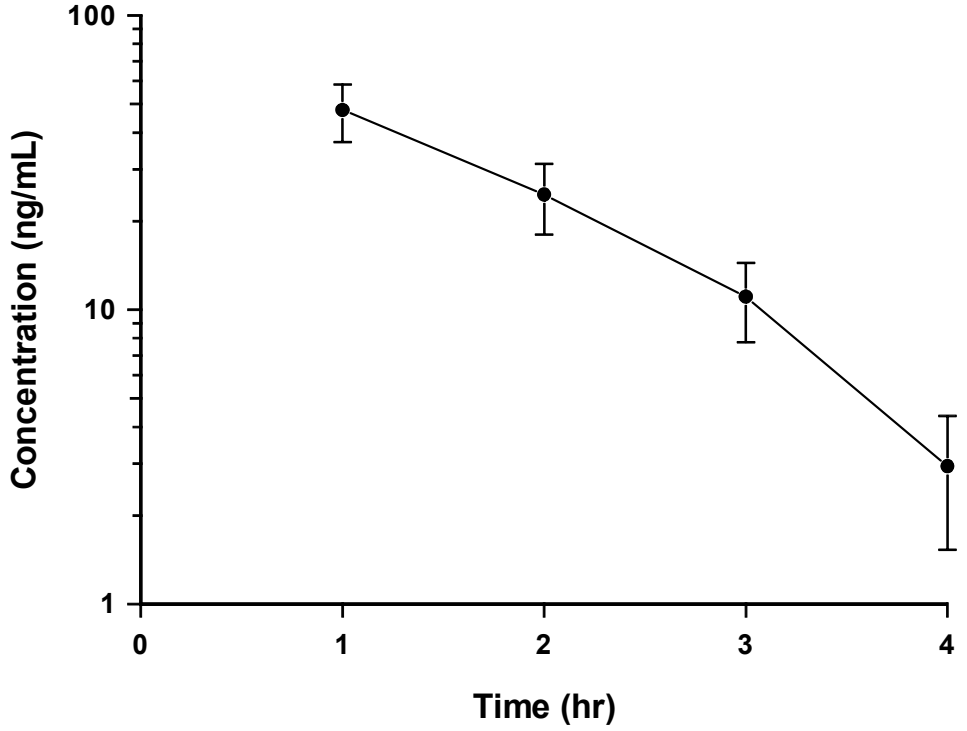

FIGURE 5.5. Mean ± SE plasma concentration vs. time following 1 mg/kg morphine sulfate administered as an i.v. bolus to six healthy dogs.

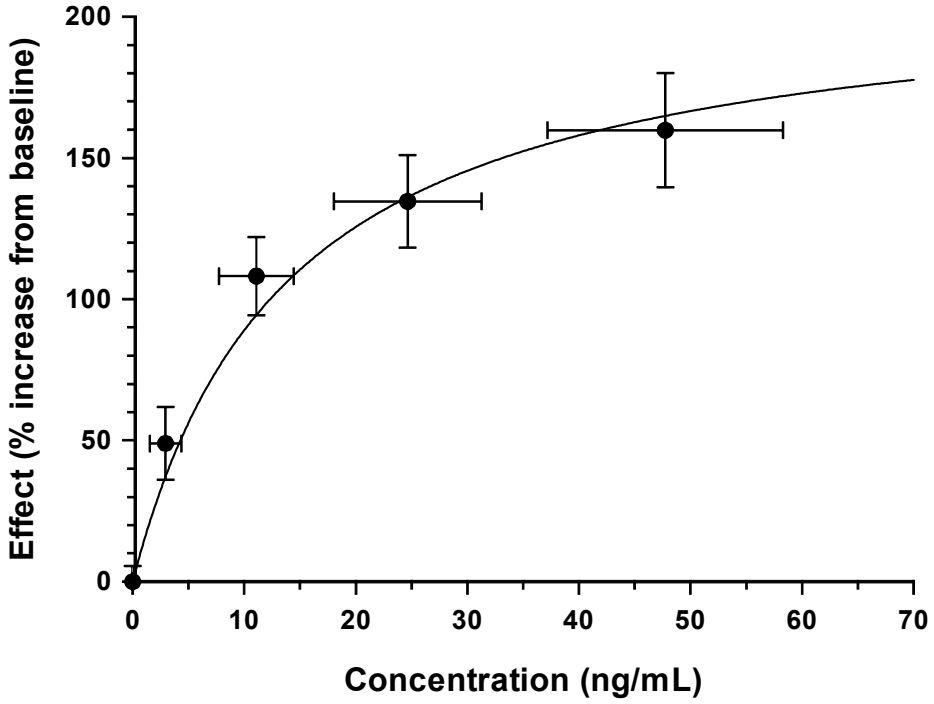

FIGURE 5.6. Pharmacodynamic model of morphine sulfate administered 1 mg/kg as an i.v. bolus to 6 dogs. The calculated values for E_{MAX} and EC_{50} were 213 ± 43 % and 13.92 ± 2.39 ng/mL, respectively. Values are expressed as mean ± SE.

PHARMACOKINETICS AND PHARMACODYNAMICS OF MORPHINE IN DOGS FOLLOWING INTRAVENOUS INFUSION AND MULTIPLE INTRAVENOUS DOSES

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6.1 ABSTRACT

Objective - The purpose of this study was to evaluate the pharmacokinetics of morphine following i.v. infusion and multiple i.v. doses, and the pharmacodynamics using a device to produce a nociceptive stimulus.

Animals - Six dogs were utilized in this study.

Procedure – A three way crossover consisting of a tiered morphine infusion targeting plasma concentrations of 10, 20, 30, and 40 ng/mL, a saline infusion, and three doses of morphine sulfate, 0.5 mg/kg i.v every 2 hr. Blinded von Frey (vF) thresholds were measured hourly for eight hours. Morphine plasma concentrations were measured by high-pressure liquid chromatography

Results - The vF device was well tolerated and caused no tissue damage. No significant changes ($p>0.05$) in thresholds were observed from the saline infusion. The morphine infusion produced significantly elevated vF thresholds ($p<0.05$) from 5 to 8 hrs, corresponding to targeted morphine plasma concentrations >30 ng/mL and a 0.15±0.02 mg/kg•hr infusion rate. Plasma morphine concentrations were variable during the infusion. The maximal effect (E_{MAX}) was 78±11% (change from saline baseline) and the plasma concentration to achieve 50% maximal effect (EC_{50}) was 29.5±5.4 ng/mL. Thresholds were significantly elevated from 1–7 hrs during the multiple dose phase with the E_{MAX} and EC_{50} being 180±67% and 27.3±7.4 ng/mL, respectively. No differences in half-life, volume of distribution, or clearance between the first and last dose were present.

Conclusions and clinical relevance – Intravenous morphine infusion (0.15±0.02 mg/kg•hr) and multiple i.v. morphine doses (0.5 mg/kg) every 2 hours maintained significant antinociceptive effects.

6.2 INTRODUCTION

Morphine has been frequently recommended as an analgesic in dogs (Thurman, et al, 1996; Carrol, 1999; Wagner, 2002). In addition to morphine's antinociceptive effects, other effects such as sedation, miosis, dysphoria, panting, bradycardia, salivation, hypothermia, vomiting, and constipation have been reported (Thurman, et al, 1996; Carrol, 1999; Wagner, 2002; KuKanich, et al 2005a, KuKanich, et al 2005b). Assessing morphine as an analgesic has been difficult. The accuracy in assessing nociception and the antinociceptive effects of opioids has been complicated by the side effects and the difficulty and individual variability in subjective visual scoring systems, such as Visual Analogue Scales (VAS) and Numerical Rating Scales (NRS) (Grisneaux, et al, 1999; Reese, et al, 2000; Lemke, et al, 2002).

A study utilizing a von Frey (vF) device conducted in this laboratory evaluated the antinociceptive effects (pharmacodynamics) following a single i.v. bolus of morphine sulfate (1 mg/kg) (KuKanich, et al, 2005a). The device was well tolerated, with no evidence of tissue damage, learned behavior, or aversion by the dogs. Antinociceptive thresholds measured in untreated animals were consistent and repeatable, and did not vary with time. Significant elevations in thresholds were observed following administration of morphine. The calculated effective concentration to elicit a 50% maximal response (EC_{50}) was 13.92 ± 2.39 ng/mL (KuKanich, et al, 2005a). By comparison, plasma morphine concentrations in people associated with analgesia have ranged from 9.1 to 40 ng/mL in postoperative patients and experimental subjects (Skarke, 2003; Gourlay, 1986; Graves, 1985; Dahlstrom, 1982; Sarton, 2000; Eisenach, 1989).

The purpose of this study was to: 1. evaluate the antinociceptive effects using vF threshold measurements during morphine intravenous infusion, 2. evaluate the antinociceptive effects using vF threshold measurements when administered morphine as multiple i.v. boluses. 3. evaluate pharmacokinetic parameters from the first and last dose during multiple i.v. doses for differences. Even though the target organs for the antinociceptive effect of morphine are primarily the spinal cord and brain (CNS), plasma morphine concentrations were assessed as a surrogate marker for CNS concentrations, as plasma is more readily and humanely sampled than CNS tissue.

6.3 Materials and Methods

6.3.1 Animals

Six healthy Beagle dogs (3 male, 3 female) aged between 4 - 5 years and weighing 7 - 15 kg were used in the study. The animals were housed in runs except for the study period when they were housed in an isolated, noise-free, and temperature controlled room. The Institutional Animal Care and Use Committee at North Carolina State University approved the study.

6.3.2 Pharmacodynamic Measurements

Pharmacodynamic (PD) measurements, vF thresholds, were measured hourly with a custom built vF device (Model 2290-4 (modified), IITC Life Science, Woodland Hills, CA). The vF device consisted of a 0.5 mm diameter solid tip, an electronic load cell calibrated from 100 to 1000 g, and a recording device as previously described, (KuKanich, et al 2005a). The operator (BK) of the vF device was blinded to the actual readings and an assistant recorded the measurements and stopped procedure if thresholds reached the maximum (1,000 g). Von Frey threshold measurements were recorded from each of the six dogs. During the procedure they kept a standing posture and the measurement was repeated 3 times on each carpal pad, for a total of 6 measurements per hour, with at least 1 minute between the replicates. Pressure was applied to the carpal pad, through the tip of the vF device, until a withdrawal / escape movement occurred or the dog vocalized. Pressure was discontinued if maximum pressure was applied 5093 kg/m^2 (equivalent to 1000 g reading on the load cell) was attained. A previous study demonstrated that this maximal pressure did not cause tissue injury to the carpal pads of

dogs (KuKanich, et al 2005a). A simple withdrawal reflex upon the tip first making contact with the carpal pad, indicative of a tactile stimulus, was not considered an endpoint. Following each vF measurement and for 24 hours after the end of the study, the foot pads were examined for redness, swelling, bleeding or exudate, and pain upon palpation. Additionally, the dogs were assessed for lameness during the study and for 24 hours following the completion of the study.

6.3.3 Study Design

A three-way crossover design was utilized. The first two crossovers consisted of a blinded and randomized design with administration of morphine or saline as an i.v. infusion. Loading doses and infusion rates were calculated to achieve targeted morphine plasma concentrations of 10 ng/mL from 0 - 2 hrs, 20 ng/mL from 2 - 4 hrs, 30 ng/mL from 4 - 6 hrs, and 40 ng/mL from 6 - 8 hrs, and were calculated from the individual dog's pharmacokinetic parameters (KuKanich, et al, 2005b). Infusion rates were 15 mL/hr for the first 2 hours, and then increased by 15 ml/hr every 2 hours to a final infusion rate of 60 mL/hr. Morphine sulfate (Baxter Healthcare, Deerfield, IL) infusion was administered mixed with 500 mL 0.9% saline solution. The fluid bags were covered with black plastic to protect them from light. Infusion pumps were calibrated prior to use. A loading dose was administered prior to increasing the infusion rate at times 0, 2, 4, and 6 hrs, following vF thresholds. Blood was collected for plasma morphine measurement during each timed interval. The saline group was treated identically to the morphine group with the exception that morphine was not administered. Von Frey thresholds were taken hourly starting at 1 hour and finishing at 8 hours. Jugular catheters, 2 per dog, were

placed prior to the study and identified as either sampling or infusion. Blood samples, 7 mL per time point from the sampling catheter, were taken prior to the infusion and hourly from 1 to 10 hours. Sampling catheters were flushed with 3 mL 0.9% saline to maintain patency following blood sampling.

The third crossover consisted of multiple i.v. doses of morphine. Morphine sulfate, 0.5 mg/kg i.v., was administered through an aseptically placed jugular or saphenous catheter which was then flushed with 10 mL sterile 0.9% saline solution. Morphine sulfate was administered every 2 hours for 3 doses. Von Frey thresholds were measured prior to drug administration and hourly for 7 hours for a total of 8 measurements. Von Frey thresholds were measured prior to the morphine sulfate administration if a dose was scheduled at the same time point. Blood samples, 5 ml per time point, were collected through an aseptically placed jugular catheter, dedicated to sampling only, and rinsed with an equal volume of saline solution following collection. Blood samples were collected prior to dosing (time 0) at times (hh:mm) 0:05, 0:10, 0:15, 01:00, 01:30, 02:00, 02:05, 03:00, 04:00, 04:05, 04:10, 04:15, 05:00, 05:30, 06:00, and 07:00. Blood samples were collected prior to morphine sulfate administration if during an hour when a dose of morphine was administered. The packed cell volume (PCV) and total solids (TS) were measured in 4 of the dogs to assess the changes associated with sample collection by a microcard reader and refractometer. The refractometer was calibrated with distilled water prior to use. Because one dog was difficult to restrain, it was sedated with i.m. (epaxial) medetomidine (0.75 mg/m^2) and subsequently reversed with i.m. (epaxial) atipamezole (3.75 mg/m^2), injected with a tuberculin syringe with a 25 g, 5/8” needle, 18 hours prior to the study to place jugular catheters.

6.3.4 Statistical Analysis

Statistical analysis was performed with a computer program (Sigma Stat 3.0, Systat Software, Inc., Point Richmond, CA). The significance level was $p < 0.05$ for all statistical analyses.

Saline infusion vF thresholds

The vF thresholds for each dog during the saline infusion were expressed as a percent change from the baseline vF thresholds by dividing the actual vF threshold by the mean saline baseline threshold. Each time point was then expressed as pooled data for all six dogs. The percent change vF thresholds from the saline infusion group and the time 0 multiple dose group, inclusive, were evaluated for differences with a Kruskal - Wallis analysis of variance (K-W ANOVA).

Morphine vF Thresholds

The morphine infusion and multiple dose vF thresholds for each dog were expressed as a percent change from the saline baseline vF thresholds as described previously. Each time point was then pooled for all six dogs. The pooled morphine vF thresholds from each method of administration were compared to the corresponding time point in the saline infusion using the Mann - Whitney Rank sum test.

Pharmacokinetics

Plasma concentrations (maximum and minimum) after multiple bolus doses were compared for differences using a K-W ANOVA. The Area Under the Curve (AUC), clearance (total body), volume of distribution (area method), and elimination half-life for the first and third (last) dose of the multiple dose phase were compared for differences with the Mann - Whitney Rank Sum test.

Packed cell volume and total solids - multiple dose phase

The packed cell volume (PCV) and total solids (TS) after the first blood sample (time 0) and the last blood sample (time 07:00) were compared for differences during the multiple dose phase with a paired t-test.

6.3.5 Plasma Drug Concentrations

Plasma drug concentrations were determined by high-pressure liquid chromatography (HPLC) with electrochemical coulometric detection. Morphine and morphine-6-glucuronide (M6G) plasma concentrations were measured by a previously published method with a 5 and 25 ng/mL limit of quantification (LOQ), respectively (KuKanich, et al 2005a).

6.3.6 Pharmacokinetic and Pharmacodynamic Modeling

Pharmacokinetic and pharmacodynamic modeling were performed using a computer program (WinNonlin, 4.0, Pharsight Corporation, Mountain View, CA). The pharmacokinetics were calculated with noncompartmental modeling from equations published elsewhere (Gibaldi & Perrier, 1982). The area under the curve (AUC) for first

and third dose was calculated with the log-linear trapezoidal rule. Pharmacodynamic modeling during the infusion phase calculated a concentration - effect relationship expressed by the equation:

$$E = \frac{E_{MAX} \bullet C^{\gamma}}{C + EC_{50}{}^{\gamma}}$$

where E is the effect (% change in vF threshold), E_{MAX} is the maximum effect, C is the concentration of morphine (ng/mL), γ is the shape parameter, and EC_{50} is the concentration of morphine which elicits a 50% maximal response.

Pharmacodynamic modeling after multiple doses produced a concentration - effect relationship expressed by the equation:

$$E = \frac{E_{MAX} \bullet C}{C + EC_{50}} .$$

6.4 RESULTS

The vF device was well tolerated with no evidence of aversion or learned behavior to its application. There was no evidence of tissue injury following any phase of the study. The most common response to the nociceptive stimulus of the vF device was active withdrawal of the leg, although vocalization also occurred occasionally. No significant within or between dog differences were observed between time points within the saline-treated group and time 0 of the multiple dose group, inclusive.

All 6 dogs vomited following the initial loading dose during the morphine infusion study, whereas no vomiting occurred in the saline infusion group. Von Frey thresholds were significantly elevated ($p<0.05$) at 5, 6, 7, and 8 hours during morphine infusion compared to saline infusion, corresponding to morphine plasma concentrations (mean ± SE) of 33.4 ± 5.9, 31.3 ± 6.0, 34.6 ± 8.3, and 36.8 ± 6.0 ng/mL, respectively (figures 6.1 and 6.2). The actual rates of morphine administration which targeted plasma concentrations of 10, 20, 30, and 40 ng/mL were 0.05 ± 0.01, 0.10 ± 0.02, 0.15 ± 0.02, and 0.20 ± 0.03 mg/kg•hr, respectively. Morphine-6-glucuronide was not detected in any plasma sample during morphine i.v. infusion. The elimination half-life of morphine after i.v. infusion was 0.61 ± 0.08 hr. Pharmacodynamic modeling calculated an EC_{50} and E_{MAX} of 29.5 ± 5.4 ng/mL and 78 ± 11% change from baseline, respectively (figure 6.3). Only 5 of the 6 dogs were included in pharmacodynamic modeling, because data from one dog could not be fit to a model.

Four of the dogs began salivating, licking, and retching, and 1 of those dogs vomited following the first morphine dose during the multiple dose phase. None of the dogs displayed salivation, licking, retching, or vomiting on either of the subsequent

doses. All dogs were moderately to markedly sedate by the end of the study (8 hours). The baseline (time 0) vF threshold was not statistically different from any time point in the saline group. Percent change vF thresholds were significantly elevated ($p<0.005$) from 1 through 7 hours, as compared to the saline infusion (Figure 6.4).

Morphine - 6 - glucuronide was not detected in any sample during the multiple dose phase. There were no significant differences among the trough or peak plasma morphine concentrations (figure 6.5 and table 6.1). There was no significant difference in the elimination half-life, volume of distribution, clearance, or the AUC between the first and last (third) dose of morphine in the multiple dose phase (table 6.1).

Pharmacodynamic modeling of morphine administered as multiple doses calculated an EC_{50} and E_{MAX} of 27.3 ± 7.4 ng/mL and 180 ± 67 % change from baseline, respectively (figure 6.6). The PCV for time 0 and 7 hours was 48.0 ± 0.4 and 45.3 ± 1.3%, respectively and not statistically different. The TS for time 0 and 7 hours was 7.2 ± 0.2 and 7.5 ± 0.1, respectively and was statistically different ($p=0.023$).

6.5 DISCUSSION

Morphine is the prototypical opiate analgesic and has frequently been recommended for use in dogs. The concentration - effect relationship of multiple doses or i.v. infusions of morphine has not been evaluated. Multiple doses of morphine sulfate (0.5 mg/kg q2h) and infusion rates of 0.15 ± 0.02 mg/kg•hr and higher, produced significant antinociceptive effects in response to the vF device. However, morphine infusion rates greater than 0.10 ± 0.02 mg/kg•hr resulted in variable plasma concentrations, whereas plasma concentrations were more consistent and predictable during multiple i.v. doses.

Following intravenous morphine infusion, plasma concentrations were variable despite the fact infusion rates were calculated from the individual dog's pharmacokinetic parameters. At low infusion rates, the actual concentrations were close to the targeted 10 and 20 ng/mL concentration, but increases in the infusion rate resulted in variable and inconsistent increases in the plasma concentrations.

Increases in clearance and / or the volume of distribution of morphine could have occurred during the higher infusion rates, which resulted in the variable and lower than expected plasma concentrations at the higher infusion rates. In comparison, plasma concentrations following multiple doses of morphine were not as variable and more predictable.

Previous studies have demonstrated variable plasma concentrations during morphine infusions similar to the results seen in the current study. Postoperative dogs receiving morphine 0.12 mg/kg•hr as a constant rate intravenous infusion had a range of plasma concentrations from 22 ± 6 ng/mL to 36 ± 3 ng/mL (Lucas, 2001). Subcutaneous

infusion of morphine via miniosmotic pumps at a constant rate of 0.1 mg/kg•hr yielded plasma concentrations ranging from 19.3 ± 1.3 to 24.9 ± 2.8 ng/mL 12 hours after implantation (Yoshimura, 1993). Subcutaneous administration of morphine via preset implantable infusion pumps at a constant rate of 0.5 mg/kg•hr in 4 dogs yielded plasma concentrations ranging from 50 to 100 ng/mL 12 hours after implantation (Gilberto, 2002).

In comparing the actual vF thresholds in the saline infusion, there were no significant within dog differences, but between dog differences were observed. This was not unexpected as between dog differences in response to noxious stimuli, such as the vF device, has been demonstrated previously (Lascelles, et al, 1997; KuKanich, et al, 2005a). Therefore in order to compare the dogs as a group the data were transformed to a percent change from the mean saline infusion values for each group to account for the between dog baseline differences. When the value of percent change was used as a comparison, no significant differences were observed within or between dogs.

Von Frey thresholds were significantly elevated from 5 to 8 hours during the morphine infusion. The lowest mean plasma concentration associated with significantly elevated vF thresholds was 31.3 ± 6.0 ng/mL. The infusion rate (0.15 ± 0.02 mg/kg•hr) to maintain 31.3 ± 6.0 ng/mL plasma concentrations is slightly higher than a previous study utilizing surgical (laparotomy) dogs (Lucas, et al, 2001). Many factors could have accounted for the differences between these studies, including the anesthetic regimen in the study reported by Lucas et al, (2001).

We calculated an EC_{50} (29.5 ± 5.4 ng/mL), from the pharmacodynamic model during the infusion phase that was similar to the multiple dose phase (27.3 ± 7.4 ng/mL).

However the E_{MAX} for the multiple dose phase (180 ± 67%) was markedly higher than the infusion phase (78 ± 11%), but similar to that calculated from a previous study (213 ± 43 %) (KuKanich, et al 2005a). A flaw in our design of the infusion portion of the study is that a maximum response (E_{MAX}) was not attained because we were reluctant to administer high infusion rates of morphine sulfate. The peak plasma morphine concentrations during the multiple dose phase (197.0 ± 15.2 ng/mL) were higher than the highest concentration in the infusion phase (67 ng/mL). Higher infusion rates to produce higher plasma concentrations would be necessary to confirm the maximal effect (plateau of the model) is correct. Additionally, the model did not fit the data from one dog and was excluded from pharmacodynamic modeling because there was only a small range of plasma concentrations in this animal.

There are no published reports on the pharmacokinetics of morphine following multiple i.v. doses. Following morphine sulfate administration, 0.5 mg/kg i.v. for 3 doses, there were no statistical differences in the elimination half-life, clearance, volume of distribution, AUC, peak, and trough levels between the first and last dose indicating no evidence of pharmacokinetic alterations. An increasing trend was observed for the peak and trough plasma concentrations, which would be expected due to drug accumulation, but was not significantly different. The accumulation index can be calculated from the following equation:

$$AI = \left(1 - e^{-(\frac{0.693 \bullet \tau}{t_{1/2}})} \right)^{-1}$$

where AI is the accumulation index, e is the natural logarithm, τ is the dosing interval, and $t_{1/2}$ is the elimination half-life (1.05 h). Therefore plasma steady state concentrations

for morphine administered every 2 hours would accumulate 1.4 times the first dose's concentration when at steady state. In the multiple dose phase, trough morphine plasma concentrations following the first and last dose were 19.0 ± 7.7 and 27.5 ± 6.1 ng/mL, respectively, demonstrating an accumulation of 1.5 similar to the predicted accumulation index for plasma. If additional repeated doses were administered at this rate, the plasma concentration would not be expected to increase further as the expected accumulation has already occurred.

An increase in the vF thresholds also occurred with the second and third doses that corresponded to increasing plasma concentrations. In comparison to the saline infusion, vF thresholds were significantly elevated ($p<0.005$) from from 1-7 hours during the multiple dose phase indicating antinociceptive effects were maintained with morphine sulfate administered 0.5 mg/kg i.v. every 2 hours.

A previous study using the von Frey device calculated an EC_{50} of 13.9 ± 2.4 ng/mL (KuKanich, 2005a). However the study was conducted following a single i.v. morphine bolus. In contrast the EC_{50} of morphine was 29.5 ± 5.4 and 27.3 ± 7.4 ng/mL following infusion and multiple dosing, respectively. A longer elimination half-life (mean ± SE) from the CSF (121.0 ± 5.6 min.) as compared to the plasma (65.4 ± 23.4 min) was present in a pharmacokinetic study of morphine (Hug, et al, 1981). However, CSF concentrations do not necessarily correlate with CNS drug concentrations (Shen, et al, 2004). Nevertheless, after a single dose of morphine, plasma concentrations probably decline more rapidly than the CNS concentrations leading to an underestimation of the effective plasma concentrations following a single dose. This would not have been as

likely after the second and third dose, which were used for the PD model (figure 6.6) or during an infusion of morphine.

The PCV and TS were measured prior to and at the completion of the multiple dose phase to ensure volume depletion did not occur due to sampling. There was no significant difference in the PCV prior to or at the completion of the study. The TS was significantly higher at the completion of the multiple dose phase as compared to before the phase was started. This may have occurred because of mild dehydration. Although vomiting was noted in one dog, it was transient and resolved within 5 minutes. Water was freely available, but no dog was seen drinking during the study. Intravenous fluid administration only replaced the volume of blood taken from sampling (administered as a catheter flush) and was not intended as a maintenance fluid rate. This mild change in TS did not appear to affect pharmacokinetics between first and third doses.

In conclusion, we compared the antinociceptive effect of morphine given as an intravenous bolus and repeated i.v. bolus injections with the EC50 being 27.3 ± 7.4 and 29.5 ± 5.4, respectively. Relevance to a clinician is that repeated i.v. injections are just as effective, and perhaps more effective than i.v. infusions. Moreover, when i.v. infusions were administered, E_{MAX} was not attained, and plasma concentrations were more variable among dogs. The lowest infusion rate to maintain significantly elevated antinociceptive effects was 0.15 ± 0.02 mg/kg•hr. Morphine sulfate, 0.5 mg/kg i.v. every 2 hours maintained significantly increased antinociceptive thresholds in dogs throughout the testing period. When multiple doses are administered, we confirmed, that in healthy dogs, the pharmacokinetics did not vary between the first and last of three consecutive doses. These results provide a basis from which clinical studies may be designed in

patients with pain.

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Table 6.1. Pharmacokinetic parameters from the multiple dose phase following morphine sulfate 0.5 mg/kg i.v. every 2 hours.

PARAMETER	UNITS	FIRST DOSE	SECOND DOSE	THIRD DOSE
Half-life (elimination)	**hr**	0.87 ± 0.05	N/A	1.05 ± 0.09
Clearance (total body)	**mL/min•kg**	48.2 ± 4.9	N/A	45.1 ± 4.6
Volume of Distribution (area method)	**L/kg**	3.6 ± 0.4	N/A	4.0 ± 0.4
Area Under the Curve (per dose)	**hr•ng/mL**	138.1 ± 16.1	N/A	148.3 ± 19.0
C_{MAX}	**ng/mL**	164.9 ± 9.7	188.4 ± 21.8	197.0 ± 15.2
C_{MIN}	**ng/mL**	19.0 ± 7.7	26.7 ± 5.3	27.5 ± 6.1

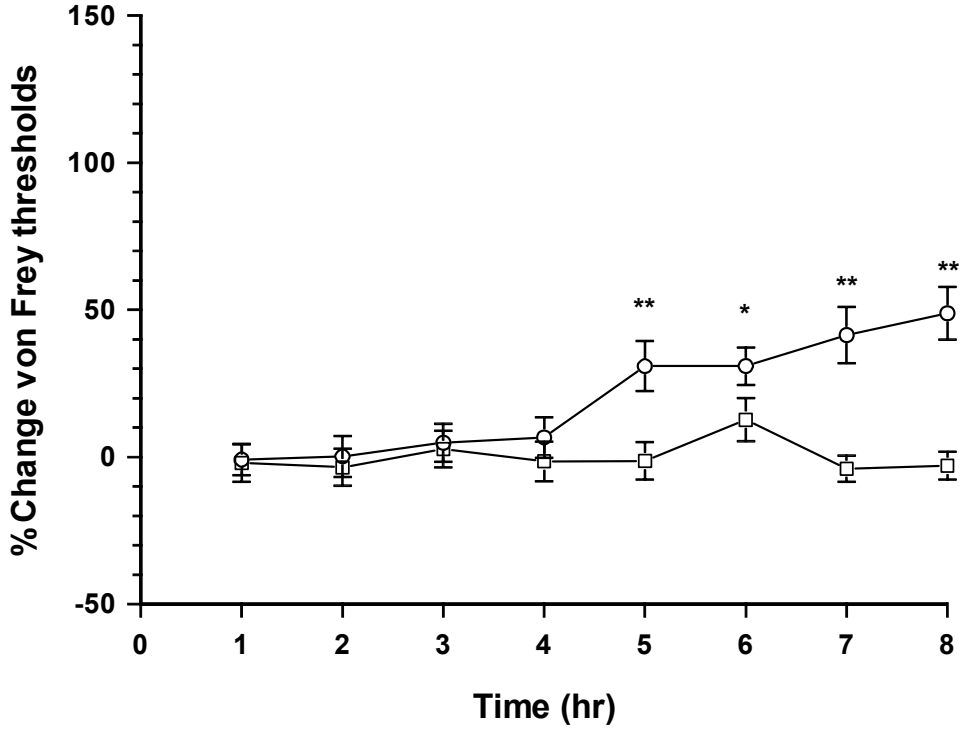

Figure 6.1. Mean ± SE change in von Frey thresholds following i.v. morphine infusion compared to saline infusion. * = $p<0.05$. ** = $p<0.005$ (□) saline infusion group. (○) morphine infusion group.

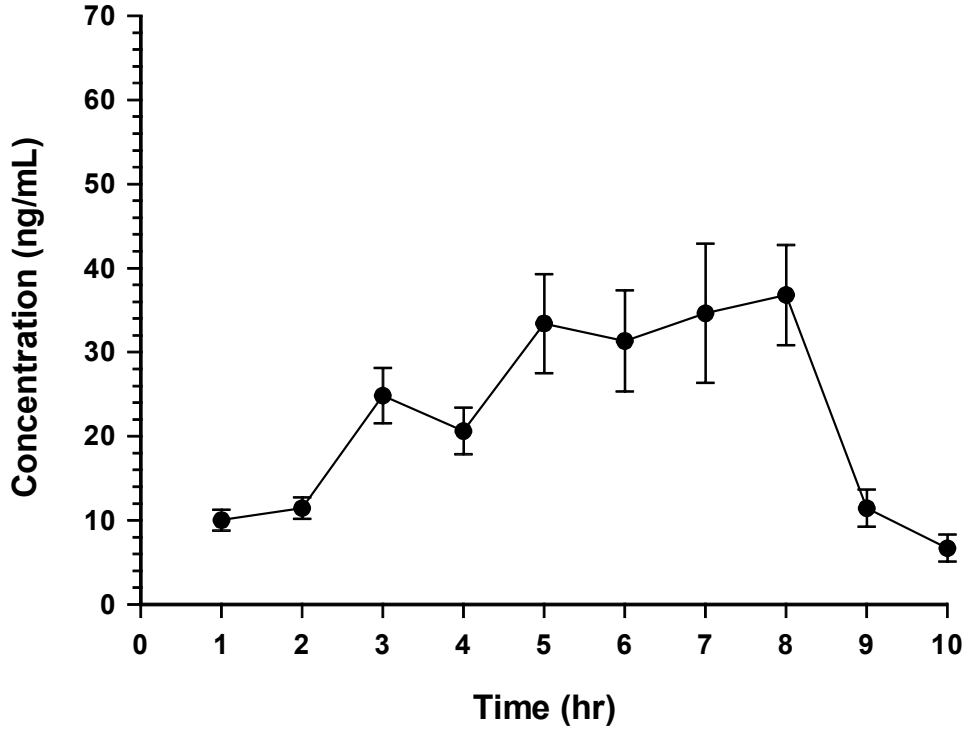

Figure 6.2. Mean ± SE plasma morphine concentrations following i.v. infusion of morphine. Targeted plasma concentrations were 10 ng/mL for 1-2 hours, 20 ng/mL for 3-4 hours, 30 ng/mL for 5-6 hours, and 40 ng/mL for 7-8 hours.

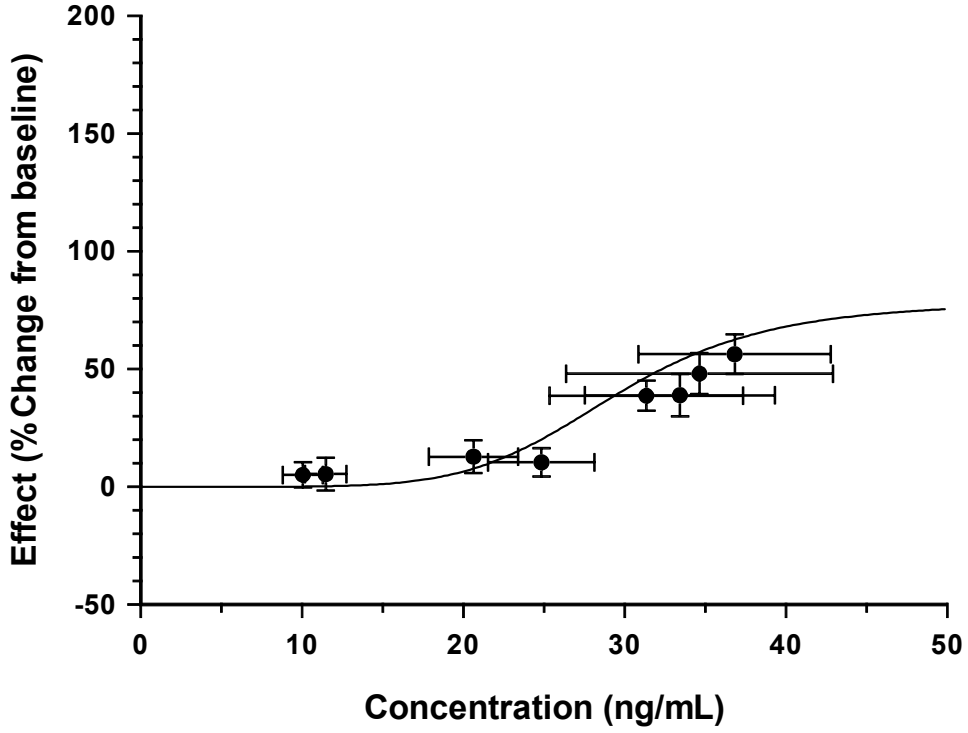

Figure 6.3. Mean ± SE pharmacodynamic model following i.v. infusion of morphine. 5/6 dogs are included.

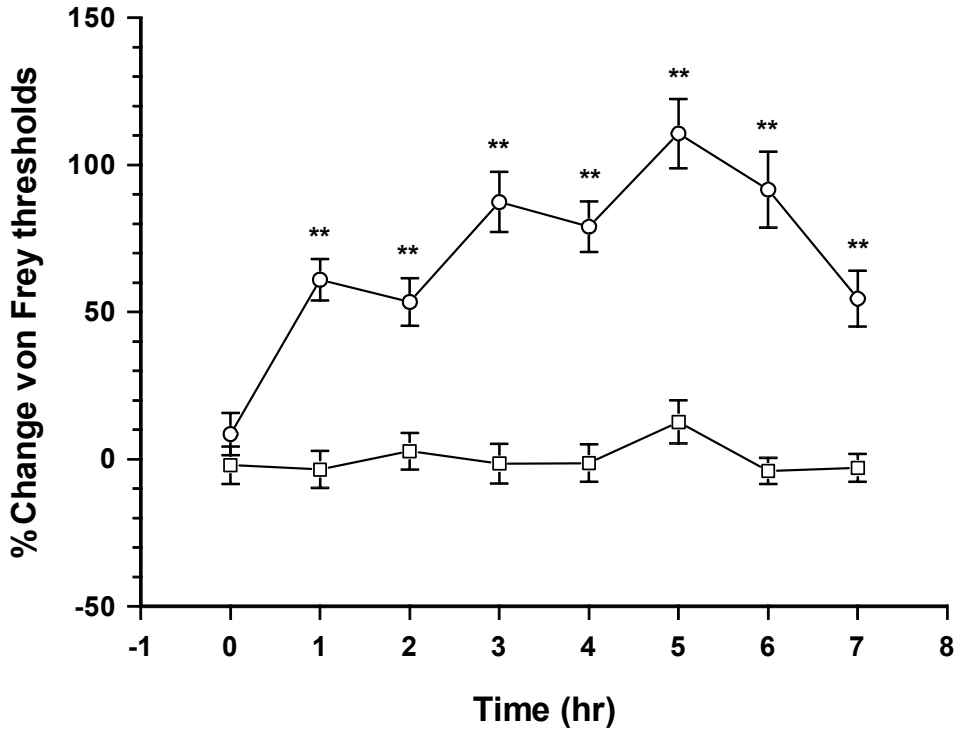

Figure 6.4. Mean ± SE change in von Frey thresholds following 0.5 mg/kg morphine sulfate administered i.v. every 2 hours compared to saline infusion. ** = $p<0.005$. (□) saline infusion. (○) morphine multiple dose group.

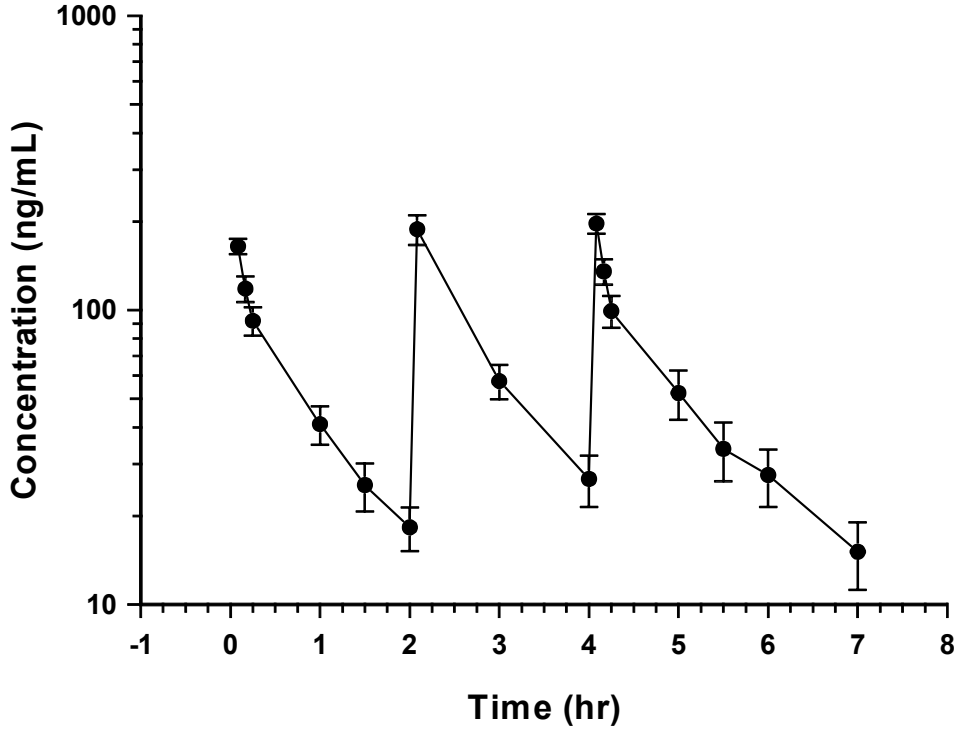

Figure 6.5. Mean ± SE morphine plasma concentrations following 0.5 mg/kg morphine sulfate administered i.v. every 2 hours.

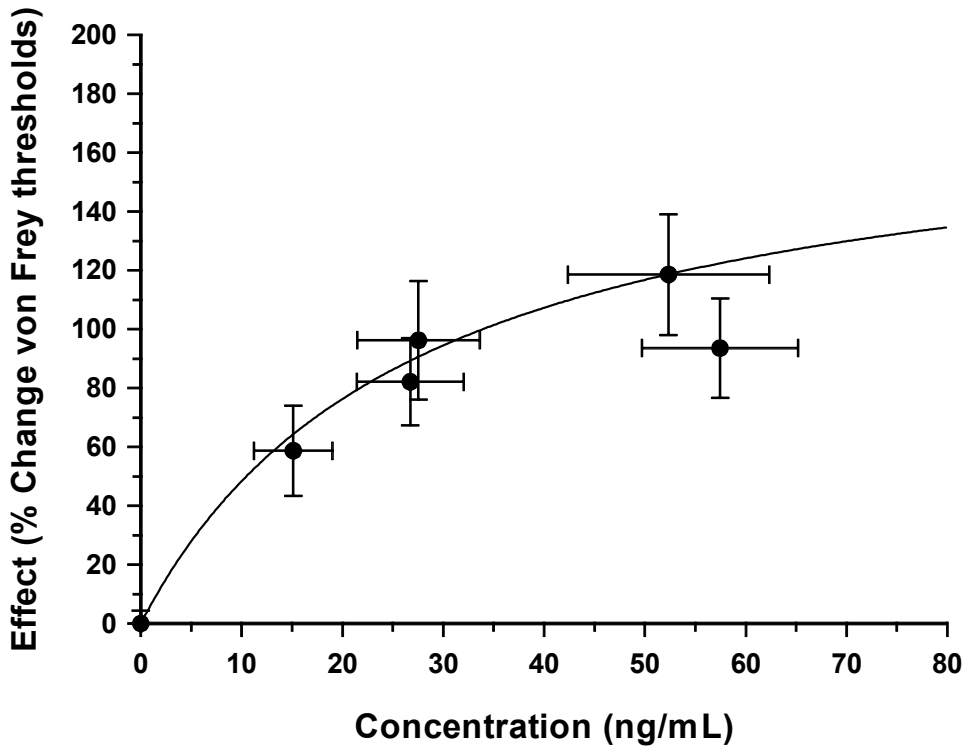

Figure 6.6. Mean ± SE pharmacodynamic model following 0.5 mg/kg morphine sulfate administered i.v. every 2 hours. The second and third dose are demonstrated.

INHIBITION OF CYTOCHROME P450 3A, P-GLYCOPROTEIN, AND GASTRIC ACID SECRETION DO NOT ALTER ORAL BIOAVAILABILITY OF METHADONE IN DOGS.

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Submitted to Drug Metabolism and Disposition

7.1 ABSTRACT

Methadone is an opioid, which has a high oral bioavailability (> 70%) and a long elimination half-life (> 20 hrs) in people. Methadone is primarily metabolized in people by cytochrome P-450 3A (CYP3A). Ketoconazole is a CYP3A and p-glycoprotein (p-gp) efflux pump inhibitor. Omeprazole is a H^+, K^+ -ATPase proton pump inhibitor and has increased the oral bioavailability of methadone by increasing gastrointestinal pH. The purpose was to evaluate the effects of CYP3A, p-gp, and gastric acid inhibition on methadone bioavailability in dogs.

Six healthy dogs were used in a 4-way crossover design. Methadone was administered i.v. (1 mg/kg), orally (2 mg/kg), orally following oral ketoconazole administration (10 mg/kg q 12 hrs for 2 doses), and orally following omeprazole (1 mg/kg p.o. q 12 hrs for 5 doses). Plasma concentrations of methadone were analyzed via high-pressure liquid chromatography or fluorescence polarization immunoassay.

The mean ± SD for the elimination half-life, volume of distribution, and clearance were 1.75±0.25 hrs, 3.46±1.09 L/kg, and 25.14±9.79 mL/min/kg, respectively. Methadone was not detected in any sample following oral administration alone (actual dose 1.90±0.15 mg/kg of the base) or oral administration with omeprazole (actual dose 1.05±0.24 mg/kg). Ketoconazole (actual dose of 11.11±2.13 mg/kg) produced detectable concentrations in one dog with a 29% bioavailability. MDR-1 genotyping, encoding p-gp, was normal in all dogs.

In contrast to people, methadone has a short elimination half-life, rapid clearance, and poor oral bioavailability and inhibition of CYP3A, p-gp, and gastric acid secretion have minimal effects on oral bioavailability in dogs.

7.2 INTRODUCTION

Methadone is a synthetic opioid currently marketed in the United States for analgesia and treatment of heroin addiction. It exists as a 50:50 racemic mixture with *l*-methadone exerting the pharmacologic effect. Methadone interacts primarily with μ opiate receptors, but also binds with variable affinity to n-methyl-d-aspartate (NMDA) and α-2 adrenergic receptors (Chizh, et al 2000; Gorman, et al 1997; Callahan, et al 2004; Codd, et al 1995). In people, methadone has a long elimination half-life (20 - 42 hours) and high oral bioavailability (70 - 100%) (Eap, et al 2002; Dale, et al 2002). Therapeutic plasma concentrations of methadone for acute pain have been reported to be between 48 to 60 ng/mL (Gourlay, et al 1982; Gourlay, et al 1984; Gourlay, et al, 1986). The pharmacokinetics and pharmacodynamics of methadone following parenteral administration have been evaluated in dogs (Schlitt, et al 1978; Garret, et al 1985; Schmidt, et al, 1994; Stanley, et al, 1980). It has exhibited variable pharmacokinetics, with the elimination half-life ranging from 1.75 – 4 hours following i.v. and s.c. administration (Schlitt, et al, 1978; Garret, et al, 1985; Schmidt, et al, 1994). There are no reports of oral administration of methadone to dogs.

Methadone is metabolized primarily by cytochrome P-450 3A4 (CYP3A4) in human hepatocytes (and enterocytes), but CYP2C8 and CYP2D6 also contribute to the metabolism (Iribarne, et al 1996; Foster, et al 1999; Oda & Kharasch, 2001; Wang & DeVane, 2003). Ketoconazole, a reversible CYP3A4 inhibitor, decreased in vitro methadone metabolism by 70 - 80% in clinically relevant concentrations (Foster, et al 1999; Wang & DeVane, 2003). In vivo inhibition of CYP3A4 also resulted in inhibition

of methadone metabolism and increased oral bioavailability in people (Benmebarek, et al, 2004).

CYP 3A12 is the predominant isoform of CYP3A in dogs as apposed to CYP3A4 being the predominant isoform in people. Ketoconazole is a CYP3A12 inhibitor in dogs, which causes in vivo inhibition of CYP3A12 with as few as two doses (Dahlinger, et al, 1998; Kuroha, et al, 2002a; Kuroha, et al 2002b). A dose titration of orally administered ketoconazole to dogs significantly inhibited CYP3A12, with doses above 10 mg/kg not producing a more significant effect than 10 mg/kg (Myre, et al, 1991).

Ketoconazole has also been shown to inhibit the p-glycoprotein (p-gp) efflux pump (also known as the ATP-binding cassette subfamily B member 1, ABCB1) encoded by the gene MDR-1 in the monkey (Ward, et al 2004). Methadone, when administered to people with a p-gp inhibitor, produced significantly higher plasma concentrations compared to a crossover without an inhibitor (Kharasch, et al 2004). The inhibitor had no effect on intravenous pharmacokinetics or elimination half-life, indicating that the increased plasma concentrations were caused by increased absorption (Kharasch, et al 2004). Inhibition of p-gp in dogs has increased the oral bioavailability of various p-gp substrates (Mealey, 2004). No reports are available to show whether or not ketoconazole also inhibits p-gp in dogs, but may increase the oral bioavailability of cyclosporine in dogs in part to p-gp inhibition (Mealey, 2004). Additionally, homozygous or heterozygous deletions of MDR-1, with a decreased expression of p-gp, have been shown to increase the effect of p-gp substrates in the dog, including the opiate loperamide, however it is unknown whether p-gp affects methadone absorption or effect in dogs (Mealey, et al, 2001; Mealey, et al 2003; Sartor, et al 2004).

Omeprazole, an irreversible inhibitor of the H^+, K^+ -ATPase (proton pump), has been shown to increase the oral bioavailability and rate of absorption of methadone in rats (De Castro, et al, 1996; Carlos, et al, 2002). Omeprazole is also an inhibitor of CYP2C19, but concurrent administration with methadone did not change the elimination rate of methadone, and oral bicarbonate administration also increased methadone bioavailability (De Castro, et al, 1996; Ko, et al, 1997; Carlos, et al, 2002). It was concluded the effect was via increased gastrointestinal pH, because it did not change the pharmacokinetics of i.v. methadone (with omeprazole). Omeprazole has been shown to significantly increase the gastric pH when given to dogs at a dose of 1 mg/kg once daily [a].

The purpose of this study was to evaluate the effects of ketoconazole administration on the oral bioavailability of methadone in dogs. An increase in methadone oral bioavailability following concurrent administration with ketoconazole could indicate methadone is either a CYP3A12 and / or p-gp substrate in the dog. A second purpose was to evaluate the effects of omeprazole administration on methadone oral bioavailability in dogs. Increased oral bioavailability of methadone with concurrent administration omeprazole could indicate oral absorption is affected by gastric pH and / or inhibition of CYP enzymes (by omeprazole) that metabolize methadone.

7.3 Materials and Methods

7.3.1 Animals – Six healthy beagle dogs (3 male, 3 female), ranging in age from 4 – 5 years and weight from 7.3 – 13.0 kg were used in a crossover study. The North Carolina State University Institutional Animal Care and Use Committee approved the study.

7.3.2 Study Design – A four-way crossover study design was used. The first crossover consisted of 1 mg/kg methadone base (Sigma, St. Louis, MO) administered as an i.v. bolus. A 24-hour washout was deemed acceptable because pilot studies showed us that the drug would be washed out by 24 hours, therefore carry-over effects were not anticipated. The second crossover, conducted 24 hours after the first crossover, consisted of methadone HCl tablets (Roxane Laboratories, Columbus, OH) dosed orally with a targeted dose of 2 mg/kg. The third crossover, 24 hours after the second crossover, consisted of 2 doses of ketoconazole 200 mg tablets (Mutual Pharmaceutical Company, Inc., Philadelphia, PA), 10 mg/kg p.o., to the nearest 100 mg, dosed 12.5 and 0.5 hours prior to oral methadone HCl (2 mg/kg) administration. The final crossover, conducted at least 8 weeks after the third crossover, consisted of 10 mg omeprazole delayed-release tablets (Mylan Pharmaceuticals, Inc., Morgantown, WV) given orally with a target dose of 1 mg/kg, administered every 12 hours for 5 doses with the last dose 1 hour prior to orally administered methadone HCl (2 mg/kg). Intravenous methadone was injected through a 20 gauge cephalic catheter (Angiocath, Becton Dickinson, Sandy, Utah) and flushed with 10 mL of 0.9% saline solution following injection. The cephalic catheter was removed following injection. Methadone administered orally was followed by ½ of

the dog's standard meal of commercially available dog food in all crossovers (Hill's d/d, Topeka, KS). Ketoconazole was also administered with food.

Jugular catheters (19 gauge, Intracath, Becton Dickinson, Sandy, Utah) were inserted prior to drug administration and used for blood collection. Blood was collected into evacuated glass tubes containing lithium heparin (BD Vacutainer, Franklin Lakes, NJ) and placed on ice until centrifugation. The samples were centrifuged for 10 minutes at 1000 g, plasma was separated, and stored frozen at –80° C prior to analysis. Blood samples were collected prior to and at 10, 20, and 30 minutes and at 1, 2, 4, 6, and 8 hours after intravenous methadone administration. Blood samples were collected prior to, and at 15, 30, and 45 minutes and at 1, 2, 4, and 6 hours following all crossovers of orally administered methadone.

7.3.3 Plasma analysis – Plasma was analyzed using high-pressure liquid chromatography (HPLC) with ultraviolet detection (LOQ 20 ng/mL) or via fluorescence polarization immunoassay (FPIA) (LOQ 25 ng/mL) as previously published (KuKanich, et al, 2005a).

7.3.4 Pharmacokinetic Analysis – Compartmental and noncompartmental analyses were performed in a standard two-stage design using commercially available computer software (WinNonlin, 4.0, Pharsight Corporation, Mountain View, CA). Compartmental and noncompartmental parameters were calculated from previously published equations (Gibaldi & Perrier, 1982). A weighting factor (1), was applied to the pharmacokinetic analysis.

Noncompartmental Pharmacokinetic Analysis - The variables for area under the curve from time 0 to infinity ($AUC_{0-\infty}$), area under the first moment curve from time 0 to infinity ($AUMC_{0-\infty}$), total body clearance (Cl_T) , apparent volume of distribution at steady state and area (Vd_{ss}, Vd_{area}), first-order rate constant (λ_z), elimination half-life ($t_{½}\ \lambda_z$), and mean residence time (MRT) were calculated using noncompartmental analysis. The concentration at time 0 (C_0) was calculated by log-linear regression using the first two time points. The $AUC_{0-\infty}$ was calculated using the log-linear trapezoidal method.

Oral bioavailability (%F) was calculated following oral administration from noncompartmental parameters with the equation:

$$\% \text{ F} = \frac{100 \bullet AUC_{p.o.} \bullet Dose_{i.v.}}{AUC_{i.v.} \bullet Dose_{p.o.}}$$

where $AUC_{p.o.}$ is the area under the curve from time 0 to infinity following oral administration, $Dose_{i.v.}$ is the dose administered i.v., $AUC_{i.v.}$ is the area under the curve from time 0 to infinity following i.v. administration, and $Dose_{p.o.}$ is the dose administered orally.

Compartmental Pharmacokinetic Analysis - Residual plots and Aikake Information Criterion (AIC) were used to discriminate the best-fit pharmacokinetic model. Compartmental analysis was used to estimate the distribution half-life ($t_{½\alpha}$), elimination half-life ($t_{½\beta}$), distribution rate constant (α), elimination rate constant (β), intercept for the distribution phase (A), intercept for the elimination phase (B), elimination rate from compartment 1(k10), rate of transfer from compartment 1 to compartment 2 (k12), rate of transfer from compartment 2 to compartment 1 (k21), half-life of the elimination phase

(k10 $t_{½}$), apparent volume of compartment 1 (V1), and apparent volume of compartment 2 (V2).

7.3.5 MDR -1 genotyping - MDR-1 genotyping was performed on all six dogs. DNA was obtained from buccal mucosal swab samples collected with a cytology brush (Cytosoft Cytology Brush # CYB-1, Medical Packaging Corp., Camarillo, CA). Genotyping was performed by a polymerase chain reaction technique described elsewhere (Hugnet, et al, 2004).

7.4 RESULTS

Intravenous methadone administration produced sedation in all dogs. None of the dogs vomited and dysphoria was not observed. There was no evidence of carryover between the crossovers as methadone was not detectable in any of the time 0 samples. The plasma profile was characterized by a rapid distribution phase followed by a slower elimination phase in 5 of the dogs, in which a two compartment model best fit. One of the dog's plasma profile was best described by a one compartment model, as a distribution phase was not observed, therefore was excluded from compartmental analysis only. The mean ± SD for the elimination half-life, volume of distribution, and clearance were 1.75 ± 0.25 hrs, 3.46 ± 1.09 L/kg, and 25.14 ± 9.79 mL/min/kg, respectively (figure 7.1, table 7.1). The compartmental pharmacokinetic parameters are presented in table 7.2.

The actual (mean ± SD) dose of orally administered methadone (base) was 1.90 ± 0.15 mg/kg when administered alone or with ketoconazole. The actual (mean ± SD) dose of methadone (base) was 1.86 ± 0.10 mg/kg when administered with omeprazole. The actual (mean ± SD) dose of ketoconazole and omeprazole were 11.11 ± 2.13 mg/kg and 1.05 ± 0.24 mg/kg, respectively. Methadone was not detected in any sample at any time when administered orally alone, with a 20 ng/mL limit of quantification (LOQ). Methadone was detected in plasma following oral administration with ketoconazole in only 1 dog with a 29% oral bioavailability, 161.21 ng/mL maximal plasma concentration, and 1.98 hr elimination half-life (figure 7.2). The corresponding i.v. elimination half-life for the same dog was 1.54 hr. When this dog was used in pilot studies (data not reported in table), ketoconazole was administered at a dose of 9.66 mg/kg twice at an 18 hour interval with the last dose given 30 minutes prior to methadone administration. This pilot

trial also produced detectable levels of methadone (figure 7.3). Methadone was not detected (25 ng/mL LOQ) in any sample at any time when administered orally with omeprazole. MDR-1 genotyping was normal in all dogs tested (no mutant alleles).

7.5 DISCUSSION

Methadone is an opioid that acts as a μ agonist, an NMDA antagonist, and an α-2 adrenergic agonist. Intravenous methadone administration resulted in marked sedation in all dogs. However, there were no dysphoric reactions, vomiting, or excitement observed after IV administration, which has been reported after administration of other opioids (KuKanich, et al 2005b; KuKanich, et al 2005c). In contrast to people, methadone exhibited a rapid clearance and poor oral bioavailability. Omeprazole had no apparent effect on the bioavailability of methadone, and ketoconazole, a potent CYP3A12 inhibitor and p-gp inhibitor, only increased the oral bioavailability in one dog.

Methadone clearance (25.14 ml/min/kg) is similar to the reported hepatic blood flow (30 ml/min/kg) in dogs (Davies, & Morris, 1993). The rapid total body clearance is suggestive of a high extraction drug, which would be expected to produce a low oral bioavailability due to a high first pass effect. Studies in people have reported hepatic blood flow values (20 ml/min/kg) similar to dogs, but a markedly slower methadone clearance (1.08 – 3.10 ml/min/kg), which is more suggestive of a low extraction drug (Eap, et al 2002; Dale, et al 2002). The clearance of morphine (62.46 ml/min/kg) and tramadol (54.63 ml/min/kg) in dogs, when investigated in our lab also exhibited a more rapid clearance as compared to people, (21.1 ml/min/kg for morphine and 3.73 ml/min/kg for tramadol) (KuKanich, et al 2005b; KuKanich & Papich, 2004; Hasselstrom & Sawe, 1993; package insert, Ultram, Ortho-McNeil, Raritan, NJ, USA). However, the clearance of fentanyl (28 ml/min/kg) in dogs was similar to people (33.5 ml/min/kg) exhibiting properties suggestive of a high extraction drug in both species (Kyles, et al 1996; Ariano,

et al 2001). In general, opioids and derivatives tend to be rapidly cleared in dogs as compared to people.

Ketoconazole administration, 2 doses prior to methadone administration, resulted in an increased oral bioavailability in only one dog. The same dog was administered ketoconazole prior to 2 doses of oral methadone, as part of a pilot study, which also produced detectable methadone plasma concentrations, demonstrating repeatability in the same dog. Ketoconazole has been shown to significantly inhibit CYP3A12 in dogs with as little as two doses (Dahlinger, et al, 1998; Kuroha, et al, 2002a). We were not able to determine why this effect was limited to a single dog in our study group.

In people, methadone is primarily metabolized by CYP3A4 to 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP) and further metabolized to 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EDMP) (Karasch, et al, 2004). A study in 4 dogs demonstrated EDDP and EDMP to be minor metabolites and it was hypothesized CYP3A (unstated isoform) may not be a major metabolizing enzyme of methadone in dogs (Garrett, et al, 1985). The results of the current study agree with the hypothesis that CYP3A12 is not a major metabolizing enzyme in dogs because administration if an inhibitor had no observable effect in 5/6 dogs.

Ketoconazole also has inhibitory effects on p-gp, but this effect has not been conclusively demonstrated in dogs. Inhibition of p-gp resulted in greater methadone absorption in people and may have been a factor in the increased absorption in one dog (Karasch, et al, 2004). Genotyping of all of the dogs were normal with no MDR-1 mutations present, with presumably normal expression of p-gp, excluding genetic variability of p-gp as a reason for the increased absorption in one dog.

Drug absorption from the gastrointestinal tract (GIT) is dependent on many factors including the pH of the mucosal surface and the pKa of the drug. Drug ionization is a factor in passive GIT absorption because the unionized fraction of a drug is more permeable to the GIT mucosa than the ionized fraction (Riviere, 1999). The Henderson-Hasselbalch equation for weak bases (such as methadone, pKa 9.2) shows that at an intestinal pH of 5.3 at the mucosal surface only 0.01% of methadone would be unionized (Kaunitz & Akiba, 2001). The majority of intestinal drug absorption occurs in the duodenum and jejunum, therefore increasing pH in the duodenum can result in greater absorption of basic drugs (Lin, 1995). Omeprazole was administered in this study to increase intestinal pH and enhance permeability and oral absorption, however no effect was observed.

The gastrointestinal anatomy and physiology of the dog is markedly different from that of people, but is unlikely to the reason for decreased oral absorption in dogs. Dogs have a shorter absolute intestinal length and relative intestinal to body length in comparison to people and consequently a shorter gastrointestinal transit time (Martinez, et al, 2004). However, dogs have a higher relative surface, due to longer intestinal villi, leading to higher absorption of most drugs in dogs compared to people (Martinez, et al, 2004). Therefore, the gastrointestinal anatomy and physiology differences in the dog are unlikely to cause a decreased oral bioavailability of methadone.

In contrast to people, methadone is rapidly cleared from the plasma in dogs, exhibits a short elimination half-life, and poor oral bioavailability. Inhibition of CYP3A12 and p-gp by concurrent administration of ketoconazole resulted in increased oral bioavailability in only one dog. Poor oral bioavailability is most likely caused by

extensive pre-systemic metabolism, presumably by an enzyme system other than CYP3A12. Alterations in gastric pH by administration of omeprazole did not affect oral bioavailability.

7.6 ACKNOWLEDGEMENTS

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7.7 FOOTNOTES

[a] Bersenas, AME & Mathews, KA. Efficacy of Gastric acid lowering therapy in the dog. 10th International Veterinary Emergency and Critical Care Symposium. San Diego, CA. 2004.

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Table 7.1. Mean ± SD of calculated noncompartmental pharmacokinetic parameters following i.v. methadone (1 mg/kg base) to 6 healthy dogs.

Variable	Value
λ_z (1/hr)	0.40 ± 0.06
$t_{½}$ λ_z (hr)	1.75 ± 0.25
MRT (hr)	2.36 ± 0.31
Cl_T (ml/min*kg)	25.14 ± 9.79
Vd_{ss}(L/kg)	3.46 ± 1.09
Vd_{area}(L/kg)	3.67 ± 1.06
$AUC_{0-\infty}$ (hr*ng)/ml	742.63 ± 249.83
$AUC_{0-\infty}$ % extrapolated	9.07 ± 6.08
$AUMC_{0-\infty}$ (hr*hr*ng)/ml	1796.21 ± 752.79
C_0 (ng/ml)	388.97 ± 149.29
λ_z = first-order rate constant. $t_{½}$ λ_z = half-life of the terminal portion of the curve. MRT = mean residence time. Cl_T = total body clearance. Cl_T /F = apparent total body clearance following oral administration corrected for bioavailability. Vd_{ss} = volume of distribution at steady state. Vd_{area} = volume of distribution of the area during the elimination phase. $AUC_{0-\infty}$ = area under the curve from 0 to infinity. $AUMC_{0-\infty}$ = area under the first moment curve from 0 to infinity. C_0 = Plasma concentration at time 0.	

Table 7.2. Mean ± SD of calculated compartmental pharmacokinetic parameters following i.v. methadone (1 mg/kg base) to 5 healthy dogs.

Variable	Value
$t_{½\alpha}$ (hr)	0.15 ± 0.08
$t_{½\beta}$ (hr)	1.92 ± 0.42
α (1/hr)	5.97 ± 2.82
β (1/hr)	0.37 ± 0.07
A (ng/ml)	240.52 ± 171.05
B (ng/ml)	270.83 ± 77.27
K10 (1/hr)	0.66 ± 0.21
K12 (1/hr)	2.16 ± 1.65
K21 (1/hr)	3.52 ± 1.62
K10 $t_{½}$ (hr)	1.12 ± 0.31
V1 (L/kg)	2.14 ± 0.72
V2 (L/kg)	1.30 ± 0.63
$t_{½\alpha}$ = distribution half-life. $t_{½\beta}$ = elimination half-life. α = rate constant associated with distribution. β = rate constant associated with elimination. A = intercept for the distribution phase. B = intercept for the elimination phase. K10 = elimination rate from compartment 1. K12 = rate of movement from compartment 1 to compartment 2. K21 = rate of movement from compartment 2 to compartment 1. K10 $t_{½}$ = half-life of the elimination phase. V1 = volume of compartment 1. V2 = volume of compartment 2	

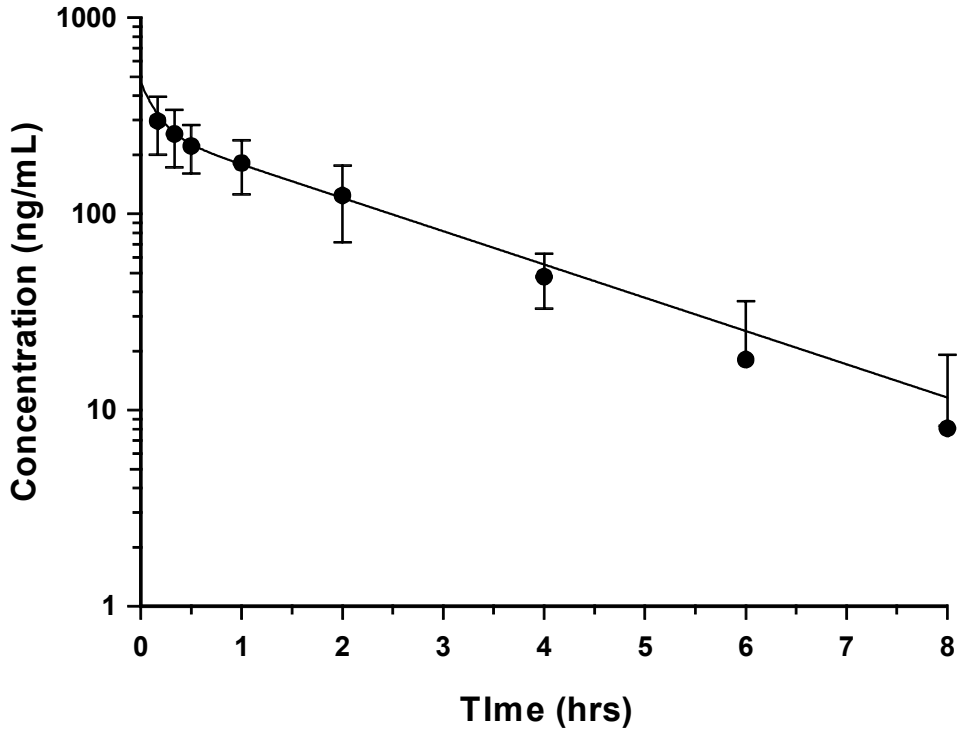

Figure 7.1. Predicted (—) and mean ± SD actual (●) methadone plasma concentrations following 1 mg/kg (base) administered i.v. to 6 healthy dogs.

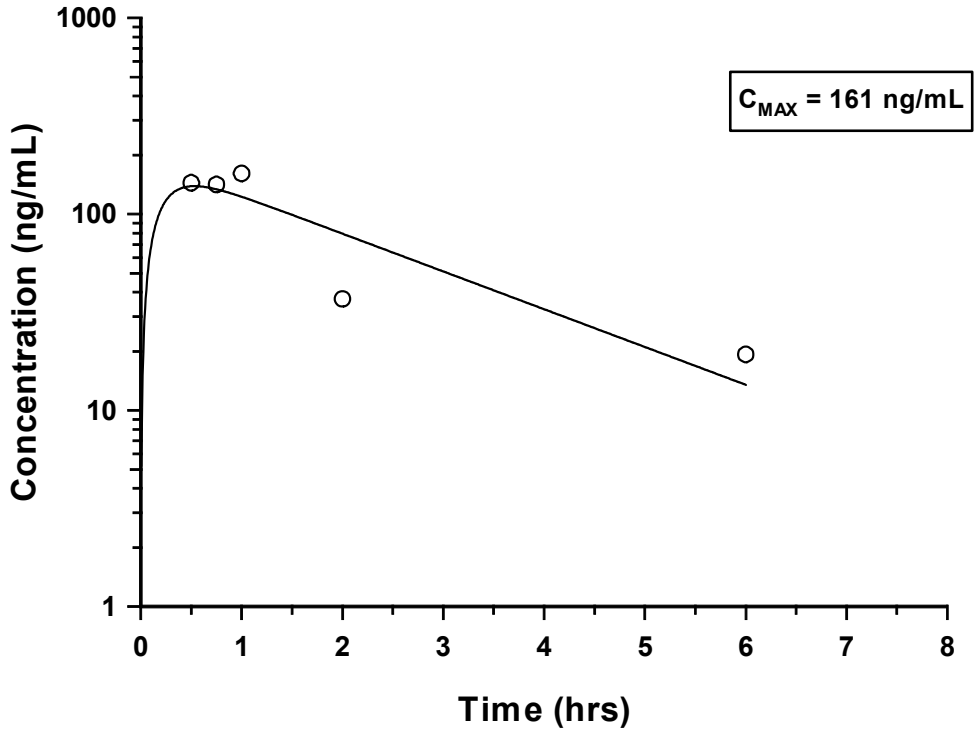

Figure 7.2. Methadone actual (o) and predicted plasma profile (—) in one dog dosed methadone orally with ketoconazole. Only one dog absorbed detectable levels of methadone.

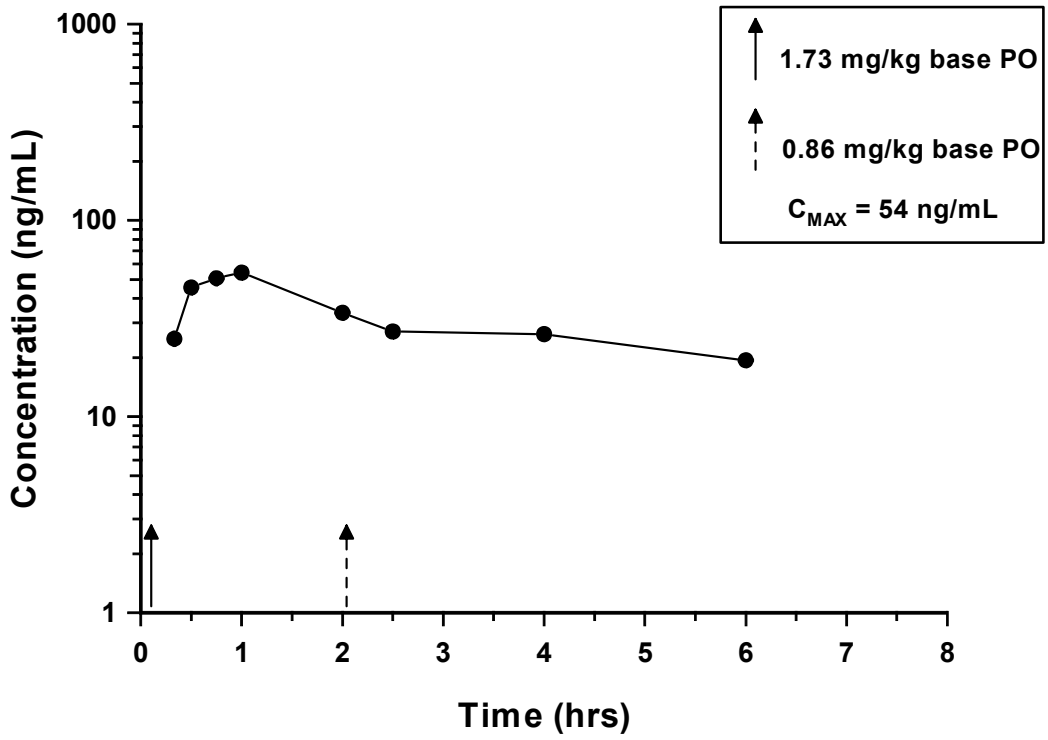

Figure 7.3. Pilot data from same animal in figure 7.2, given 2 doses of methadone following 2 doses of ketoconazole 9.66 mg/kg p.o. separated by 18 hours.

8. CONCLUSIONS

The pharmacokinetic parameters following intravenous morphine administration to healthy dogs obtained in the current study were similar to previous studies (Hug, et. al., 1981; Jacqz, et. al., 1986; Dohoo, et. al., 1994; Dohoo, 1997 Dohoo & Tasker, 1997; Barnhart, et. al., 2000). However, the advantage of the studies reported in this paper is that the HPLC assay was more specific then the previous studies. We also were able to detect presence/absence of the glucuronide, which was not specifically characterized in previous studies.

Morphine was rapidly distributed and eliminated in the dogs of our study. The administration was associated with sedation, dysphoria, and vomiting as adverse effects. Following oral administration of morphine tablets (extended release), the absorption was found to be poor and erratic in contrast to previously published studies (Dohoo, et al, 1994; Dohoo & Tasker, 1997; Doohoo, 1997). The previous studies on oral morphine analyzed plasma samples using a radioimmunoassay, which was not specific for morphine and showed cross reactivity with metabolites and may have overestimated actual plasma concentrations. The high-pressure liquid chromatography method used in the current study does not cross react with metabolites yielding more specific determinations of actual plasma concentrations. In both the i.v. and oral morphine studies, plasma concentrations of morphine-6-glucuronide (M-6-G) were assessed because it is an active metabolite and may contribute to the antinociceptive effects of morphine, as it apparently does in people. However, M-6-G was not detected in any plasma sample following either route of administration indicating that it is unlikely to

contribute to the antinociceptive effects of morphine administration in dogs. Other metabolites of morphine may possess antinociceptive effects, but this has not been demonstrated. The implication of this observation is that it may require higher levels of morphine in dogs to produce an equivalent level of antinociception compared to other animals – including humans – because of an inability of dogs to produce an active metabolite that produces additive or synergistic effects.

Plasma profiles and pharmacokinetic parameters of morphine in dogs describe the disposition of morphine, but do not provide information about the efficacy of morphine as an antinociceptive agent. Plasma morphine concentrations in people have been correlated to morphine's antinociceptive effects and can serve as a guideline to effective concentrations in dogs. However species differences in the effects of morphine may be present resulting in inaccurate assessment of the antinociceptive effects of morphine in dogs when effective plasma concentrations are derived from human literature. In addition, as mentioned above, dogs may require higher concentrations than humans because of the lack of an active metabolite.

The von Frey (vF) device is a mechanical stimulus, which can be used to quantitate the pressure thresholds in dogs and compare changes in thresholds in dogs treated with morphine and placebo. Application of the vF mechanical stimulus activates the same nociceptors that are stimulated in clinical pain. Evaluation of the change in vF thresholds following morphine administration may predict the antinociceptive effects of morphine in dogs.

Initially, the vF device was evaluated for learned behavior, tolerance, aversion, and avoidance in untreated dogs to assess the repeatability. No changes were present in

the untreated (control) dogs for 8 hours with hourly measurements (Chapter 5). Additionally, during a blinded morphine and saline infusion crossover study there were no changes in the saline thresholds values over 8 hours (Chapter 6). In comparing the untreated vF thresholds and the saline infusion thresholds, no significant differences were present at any time point in both groups, indicating vF thresholds were repeatable over time and day to day.

The next step assessed the ability of the vF device to detect differences in vF thresholds following administration of morphine sulfate (1 mg/kg) intravenously. Based on prior clinical use in dogs, this dose was expected to produce antinociceptive effects. Significant elevations in vF thresholds were observed when the dogs were evaluated for 4 hours following morphine injection. When the dogs were assessed individually, the mean ± SE time for vF thresholds to decrease to values not significantly different from baseline was 2.8 ± 0.6 hours (Chapter 5). The plasma concentration to achieve 50% maximal effect (EC_{50}) was 13.92 ± 2.39 ng/mL and the maximal effect (E_{MAX}) was 213 ± 43 %, change from baseline values (Chapter 5). The vF device was able to discriminate the antinociceptive effects of morphine given as a single i.v. injection from the untreated group.

The final phase evaluated the antinociceptive effects of morphine following multiple i.v. injections and as an i.v. infusion. Morphine was administered as repeated i.v. injections of 0.5 mg/kg morphine sulfate every 2 hours for 3 doses. Von Frey thresholds were measured hourly beginning prior to the first injection and then subsequently for 7 hours after the injection. Significant elevations in vF thresholds were maintained throughout the study period as compared to baseline (Chapter 6). Pharmacodynamic

modeling calculated an EC_{50} and E_{MAX} of 27.3 ± 7.4 ng/mL and 180 ± 67% (change from time 0), respectively (Chapter 6). Pharmacokinetic parameters from the first dose and the last dose were assessed for differences and no significant differences were found.

In an attempt to assess the antinociceptive effect resulting during morphine infusion, morphine was administered as an i.v. infusion targeting 10 ng/mL from 0-2 hours, 20 ng/mL from 2-4 hours, 30 ng/mL from 4-6 hours, and 40 ng/mL from 6-8 hours in a blinded randomized crossover with saline. No significant differences in vF thresholds over time were observed during the saline infusion. Significant elevations were present in vF thresholds at infusion rates that attained plasma concentrations targeting 30 ng/mL and greater. The lowest plasma concentration in which significantly elevated vF thresholds were present was 31.3 ± 6.0 ng/mL. The E_{MAX} was 78 ± 11% (increase from saline baseline) and the EC_{50} was 29.5 ± 5.4 ng/mL during the morphine infusion.

Despite differences in design and method of morphine administration the pharmacodynamic effect was repeated, with almost identical values from multiple dose and infusion phases (27.3 vs 29.5 ng/mL). This demonstrates the robustness of our model for identifying antinociceptive plasma concentrations of morphine. However the EC_{50} from the single i.v. bolus (13.9 ± 2.4 ng/mL) was lower than that calculated from that of the multiple dose and infusion phase (Table 8.1). This is likely due to the fact plasma concentrations of morphine were quantified, but the actual effect of morphine occurs primarily in the CNS. Plasma samples are more readily and humanely obtained in comparison to brain and spinal cord samples. A steady state concentration between the plasma and CNS is more likely to occur following multiple doses and infusion, therefore is more likely predicative of CNS concentrations necessary to elicit the antinociceptive

effects. Plasma elimination of morphine is more rapid in dogs in comparison to CSF elimination. Therefore the EC_{50} would be underestimated in conditions where steady state concentrations are not present. Morphine concentrations in the CSF do not directly measure CNS concentrations, but may represent a slower release from the CNS. Hysteresis is the condition in which the concentration - time profile is not in phase with the effect - time profile. Hysteresis appears to occur following a single i.v. injection of morphine, but it is unknown whether hysteresis would occur if CNS concentrations were assessed following a single dose.

The vF device successfully evaluated the antinociceptive effects of morphine in dogs. The calculated EC_{50} following i.v. injection, repeated i.v. injections, and i.v. infusion are in the same range of plasma concentrations considered effective in people. The vF device was well tolerated with no evidence of learned behavior, tolerance, avoidance, or aversion to its use and caused no visible tissue damage.

The oral bioavailability of methadone was assessed with and without concurrent administratation of ketoconazole or omeprazole. The oral bioavailability remained poor regardless of concurrent medication. Neither morphine or methadone when dosed orally are consistantly absorbed and plasma concentrations remain low and are unlikely to provide antinocicpetive effects dosed orally.

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Table 8.1 Comparison of the single i.v. bolus, multiple dose, and infusion EC_{50} of morphine in dogs.

	1 mg/kg IV bolus	**0.5 mg/kg multiple dose**	**Infusion**
EC_{50} (ng/mL)	13.9 ± 2.4	27.3 ± 7.4	29.5 ± 5.4

9. FUTURE DIRECTIONS

These studies have focused on the pharmacokinetic and pharmacodynamic modeling of morphine in dogs using a non-invasive von Frey (vF) device to produce a nociceptive stimulus. The vF device was well tolerated, produced no adverse effects, and produced a nociceptive stimulus that could be correlated the plasma concentrations of morphine in healthy Beagle dogs. Additionally, it was demonstrated that dogs do not produce sufficient levels of morphine's active metabolite, morphine - 6 - glucuronide, to contribute to morphine's antinociceptive effect. However, it is likely that dogs produce other metabolites that we presume are inactive. We assume that there are other metabolites produced because clearance is very high in dogs, and cannot be accounted for simply by renal elimination of unchanged drug. The presence of metabolites is the likely reason that previous studies that have used non-specific assays (eg, radioimmunoassay) have produced invalid results.

Now that (a) a validated assay exists for the specific parent drug and (b) a validated system for testing nociception has been reported, these results can be applied to other studies. An extension of the current studies could be carried out on hospitalized patients that are in need of analgesic medications. Population pharmacokinetics studies involve large numbers of animals with a limited number of samples collected from each animal. Population pharmacokinetic studies of morphine may predict pharmacokinetic differences in different disease or physiologic states. The influence of concurrently administered medications, organ dysfunctions, gender, breed, age, body condition, and genetic variability (such as expression of the P-glycoprotein efflux pump encoded by the

multi drug resistance gene, MDR-1) could also be assessed and whether or not interactions occur with the pharmacodynamics of morphine.

The pharmacodynamics of other opioids, such as fentanyl, hydromorphone, oxymorphone, meperidine, and buprenorphine are unknown, despite the common use of these drugs. Designing studies similar to the current study to assess the pharmacokinetics and pharmacodynamics of opioids commonly used in veterinary medicine would allow more accurate dosage recommendations to be implemented. The application of our techniques can also be used to study new, yet unregistered, pain medications for dogs being developed by pharmaceutical sponsors. These techniques would provide drug sponsors with a relatively simple and repeatable means to screen new drugs and estimate therapeutic dosages.

The pharmacodynamics and interactions of concurrently administered analgesic drugs have not been well evaluated in veterinary patients. As detailed in chapter 2, many different classifications of drugs have been demonstrated to provide antinociceptive effects. Drugs targeting different receptor systems may be used in combination with opioids to decrease the concentration of the opioid required to maintain their antinociceptive effects, which may decrease the adverse effects associated with the use of opioids. Combinations of drugs other than opioids could be assessed which may prove to be more useful due to increased antinociceptive effects or decreased adverse effects. Receptor systems, enzymes, and molecules that have demonstrated an ability in experimental and clinical models to provide antinociceptive effects include: norepinephrine, serotonin, GABA, glutamate (AMPA and NMDA receptors), phospholipase, cyclooxygenase (COX), and lipoxygenase (LOX). By examining the

interactions of combinations of drugs which affect these targets, new strategies may be developed to increase analgesia and decrease the adverse effects of these drugs. Conversely, drug combinations may be found which antagonize their antinociceptive effects or the adverse effects of combination therapy may outweigh any benefit on their antinociceptive properties.

The vF device could be examined in other veterinary species commonly treated for pain including cats, horses, cattle, small ruminants, and camelids. Similar to the lack of reliable pharmacodynamic knowledge of opioids in dogs, little is known about the pharmacodynamics of opioids in other commonly treated veterinary species. In addition to the antinociceptive effects of opioids, drug combinations could be assessed in other veterinary species to decrease adverse effects and increase analgesia.

There is even less information regarding the antinociceptive effects of drugs on exotic animal species. Ferrets, psittacines, raptors, reptiles, turtles, and fish are common veterinary species as pets, wildlife rehabilitation programs, and even food animal species. With increased awareness of animal welfare, increased efforts are directed at increasing the veterinary care of exotic species including emphasis on pain management. With the exception of laboratory rodents, few studies have evaluated the antinociceptive effects of drugs on exotic species, primarily due to the difficulty in evaluating these animals.

Determination of rational dosage regimens for analgesic drugs involves both pharmacokinetic and pharmacodynamic modeling. The von Frey device is a non-invasive device that could be used in a variety of species to assess the antinociceptive effects of various drugs and drug combinations in both experimental and clinical models.