Analgesic efficacy and pharmacokinetics of combinations of morphine, dexmedetomidine and maropitant in dogs

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Sandeep Raj Karna

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ABSTRACT

Multimodal analgesia is gaining popularity in veterinary medicine. It is an approach that involves the administration of two or three classes of analgesic drugs with different modes of actions to enhance the analgesic effects and lower the adverse effects associated with high dose of a single drug. In a series of experiments conducted in this thesis, the combinations of morphine, dexmedetomidine and maropitant were evaluated using different pain models with the aim of using them in a multimodal strategy in dogs undergoing ovariohysterectomy or other surgical procedures.

Firstly, a pilot study evaluating the efficacy of combinations of the test drugs was performed using a hot-plate test and tail-flick test in rats. The combination of morphine and maropitant showed a significantly higher ($p < 0.0001$) tail-flick latency compared to all other treatment groups indicating a supra-additive effect of spinal analgesia between morphine and maropitant.

A pharmacokinetic study to investigate the disposition of the test drug combinations after intramuscular (IM) administration in dogs under anaesthesia was conducted. The results showed that the elimination half-life of morphine was higher and the clearance rate was lower when combined with dexmedetomidine compared to morphine and maropitant combination or morphine alone at higher doses. This effect may have a clinical advantage of prolonging the dosing interval of morphine.

A study to evaluate and compare the analgesic efficacy of the combination of morphine, dexmedetomidine and maropitant in dogs undergoing ovariohysterectomy was conducted. The study showed that dogs receiving the combination of morphine and

dexmedetomidine had significantly lower ($p < 0.05$) pain scores, obtained by the short form of the Glasgow composite measure pain scale and visual analogue pain scale, in the postoperative period. All dogs that received dexmedetomidine showed arrhythmia and second-degree heart block immediately after IM administration.

Finally, the efficacy of the test drug combinations was evaluated using changes in electroencephalographic indices of nociception (median frequency, spectral edge frequency and total power) in anaesthetised dogs subjected to a noxious electrical stimulus. The combination of morphine and dexmedetomidine showed a significantly lower change in the post stimulation median and spectral edge frequencies compared to all other treatment groups. The dogs receiving dexmedetomidine also demonstrated arrhythmia and second-degree heart block.

In conclusion, the combination of morphine and dexmedetomidine showed a superior analgesic effect compared to morphine alone at higher dose and appeared to be the most effective combination among other combinations of morphine, dexmedetomidine and maropitant. The cardiovascular changes produced by the test drugs may be clinically insignificant in fit and healthy dogs. In future, the efficacy of the combination of morphine, dexmedetomidine and maropitant at other different doses rates and ratios should also be evaluated.

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DECLARATION

Each experimental chapter (Chapter 3-6) in this thesis is in the form of a paper for different journals where it is published or intended to be published. Consequently, inconsistencies exist in the format, writing and referencing styles between the chapters. Also, there are some repetitions in introduction and methodology sections between the chapters. Although co-authors of the publications contributed to the various aspects of the studies involved in thesis, my input was greatest which is confirmed by the DRC-16 form attached at the end of Chapter 3-6.

LIST OF ABBREVIATIONS

ASA	American society of anesthesiologists
AUC	Area under the curve
CCN	Center for canine nutrition
CL	Clearance rate
C_{max}	Maximum plasma concentration of a drug
CMPS-SF	Composite measure pain scale-short form
CNS	Central nervous system
COX	Cyclooxygenase
ECG	Electrocardiogram
EEG	Electroencephalography
EtCO2	End-tidal CO_2 tension
EtHal	End-tidal halothane tension
F50	Median frequency
F95	Spectral edge frequency
FFT	Fast Fourier transformation
*f*R	Respiratory rate
GABA	Gamma aminobutyric acid
GLM	Generalized linear model
GPCR	G-protein coupled receptor
HP	Hot plate
HR	Heart rate
IM	Intramuscular

ISAP	International association for study of pain
IV	Intravascular
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LOQ	Lower limit of quantification
LRF	Lateral reticular formation
LSMEANS	Least squares means
M-3-G	Morphine-3-glucuronide
M-6-G	Morphine-6-glucuronide
MAC	Minimum alveolar concentration
MAM	Minimal anaesthesia model
MRT	Mean residence time
N/OFQ	Nociceptin/orphanin FQ
NDS	Numerical descriptive scale
NK1	Neurokinin 1
NRS	Numerical rating scale
NSAID	Nonsteroidal anti-inflammatory drugs
NTT	Nociceptive threshold testing
OHE	Ovariohysterectomy
PAG	Periaqueductal grey matter
PCV	Packed cell volume
P_E'CO2	End-tidal partial pressure of carbon dioxide
PK-PD	Pharmacokinetics-pharmacodynamics
PP	Plasma protein
Ptot	Total power
RSD	Relative standard deviation

RVM	Rostral ventromedial medulla
SC	Subcutaneous
SD	Standard deviation
SDS	Simple descriptive scale
SE	Standard error
SpO2	Arterial haemoglobin oxygen saturation
$T_{1/2}$	Elimination half life
TF	Tail flick
T_{max}	Time to reach the maximum concentration of a drug
VAS	Visual analogue scale
Vd_{ss}	Volume of distribution at steady state
V_z	Terminal slope

CHAPTER 1 GENERAL INTRODUCTION

Pain is an important aspect of determining well-being and overall health in animals. Pain from surgery and other clinical or non-clinical conditions such as disease, injury, routine husbandry practices etc. has significant negative effects on animal welfare and can lead to prolonged recovery, increased risk of infection, delayed wound healing, decreased food and water intake and alteration in sleep patterns and behaviour (Tranquilli *et al.* 2004). Therefore, it is an ethical responsibility of veterinary professionals to prevent the occurrence of pain or to reduce it significantly if it occurs.

With the expansion in the knowledge of pain physiology and the effect of different analgesic drugs, the number of drugs being available to treat pain in veterinary patients, including dogs has also increased in the recent years. In addition to the traditional analgesics (opioids, NSAIDs and local anaesthetics), several adjuvant analgesics are used in combination with mainstay analgesics especially in the companion animals (Lamont and Mathews 2007). These adjuvant analgesics are either adopted from the human studies or from the findings of the laboratory animals research or anecdotal experiences of the clinicians (Lamont and Mathews 2007; Ruel and Steagall 2019). This act of combining the drugs from different classes is termed as multimodal analgesia. In addition to the enhanced analgesic effects, combining two or more classes of drugs may also lead to decrease in adverse effects associated with higher doses of a single drug (Young and Buvanendran 2012).

Co-administration of morphine with dexmedetomidine and maropitant could be a potential multimodal analgesic protocol for providing effective peri- and post-operative analgesia in dogs. This recommendation is based on potential synergies in the mechanism of action of these drugs in blocking different pain pathways and is explained in detail in the literature review (Chapter 2).

1.1 The research problem

Drug interaction is a complex phenomenon. The outcome of using a combination of drugs could be synergistic (supra-additive), additive or sub-additive and this result can be seen for both the beneficial analgesic effects and the limiting adverse effects (Miranda *et al.* 2008; Raffa *et al.* 2010). A thorough examination of the interaction between the drugs is needed to evaluate the efficacy and safety of their combination. Although morphine, dexmedetomidine and maropitant are used in companion animal medicine, the analgesic efficacy and safety of the three drugs in combination has not been evaluated previously.

1.2 Thesis structure

This thesis consists of a series of novel experiments to evaluate and compare the efficacy and safety of different combinations of morphine, dexmedetomidine and maropitant. Following the general introduction of the research problem in this chapter, Chapter 2 presents the review of the literature pertaining to the pain physiology, pharmacology of the analgesic drugs and the pain assessment techniques relevant to this study. This is followed by four separate experimental studies (Chapters 3-6) formatted according to the guidelines of the submitted journals. Appendices, which consist of additional relevant information but not presented in the publications due to word limits

or other reasons have been placed at the end of each chapter.

Experiment 1 (Chapter 3) tested if the combinations of morphine, dexmedetomidine and maropitant enhances the analgesic effect and decreases the dose of individual drugs using rat models (hot-plate and tail-flick tests). This was a pilot study conducted to generate some indicative data about the interaction between the drugs and has been published in the journal *Veterinary Anaesthesia and Analgesia*.

Experiment 2 (Chapter 4) investigated the pharmacokinetics of the drug combinations after intramuscular administration in dogs under anaesthesia. This study was performed in combination with the EEG experiment in chapter 6. This study has been published in the *Journal of Veterinary Pharmacology and Therapeutics*.

Experiment 3 (Chapter 5) evaluated the analgesic efficacy of combination of morphine, maropitant, and dexmedetomidine in dogs undergoing ovariohysterectomy using the Glasgow composite pain scores and the visual analogue scale. Pharmacokinetics analysis of the drugs in this study was also performed. A manuscript on this study is under revision in the *New Zealand Veterinary Journal*.

Experiment 4 (Chapter 6) studied the effect of combinations of morphine, dexmedetomidine and maropitant on electroencephalographic indices of nociception in response to acute noxious electrical stimulation in anaesthetised dogs. A manuscript based on this study has been pubished in the *Journal of Veterinary Pharmacology and Therapeutics*.

Chapter 7, the final chapter of this thesis, comprises of the general discussion and conclusions from all the experiments.

CHAPTER 2 LITERATURE REVIEW

2.1 Definition and the neurophysiology of pain

The widely accepted definition of pain was given by the International Association for the Study of Pain (IASP) which defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage” (Merskey 1979). According to IASP's definition, there are two aspects of pain experience: sensory and emotional. Sensory experience, also known as nociception, involves somesthetic senses like pain, touch and heat. The emotional experience involves cognitive and emotional processing of nociceptive stimuli by the brain; the stimuli could be existing or imaginary (Abbracchio and Reggiani 2013). In summary, pain is a conscious and highly subjective experience, which is affected by memories, emotional, genetic, cognitive and pathological factors (Abbracchio and Reggiani 2013). The complex nature of pain makes it further difficult to treat in animals because they cannot verbalize the extent of discomfort they experience or the effectiveness of analgesics (Anil *et al.* 2002).

For clinical purposes and depending on duration, pain can be categorised as acute and chronic. Acute pain is short-lived and often has obvious cause such as trauma, burns, inflammation and infarctions. It is often associated with autonomic nervous signs including tachycardia, diaphoresis or elevation in the blood pressure (Gaynor and Muir 2014a). Acute pain normally disappears after the disappearance of the initial damage. However, maladaptive mechanisms may occur and pain may persist after healing of the injury leading to the transition to chronic pain (McGreevy *et al.* 2011). Chronic pain

persists beyond the normal time of healing (Loeser and Treede 2008). It is complex and often difficult to manage and is recognised as a disease itself.

2.1.1 Anatomy of the pain pathway

There are four main components of the pain process: 1) **transduction** of noxious stimuli (mechanical or heat energy) to electrical signals at free "unencapsulated" nerve endings branching from main axons in the injured tissue, 2) **transmission** of electrical signals from the site of tissue injury to the central nervous system (CNS), 3) **modulation** to reduce activity in the transmission system, and 4) **perception** of signals as pain (Gaynor and Muir 2014b).

The different components involved in processing of the pain related information are described below:

2.1.1.1 Peripheral/afferent pathway

Sherrington (1906) proposed the term and definition of "nociception" after noticing that pain occurs after tissue injury (Sherrington 1906). The mechanical, thermal or chemical stimulus that produces or has the potential to produce tissue damage is called a noxious stimulus and the naked nerve endings capable of detecting such stimulus are called as nociceptors (Loeser and Treede 2008). This process of transduction of noxious stimuli into electrophysiological activity occurs at specialized free endings of nociceptive primary afferent neurons.

Different types of nociceptors have been identified, which are classified according to the afferent sensory nerve fibres involved. In the peripheral nervous system, there are

three main types of sensory fibres: Aβ-fibres, Aδ-fibres, and C-fibres (Smith and Lewin 2009). Aβ-fibres have the fastest conductance of action potential because they are highly myelinated and are large in diameter (Rice and Albrecht 2008). They have a low activation threshold (Brown and Iggo 1967) and are responsible for the transmission of tactile information (Ikeda *et al.* 2014). Aδ-fibres are smaller in diameter and thinly myelinated and thus can conduct the information slower (5-30 m/s) than Aβ-fibres. As reported from human studies, they are associated with sharp pricking pain (Stucky *et al.* 2001; Wickremaratchi and Llewelyn 2006). C fibres are unmyelinated, slower conducting (0.5-2.0 m/s), and have the highest thresholds for activation. They are associated with the slower burning type of pain and comprise 70% of all nociceptors in rats (Stucky *et al.* 2001).

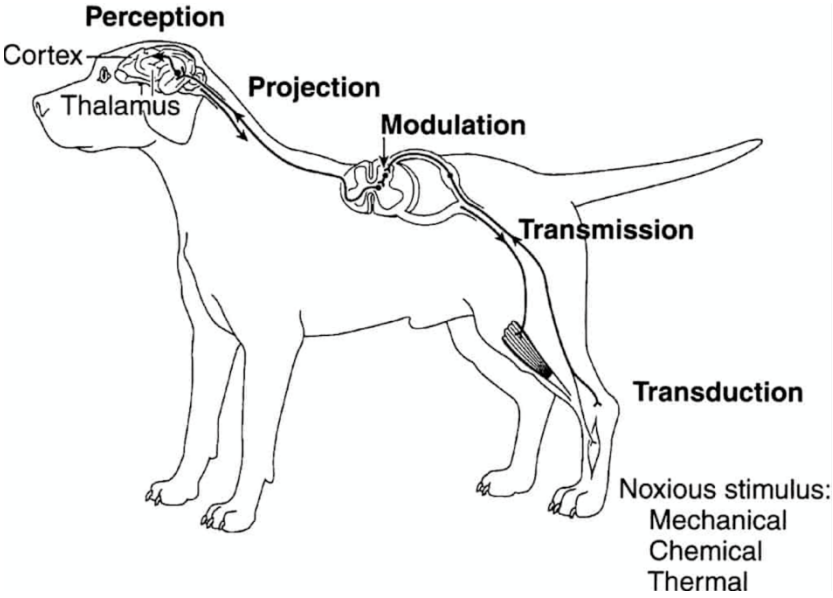


Figure 2.1 Parts of pain pathways involved in nociception in a dog.

From: Gaynor and Muir (2014b)

2.1.1.2 The spinal cord

The dorsal horn of the spinal cord is a key region where afferent information is received, modulated and transferred to the higher brain structures. The primary afferent neurons from receptors in the skin, subcutaneous tissue, muscle fascia, joint capsules and visceral organs enter dorsal horn and synapse with intrinsic spinal dorsal horn neurons (Brown 2012). The dorsal horn is organised into a series of parallel laminae (Rexed 1952). Lamina I is the marginal layer and the lamina II includes substantial gelatinosa, which is a narrow cellular band near the dorsal limit of the grey matter (Ralston and Ralston 1979). Rest of the dorsal horn consists of laminae III-VI (Rexed 1952).

Most nociceptive afferents Aδ and C-fibres terminate superficially in laminae I– II, although a small number reach deeper laminae (Sugiura *et al.* 1986; Todd 2002). The Aβ-fibres terminate in the deeper laminae (III-VI). Some afferent neurons are discovered to be entering the spinal cord through ventral horns (Brown 2012). They are found to be non-myelinated and primarily originate from visceral organs and their mode of termination in the spinal cord is not well known. After entering the spinal cord, primary afferent fibres bifurcate into ascending and descending branches and can enter the dorsal horn to the segments lower or higher than the segment of their entrance to the spinal cord (Purves *et al*. 2001).

While many compounds are proposed as potential endogenous neurotransmitters, the current knowledge suggests that the major neurotransmitters released in response to the noxious stimulus from primary afferent neurons in the dorsal horn are the excitatory amino acids: aspartate and glutamate, and the peptide: substance P (Budai 2000).

The projection neurons from Lamina I project to several areas in the brain including the thalamus, periaqueductal grey matter (PAG), lateral parabrachial area, the nucleus of the solitary tract and medullary reticular formation (Giesler *et al.* 1979; Menétrey *et al.* 1982; Cechetto *et al.* 1985). About 80 % of the lamina I projection neurons express NK1 receptors (Todd 2002). Binding of substance P to NK1 receptors has many functions including the transmission of afferent noxious input. Substance P is synthesized and transported by a subpopulation of the neurons in the dorsal root ganglions and is released in the spinal cord in response to noxious stimuli in the peripheral tissue and acts on the NK1 receptors (Hökfelt *et al.* 1975; Brimijoin *et al.* 1980). Substance P and NK1 receptors also play a pivotal role in the development of hyperalgesia (Mantyh *et al.* 1997). The Lamina I neurons also project into to rostral ventromedial medulla (RVM) from which the descending neurons project back to the dorsal horns. Projection neurons are also found in the deeper laminae III-IV which project primarily to the thalamus. This makes most spinothalamic tract and mainly carries sensory information.

2.1.1.3 Ascending nociceptive pathways to higher centres

Nociceptive information from the spinal cord is transmitted to the brain via ascending pathways (second-order neurons). Nociceptive information to the thalamus is transmitted by the contralateral spinothalamic tract and to the brain stem via spinoreticular (spinoparabrachial) and spinomesencephalic tract (Craig and Dostrovsky 1999; Schaible 2006). These tracts possess different functions depending on their origin and destination. Spinoreticular projections to the brainstem are important for the autonomic component of the pain response, which also acts as an indirect means to

transmit the nociceptive message to the forebrain after being processed by the brainstem. Studies show that brainstem plays a central role in mediating the stimuli related to pain perception in animals (Tracey and Mantyh 2007). The nociceptive information to the various cortical regions is mainly transmitted via the thalamus (Craig and Dostrovsky 1999).

2.1.1.4 Pain perception and processing in supraspinal centres

Pain perception is a complex phenomenon and much more than processing the sensory inputs. The nociceptive input does not have a linear relationship with the resultant pain experienced by a human or an animal. In humans, the pain has three facets: sensory-descriptive aspect, affective-motivational and cognitive-evaluative (Melzack and Casey 1968; Lamont *et al.* 2000; Hellyer *et al.* 2007). The sensory-descriptive aspect involves the perception of the location, intensity, and quality of pain, and is mediated by somesthetic cortex in the brain. The affective-motivational aspect consists of behavioural, emotional and autonomic responses, and involves reticular formation, hypothalamus and limbic system. This aspect is associated with suffering due to pain and there is substantial evidence that the neural pathways associated with this aspect are well developed in animals (Hellyer *et al.* 2007; Becker *et al.* 2018). Finally, the cognitive-evaluative aspect involves the effect of experience, attention and conditioning and is mediated by the higher cortical functions. The cognitive-evaluative aspect deals with the anticipation factor (complex feelings like how long will it hurt? Am I going to die?), and this is not well developed in animals (Hellyer *et al.* 2007).

2.1.1.5 Descending pathways/ signal modulation

Descending pathways originate from the brainstem and other cerebral structures and play an important role in the modulation of nociceptive information. These pathways can be inhibitory (anti-nociceptive) or facilitatory (pro-nociceptive) (Heinricher and Fields 2013). The inhibitory pathways are active during fight and flight response and opioid analgesia (Heinricher and Fields 2013). Sustained activation of facilitatory circuit is found during chronic pain. The neurotransmitters involved in inhibition are norepinephrine, serotonin, dopamine, opioids, Gamma aminobutyric acid (GABA), cannabinoids and adenosine and those involved in facilitation are substance P, glutamate, nerve growth factor and cholecystokinin (Shilo and Pascoe 2014).

Studies have shown that stimulation of higher brain centres such as periaqueductal gray (PAG) and the lateral reticular formation (LRF) in the midbrain can inhibit the firing of dorsal horn neurons in response to noxious stimuli (Abbracchio and Reggiani 2013). Interaction between different descending systems and their normal physiological function is still not well understood.

2.2 Pain assessment methods in animals

Lack of verbal communication is the major obstacle in evaluation and characterisation of pain in animals. In the absence of verbal communication, we must depend on alternative methods to evaluate pain in the animals. Some of such useful methods are: measurement of physiological parameters; behavioural methods including pain scales; and utilisation of neurophysiological techniques (Livingston 2010). These methods are discussed below:

2.2.1 Measurement of physiological parameters

Based on the assumption that pain can induce changes in the autonomic nervous system, several physiological parameters have been used in the assessment of pain (Cowen *et al.* 2015). Such tools include parameters such as heart rate, respiratory rate, blood pressure, pupillary diameter etc. Measuring physiological parameters can be easy and useful methods of pain assessment especially if the normal values of the parameters have been established (Anil *et al*. 2002). An advantage of using these parameters is that they are objective tools and are not affected by observer-based variability as in subjective methods of pain assessment (Hernandez-Avalos *et al.* 2019). However, they can be altered by other confounders such as stress, depth of anaesthesia, medication etc. Thus, use of only one of such parameters may not be reliable in the precise assessment of pain (Gaynor *et al.* 2014b).

Neurohumoral substances such as plasma concentration of adrenal corticosteroids and associated releasing hormones from the anterior pituitary have also been used in the assessment of pain. These substances do not directly assess the pain experienced by the animal but help in identification and grading of pain-induced distress (Mellor *et al.* 2000).

2.2.2 Behavioural methods

Changes in behaviour can be used to recognise and assess pain in animals (Reid *et al.* 2018). An advantage of using behavioural method is that changes in the behaviour of animals in response to pain is immediate (Le Bars *et al.* 2001). It has been shown that the changes in behaviour correlate with physiological signs of distress in various farm animals (Carlstead *et al.* 1993). Several behaviours which can be used to indicate pain

and distress in animals have been studied which includes postures, facial expressions, stereotypical movements, vocalizations etc. For reliable assessment of pain, it is important to describe the subclasses of these behaviours and describe the changes in those subclasses in response to noxious stimuli (Carlstead *et al.* 1993; Morton *et al.,* 1985).

Although the changes in behaviour reflect the presence of pain in animals, quantification of pain based on these behaviours remain a challenge (Hansen 2003). Further, any change in pain experienced may not be reflected as a proportionate alteration in the behaviour of an animal (Livingston 2010).

Several tests and pain scoring systems based on changes in behaviour in response to noxious stimulus have been developed to attempt quantification and compare analgesic drugs and regimen. The behavioural methods of pain assessments used in this PhD research were hot plate and tail-flick tests in rats and pain scoring systems in dogs which are described below:

2.2.2.1 Hot plate and tail-flick tests in rats

The hot plate (HP) and tail-flick tests (TF) are the oldest and widely used tests to measure behavioural response to pain, and to study analgesic efficacy of drugs in rats and mice (D'Amour and Smith 1941; Eddy and Leimbach 1953). These tests are forms of nociceptive threshold testing (NTT) involving stimuli of short duration and of fixed or increasing intensity. Also, the reaction time related to the threshold is measured (Le Bars *et al.* 2001). They are easy to perform, require minimal instrumentation, and have well-defined endpoints.

Hot plate test involves introducing a rat into an open-ended cylindrical space with a floor consisting of a metallic plate that is heated by a thermode or a boiling liquid and measures the nociceptive response of the rat (Le Bars *et al.* 2001). Behaviours such as forepaw or hind paw licking and/or jumping act as the endpoint. Tail flick test measures the nociceptive threshold to infrared heat stimulus on the rat's tail. There are two variants of the tail-flick test: one involves the application of the radiant heat to a small surface of the tail and another involves immersing of the tail at the pre-set temperature. The tail-flick test using radiant heat in rats is a simplified version of a test used in humans (Hardy *et al.* 1940). The application of the heat to the tail of the rat causes pain and provokes the withdrawal of the tail by a brief vigorous movement. The reaction time after application of the heat source is called tail-flick latency (Le Bars *et al.* 2001). Although two forms of tail flick test appear to be similar, they differ in terms of the surface area of the tail stimulated.

Even though the hotplate and tail flick tests are widely used and simple to execute, they have some limitations. Given that these tests were developed and used mainly to test opioid analgesics, they may be less sensitive for non-opioid analgesics (Le Bars *et al*. 2001). The hotplate test relies upon behaviours such as forepaw or hind paw licking and/or jumping as the endpoint. The paw licking behaviour is more stereotyped for mice and is complex in rats. These behavioural endpoints may be affected differently by different drugs and this could affect the test sensitivity (Carter 1991). Another limitation of the tail flick test is that it is based on spinally integrated reflex and doesn't assess analgesia involving supraspinal sites (D'Amour *et al*. 1941).

2.2.3 Pain scoring systems

The first pain scales for the clinical studies in non-rodent species were modified and adapted from the human pain scales, particularly those used for evaluation of acute pain in the prelingual children (McGrath 1987; Hansen 2003). In human patients, the intensity of the pain experienced is self-assessed by the patients and is recorded in the scales (McGrath 1987). In veterinary patients, the behaviour related to pain is observed and scored by an experienced veterinarian or a technician. These scales rate the behaviour of the animals related to pain based on the intensity of the pain experienced by them.

The most commonly used pain scales in domestic animals are simple descriptive scale (SDS), the numerical rating scale (NRS), the visual analogue scale (VAS) and multiparametric or composite scales.

An SDS uses four or five points based on description of the pain related behaviour of an animal assessed by an experienced veterinarian or a technician (Taylor and Houlton 1984; Waterman and Kalthum 1989). For example, Taylor and Houlton (1984) used an SDS for comparison of postoperative analgesic effect of morphine, buprenorphine and pentazocine in dogs where 0= unsolicited howling, 1= resents manipulation of the operation site, 2= comfortable but slightly uneasy, and 3= very comfortable (Taylor and Houlton 1984). The advantages of this scale are that it is simple to use, it is not specific to any species and the observations are not influenced by the visual acuity. However, this scale has drawbacks such as low sensitivity and significant observer variability (Gaynor and Muir 2014b).

The **NRS** uses multiple behavioural categories which are assigned whole numbers. Each category has a descriptive definition of pain (Gaynor and Muir 2014b). NRS has been extensively used in humans and has been shown to be reliable and reproducible in measuring clinical pain in dogs (Holton *et al.* 1998a; Holton *et al.* 1998b). NRS involves thorough examination of the patients based on numerous categories related to pain, thereby having an advantage of being more sensitive than SDS. A limitation of NRS is that whole numbers are assigned to the categories and the scale assumes that there are equal differences between the categories, which may not be true. Also, this scoring system has not been shown to be sensitive in assessing surgical pain and evaluating the differences between the analgesics in treating surgical pain in animals (Gaynor and Muir 2014b).

The **VAS** is a very simple observer-based scaling system. The VAS consists of a straight line usually 10 cm long whose ends relates to the extremes in the pain intensity (Scott and Huskisson 1976). The observer is asked to mark a position corresponding to the intensity of the pain and the distance to the extreme marked with no pain is the pain score. VAS has shown to be more sensitive than SDS (Scott and Huskisson 1976; Holton *et al.* 1998b) and is proven to be satisfactory for subjective measurement of pain.

A challenge of using VAS in all species is the quantification (Holton *et al.* 1998b). The degree of pain experienced is not always correlated with the extent of the alteration in the behaviour. In dogs, like in humans, the peripheral environment can modify the pain response. The postoperative pain response shown by a dog in the recovery room of the

hospital may be different from that in their home environment. Also, this scale is prone to significant observer variability.

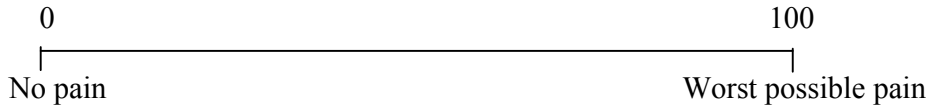


Figure 2.2: Visual analogue scale for scoring of pain.

Glasgow Composite Measure Pain Scale (CMPS), a pain scoring system developed by Holton *et al*. (2001), was the first scale utilising multiple behaviours and the assessor-animal interaction factors for the assessment of the acute pain in domestic animals in clinical and surgical settings (Holton *et al.* 2001). It is in the form of a structured questionnaire to be filled by the observer assessing evoked behaviours and clinical observations. The behaviours are assigned a score, addition of which provides a final score that is indicative of the level of pain and analgesic intervention required. CMPS is unique in veterinary medicine because it was designed using psychometric principles, which is an established process in human medicine (Reid *et al.* 2007).

The CMPS has advantages of being more accurate and involving less observer-based bias in assessing the pain compared to SDS, NRS and VAS. The observer only needs to identify the presence or absence of the behaviour related to pain and does not require specialised skills and experience (Reid *et al.* 2007; Gaynor and Muir 2014b). However, a limitation of CMPS is that it is time-consuming to complete the questionnaire, which might limit its use in a clinical practice situation. Further, the pain scale has been

validated for its use in assessing acute pain in dogs in a hospital setting but not in clinical trials after elective surgery (Holton *et al.* 2001).

For the routine clinical use, a user-friendly, short-form (CMPS-SF) was developed (Reid *et al.* 2007). The CMPS-SF considers six behavioural categories with descriptive items: vocalisation, attention to wound, mobility, response to touch, demeanour, and posture/activity (see appendix 5). The items are placed in increasing order of intensity of pain and the assessor chooses the item that best describes the dog's behaviour. Each item is assigned a score, which is summed to achieve the total score; the maximum total score is 24 if the dog is able to walk and 20 if mobility is not possible. The recommended intervention level for analgesia is 6/24 or 5/20 (Reid *et al.* 2007).

2.2.4 Neurophysiological technique: EEG

Neurophysiological techniques such as electroencephalography (EEG) have long been used to analyse the depth of anaesthesia in humans for both clinical and research purposes (Rampil and Matteo 1987; Long *et al.* 1989; Schwender *et al.* 1996).

The electroencephalogram is the summative electrical activity of the population of neurons (mainly pyramidal type) in the cerebral cortex recorded by the electrodes placed at various locations on the animal's head (Brazier 1977; Niedermeyer and da Silva 2005). Postsynaptic potentials are generated when the action potentials travelling along the nerve fibres reach the synapse, which can be excitatory or inhibitory depending on the type of neuron they are generated by. The excitatory and inhibitory postsynaptic potentials create electrical activity in the extracellular fluids immediately adjacent to the postsynaptic neuronal membrane. Because neurons are elongated in

structure, the changes in the extracellular potential along the long cell membrane of neurons are summated, resulting in an overall electrical vector. The far-field (recorded at a distance from their sources) potential of this vector, as recorded between the two electrodes, is the electroencephalogram and represents the overall activity in the cerebral cortex.

The EEG frequency spectrum can be derived from the raw electroencephalogram by a mathematical process called fast Fourier transformation (FFT), which converts the EEG signal from the time domain to the frequency domain. The frequency variables such as median frequency (F50), 95% spectral edge frequency (F95) and total power (Ptot) are obtained from the frequency spectrum. The F50 is the frequency below which 50% of the total power is located and F95 is the frequency below which 95% of the total power is located (Figure: 2.1).

During unconsciousness and anaesthesia, the brain exhibits synchronised EEG activity which is manifested as a low frequency and high amplitude waves in the electroencephalogram (Simons *et al.* 1989). The synchronisation occurs when a large group of neurons depolarise at the same frequency. The awake and conscious state and arousal are characterized by a high frequency and low amplitude waves in the electroencephalogram, also referred to as de-synchronization (Simons *et al.* 1989). Application of the acute noxious stimuli can also shift the electroencephalogram towards higher frequency and lower amplitude indicating an increase in the cortical arousal (Grint *et al.* 2015).

The electroencephalogram primarily reflects the activity of the cerebral cortex. However, brain stem and thalamus can also have a strong regulatory influence on the

cerebral cortex especially during unconsciousness and anaesthesia and thus the electroencephalogram in such states is also influenced by the lower brain centres (Simons *et al.* 1989).

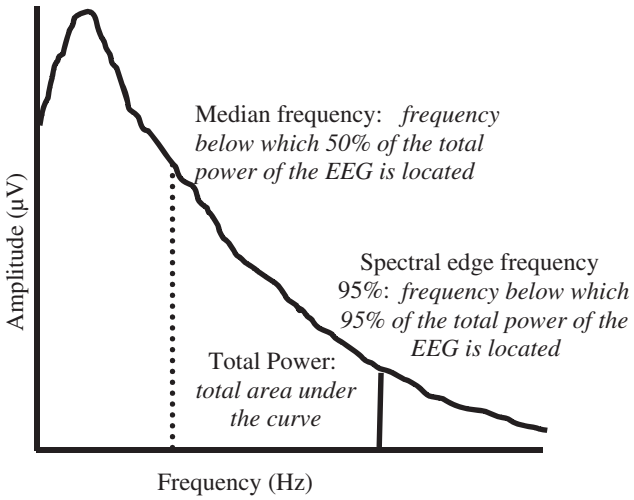


Figure 2.3: The schematic representation of the three frequencies (F50, F95 and Ptot) used in EEG spectrum analysis.

From: Murrell and Johnson (2006).

2.2.4.1 EEG for nociception studies and the minimal anaesthesia model

The cerebral cortex receives thalamic nociceptive input directly and is involved in pain processing (Schnitzler and Ploner 2000). Therefore, EEG can be a useful technique to study pain processing and nociceptive pathways. Studies show that changes in the electroencephalogram correlate with subjective pain scores in humans (Chen *et al.* 1989) and behavioural responses to a nociceptive stimulus in animals (Ong *et al.* 1997). Several studies have investigated the effect of externally applied noxious stimulus on the electroencephalogram of humans under anaesthesia. Some of them have found that a

de-synchronisation is associated with such stimulus (Rampil and Matteo 1987; Wilder-Smith *et al.* 1995; De Beer *et al.* 1996) whereas others (White and Boyle 1989; Schraag *et al.* 1998) have failed to show such association.

The minimal anaesthesia model (MAM) developed by Murrell and Johnson (Murrell *et al.* 2003; Johnson *et al.* 2005b) has been used to evaluate nociceptive responses and antinociceptive efficacy of analgesic drugs/regimes in a wide variety of mammals, including horses and ponies (Johnson *et al.* 1999; Murrell *et al.* 2003), cattle (Gibson *et al.* 2007), sheep (Johnson *et al.* 2005a), deer (Johnson *et al.* 2005c), pigs (Haga and Ranheim 2005), dogs (Kongara *et al.* 2010; Kongara *et al.* 2012; Kongara *et al.* 2014b), cats (Kongara *et al.* 2014a) and rats (Kongara *et al.* 2014c; Singh *et al.* 2018). Since the EEG can be affected by the depth of anaesthesia, the choice of anaesthetic agents and other analgesics, the MAM standardizes these conditions for the use of EEG to assess nociception. In this model, animals are kept in a "light" plane of anaesthesia that maintains unconsciousness but allows generation of the changes in EEG due to noxious stimulus. The animal studies mentioned above have shown that the noxious stimulus is associated with an increase in F50 and F95 and decrease in Ptot, with F50 being the most sensitive indicator of nociception. The MAM is also an ethical model of nociception because the animals are unconscious under a light plane of anaesthesia but still able to respond to noxious stimulation.

The MAM involves anaesthetising the animal with halothane following the induction and maintaining the animal in the low plane of anaesthesia at or near minimum alveolar concentration (MAC) of halothane for that species (Murrell *et al.* 2003; Johnson *et al.* 2005b). The baseline electroencephalogram is recorded once the target plane of

anaesthesia is achieved which is followed by a brief noxious stimulus. The post-stimulus electroencephalogram is measured which is compared with the baselines to determine the change in EEG in response to the noxious stimulus.

Halothane is used as an anaesthetic of choice because this agent causes less cortical suppression compared to other anaesthetics (Johnson and Taylor 1998). Also, halothane does not have analgesic properties (England and Jones 1992) and thus does not interfere assessing nociception and the effect of analgesics being tested using this model.

2.3 Ovariohysterectomy and pain

Ovariohysterectomy (OHE) is a routine elective surgical procedure in dogs. The Centre for Veterinary Medicine of the US Food and Drug Administration considers OHE as a moderately painful procedure (Connolly 2000; Hansen 2003). The pain during and after OHE is from the surgical incision (somatic pain), manipulation of abdominal visceral organs, stretching of the ligaments (visceral pain), and inflammation (Hansen *et al.* 1997; Guerrero *et al.* 2016). It is still unclear whether somatic or visceral pain predominates following OHE, since results from previous studies are conflicting. Carpenter *et al.* (2004) showed that intraperitoneal administration of lidocaine, a local analgesic, at the level of the manipulated viscera after OHE in dogs decreased the postoperative pain scores indicating that pain originating from visceral manipulation and stretching of the suspensory ligament is the important source of pain form OHE. Similarly, Fitzpatrick *et al.* (2010) demonstrated that that application of bupivacaine at abdominal incision sites in dogs undergoing OHE did not show improved pain, indicating that the pain from stretching of suspensory ligament might be the main source of pain during the procedure. However, Bubalo *et al.* (2008) showed no

additional analgesic benefit after lidocaine infiltration of the ovarian pedicle during OHE, which contraindicates the belief that pain from stretching of the suspensor ligament is the predominant source of pain during OHE.

2.3.1 Use of analgesics during OHE

The analgesics commonly used for management of pain from OHE and other surgical procedures in dogs belong to three major class: opioids, nonsteroidal anti-inflammatory drugs (NSAIDs), and local anaesthetics. In addition, analgesic adjuvants, the drugs which are primarily used for other purposes than pain but possess analgesic properties, are also occasionally used in combination with other analgesics for controlling pain from OHE (Lamont and Mathews 2007).

The full opioid agonists available for the use in veterinary medicine include morphine, methadone, oxymorphone, hydromorphone, pethidine, codeine, fentanyl, remifentanil, alfentanil, etorphine, sufentanil and carfentanil (Lamont and Mathews 2007; Borer-Weir 2014). Buprenorphine is a partial agonist and butorphanol and nalbuphine are classified as agonist-antagonist drugs. The opioid receptors and their mechanism of action are described below in the section on morphine.

The NSAIDs are among the most commonly used drugs to treat pain in humans and animals. Salicylates, a naturally occurring NSAID obtained from willow barks, have been used for a thousand years to treat pain (Vane and Botting 2003). The acetylated salicylic acid commonly known as aspirin formed the first synthetic NSAID. Aspirin is still the most common household analgesic in humans (Burke *et al.* 2006). The cyclooxygenase enzymes (COX-1 and/or COX-2) are involved in the synthesis of

biological mediators called prostaglandins and thromboxane (Vane and Botting 1998). Prostaglandins, in addition to their number of physiological roles, have a key role in mediating inflammation and thromboxanes are involved in blood clotting. The pharmacological action of NSAIDs is through the inhibition of cyclooxygenase enzymes resulting in inhibition of prostaglandins and thromboxane (Vane and Botting 1998). The NSAIDs available to be used in veterinary medicine include carprofen, meloxicam, ketoprofen, phenylbutazone, suxibuzone, flunixin, firocoxib, robenacoxib, mavacoxib and tepoxalin (Borer-Weir 2014).

Local anaesthetics are a class of chemically related compounds that reversibly block the sensory nerve fibres resulting in blockage of peripheral and central pathways of pain (Vandam 1987). These drugs act by blocking the Na^+ channels which result in the prevention of movement of Na^+ ions across the concentration gradient from the extracellular fluid leading to the prevention of depolarisation and blocking the transmission of the action potentials (Butterworth *et al.* 1990) . They completely block the sensory nerve fibres and prevent the development of the central sensitization of pain. The local anaesthetics available to be used for veterinary patients are procaine, amethocaine, lidocaine, prilocaine, mepivacaine, bupivacaine and ropivacaine (Borer-Weir 2014). Lidocaine and bupivacaine are used to provide local and regional analgesia during OHE (Carpenter *et al.* 2004).

Analgesic adjuvants are the diverse group of drugs that are typically newly adapted from human medicine and laboratory animal research. These drugs are basically administered with traditional analgesics (opioids, analgesics and local anaesthetics) and can enhance the analgesic efficacy of the later (Lamont and Mathews 2007). These days

several analgesics are used as adjuvants, out of which ketamine, gabapentin, alpha-2 adrenoceptor agonists, amantadine and tramadol are few popular ones (Lamont and Mathews 2007).

A survey involving 282 New Zealand veterinarians showed that opioids were used as a premedication in 95% of the anaesthesia and analgesia protocols during OHE and morphine was the most commonly (69%) used opioid (Gates *et al.* 2020). This study didn't reveal the percentage of protocols that used a combination of two or more analgesics. However, 10 most frequently reported anaesthesia analgesia protocols, which accounted for 70% of all the protocols, used only one analgesic drug. Similarly, another survey involving 320 practising veterinarians in New Zealand also showed that opioids were most frequently used analgesics in small animal practices. It was shown that 74% of dogs undergoing OHE received at least one analgesic, 39% of dogs received more than one analgesic drug and only 1% of each received more than two analgesics under multimodal strategy (Williams *et al.* 2005). Several studies in other countries also show that the perioperative use of a combination of two or more analgesics in dogs undergoing OHE was not a popular choice among veterinarians (Capner *et al*. 1999; Hewson *et al.* 2006; Hugonnard *et al.* 2004). Combining opioid analgesics with other classes of analgesics enhances the total analgesic effect through synergistic interaction between the drugs as shown in various animal model experiments (Kolesnikov 2000; Matthews and Dickenson 2002). This approach is known as multimodal analgesia (Corletto 2007). Combination with other analgesics also reduces the overall requirement of opioids (Kehlet 2004), thereby leading to less side effects, which is described in the morphine section below. One of the reasons behind the underuse of multimodal analgesia, as shown by various surveys mentioned above, is

that there are limited studies evaluating the efficacy of the combination of two or more analgesic drugs or regimens (National Research Council, 2010).

2.4 Analgesic drugs of interest in this PhD research

2.4.1 Morphine

Morphine belongs to a class of analgesic drugs known as opioids. The first report of opioids is found in the writings of Theophrastus in the third century B.C. and they have been used as a mainstay analgesic for thousands of years. Morphine is one of the most commonly used analgesics in the veterinary practice (Clarke *et al.* 2014).

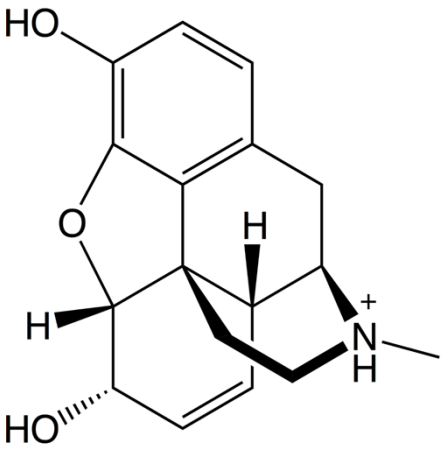


Figure 2.4 Molecular structure of morphine.
From: Cong et al. (2015)

2.4.1.1 Mechanism of action

The opioid receptors belong to a large family of G protein-coupled receptors (GPCRs) which can be activated by endogenously produced opioid peptides and exogenously administered drugs (Gutstein and Akil 2001). Binding of the opioid agonists to the

receptors via the G protein can lead to inhibition of adenylyl cyclase, activation of potassium (K^+) conductance and inhibition of calcium (Ca^{2+}) conductance (Inturrisi 2002). The net effect of these events is hyperpolarisation of the nerve cells and inhibition of release of excitatory neurotransmitters (eg: substance P from primary afferent fibres in the dorsal horn of the spinal cord) resulting in the inhibition of transmission of the nociceptive impulses (Inturrisi 2002).

The mu (μ), delta (δ) and kappa (κ) receptors (Lord *et al.* 1977) are the most extensively studied opioid receptors and nociceptin/orphanin FQ (N/OFQ) receptor is the most recently discovered member of the opioid receptor family (Waldhoer *et al.* 2004; Donica *et al.* 2013). Drugs acting as agonists on opioid receptors can produce a wide range of effects such as analgesia, changes in mood and producing reward behaviour, alteration of respiratory, cardiovascular, digestive and endocrine systems (Gutstein and Akil 2001). Morphine exerts the analgesic and adverse effects mainly through its action on μ receptors (Kieffer 1999). In the periphery, the μ receptors are found in sensory neurons in the inflamed tissue (Stein 1995, 2013). They are also widely distributed in the pre- and postsynaptic sites in the dorsal horn of the spinal cord and in the brain stem, thalamus and cortex, which constitutes the ascending pathway in pain transmission. In addition, a high distribution of these receptors is also found in the descending pain modulatory pathway, which includes the midbrain periaqueductal gray, the nucleus raphe magnus, the rostral ventromedial medulla, and the dorsal horn of the spinal cord (Lueptow *et al.* 2018).

2.4.1.2 Pharmacokinetics of morphine in dogs

2.4.1.2.1 Absorption and distribution

Morphine is a hydrophilic and lipophobic opioid. It is adequately absorbed through the gastrointestinal tract, rectal mucosa, and through subcutaneous, intramuscular, epidural and intrathecal routes (Gutstein and Akil 2001). After its oral administration, the drug undergoes a significant first-pass metabolism in the liver, which reduces the bioavailability of morphine to about 20-25% in humans (Glare and Walsh 1991) and 5-17% in dogs (Dohoo *et al.* 1994; KuKanich *et al.* 2005). The pharmacokinetic parameters observed after administration via different doses and routes in several studies are shown in Table 2.1

2.4.1.2.2 Metabolism and excretion

In humans, morphine is metabolized by glucuronidation resulting in the formation of two major metabolites: morphine-6-glucuronide (M-6-G) and morphine-3-glucuronide (M-3-G) (Yeh *et al.* 1977). M-3-G does not possess the analgesic property but the M-6-G exhibits the analgesic potency equal to or higher than morphine (Van Crugten *et al.* 1997; Murthy *et al.* 2002). In dogs, it has been shown that hepatocytes produce M-6-G in very low concentrations compared to humans (Milne *et al.* 1996; KuKanich *et al.* 2005). Very little morphine is excreted in the urine, whereas both M-6-G and M-3-G metabolites are eliminated via glomerular filtration and excreted in the urine (Hasselström and Säwe 1993).

2.4.1.2.3 Onset of action

Due to its poor lipid solubility and delayed attainment of peak concentration in the CSF, morphine has a relatively slow onset of action; the onset time is 10-15 min after

intravascular injection (IV) (Hug *et al.* 1981) and up to 30 min after IM injection (Clarke *et al.* 2014).

Table 2.1 Comparative pharmacokinetics values following morphine administration to dogs through different routes.

Author	KuKanich *et al.* (2005)	Hug *et al.* (1981)	Jacqz *et al.* (1986)	Dohoo *et al.* (1994)	Dohoo and Tasker (1997)		Barnhart *et al.* (2000)			
Dose (mg/kg)	0.5	0.3	1	0.5	15	15	0.5	1	2	1
Route	IV	IV	IV	IV	IV	Oral	IV	IM	Rectal sol	Rectal sup
$T_{1/2}$	69.6	72	66	66	52.2	51.8	94.9	81.6	65.8	90.5
Vd_{ss}	4.6	6.1	1.5	4.1	3.6	2.6	7.2	6.8	6.1	7.6
CL	63	57	51.5	41	57	54	85.2	91.2	88.4	84.6
F	100	100	100	100	100	17.9	100	119	16.5	23.4

All values are reported as means. $T_{1/2}$, elimination half-life (min); Vd_{ss}, volume of distribution at steady state (L/kg); CL, total body clearance (mL/min/kg), F, bioavailability (%); sol, solution; sup, suppository

2.4.1.3 Clinical and adverse effects of morphine

Although there are many compounds with properties similar to morphine, morphine remains the standard against which other analgesics are compared. It is typically administered via intramuscular or subcutaneous routes at fixed dosing intervals, and less commonly via intravascular routes (Lamont and Mathews 2007). The common situations in which morphine is indicated include traumatic injuries and perioperative processes. Perioperatively, it is used to produce analgesia and sedation (Clarke *et al.* 2014). Morphine can also produce anaesthetic sparing effects of the volatile inhalant

anaesthetics and a consequent improvement of the cardiovascular functions in some cases (Muir III *et al.* 2003).

Morphine is associated with significant adverse effects in all animal species including dogs. Studies have shown that IV and IM doses of morphine at or below 1 mg/kg can cause emesis (Blancquaert *et al.* 1986; Barnhart *et al.* 2000; Kongara *et al.* 2012). Morphine can also cause respiratory depression (Taylor and Houlton 1984; Mastrocinque and Fantoni 2003), and release of histamines leading to cardiovascular changes when used intravascularly (Robinson *et al.* 1988; Guedes *et al.* 2006; Maiante *et al.* 2009). One strategy to reduce its adverse effects and enhance the analgesic efficacy is by combining it with other classes of analgesics having different modes of actions (Epstein *et al.* 2015); this strategy is also known as multimodal analgesia.

2.4.2 Dexmedetomidine

Dexmedetomidine is the dextro-isomer of medetomidine ((4-[2,3] dimethylphenyl] ethyl)-I Himidazole), and the active compound in the racemic mixture. Medetomidine belongs to the class alpha-2-adrenoceptor agonists, which also consists of the other veterinary drugs xylazine, detomidine, and clonidine (Maze and Tranquilli 1991). Medetomidine is a relatively new and most potent alpha-2-adrenoceptor agonist currently available in veterinary medicine (Maze and Tranquilli 1991). The alpha-2/alpha-1 selectivity ratio of 5-10 times more than other alpha-2-adrenoceptor agonists makes it the most selective agonist (Virtanen *et al.* 1988). Dexmedetomidine is a commonly used sedative and analgesic in a variety of veterinary species (Murrell and Hellebrekers 2005).

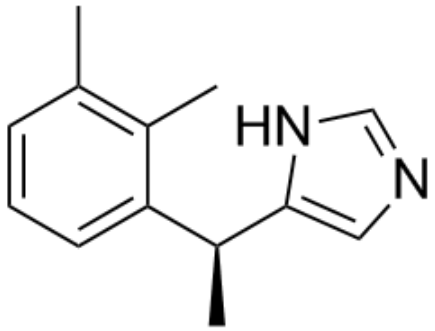


Figure 2.5: Molecular structure of dexmedetomidine.

From: National Center for Biotechnology Information, PubChem Database.

2.4.2.1 Mechanism of action

The analgesic activity of the alpha-2-adrenoceptors agonists including dexmedetomidine occurs through receptors present at various sites in the CNS as well as the periphery. The alpha-2-adrenoceptors are present in high concentration in the superficial laminae of the dorsal horn of spinal cord where synapsis of the nerve fibre occurs and brain stem where modulation of noxious stimuli takes place (Ossipov *et al.* 1989). In the spinal cord, the presynaptic activation of the alpha-2-adrenoceptors located on the primary afferents can lead to decrease in calcium influx and inhibits the release of the neurotransmitters such as substance P (Buerkle and Yaksh 1998). The activation of the postsynaptic alpha-2-adrenoceptors on projection neurons contributes to analgesia via hyperpolarization of the nerve cell by increasing the influx of potassium through G_i protein-coupled potassium channels (North *et al.* 1987). In addition, the descending neurons in the spinal cord that regulate afferent nociceptive input and use

noradrenaline as the neurotransmitter also have binding sites for the alpha-2-adrenoceptor receptors agonists (Ossipov *et al.* 1990).

In addition to the spinal action, studies have shown the involvement of several supraspinal sites in analgesia. Microinjection studies have shown that alpha-adrenergic agonists decrease the release of norepinephrine in the brain and inhibit the spontaneous firing of noradrenergic neurons of nucleus locus coeruleus (a nucleus in the pons and the principal site for brain synthesis of norepinephrine) (Svensson *et al.* 1975; Cedarbaum and Aghajanian 1976). It is also shown that intracerebral microinjections of alpha-adrenoceptor agonists into the periaqueductal grey inhibits spinal nociceptive reflex (Yaksh 1985). The sedative action of dexmedetomidine is also through the alpha-2-adrenoceptor present in locus coeruleus in the brain (Mizobe *et al.* 1996).

2.4.2.2 Pharmacokinetics of dexmedetomidine

2.4.2.2.1 Absorption and distribution

In dogs and cats, dexmedetomidine can be administered by IV or IM route. The terminal elimination half-life of intravenously administered dexmedetomidine at doses of 10 and 20 µg/kg were reported to be 0.66 and 0.78 hours and the clearance rate were 0.97 and 1.24 L/hour/Kg respectively (Kuusela *et al.* 2000).

The pharmacokinetics of IM dexmedetomidine in dogs has not been studied previously and the only one study on the pharmacokinetics of IM medetomidine in dogs exist in the literature. Following the IM injection at dose 80 µg/kg, the drug is rapidly absorbed

(within 30 min) with a rapid onset of clinical action. The elimination half-life of the drug was 1.28 hour and the clearance rate was 27.5 ml/min/kg.

2.4.2.2.2 Metabolism and excretion

The medetomidine is metabolized by hepatic hydroxylation in dogs (Salonen 1989) unlike in human where metabolization by hepatic glucuronidation is more common. The clearance of dexmedetomidine is slower than levomedetomidine (Kuusela *et al.* 2000). The cardiac depressant effect of dexmedetomidine has been attributed for its delayed elimination (Salonen 1992). The glucuronides are the major metabolites which are excreted via urine (Salonen 1989).

2.4.2.3 Clinical effects of dexmedetomidine

Dexmedetomidine can produce dose-dependent sedation, analgesia and marked cardiovascular changes. It has been shown that the analgesic effect of the drug increases with the dose but a ceiling effect is observed with the sedative effect (Kuusela *et al.* 2000). The analgesic dose of medetomidine/dexmedetomidine is believed to be much less than the sedative effect. A study shows that analgesic effect of medetomidine begins at plasma level of 1-5 ng/ml (Salonen 1992). However, another study shows that analgesic effect of medetomidine does not exist below the level of 9.5 ng/ml (Kuusela *et al.* 2000).

The cardiovascular effects shown by medetomidine/dexmedetomidine are the consequence of vasoconstriction mediated by alpha-2 receptors at the synapses and dogs are more prone to this effect (Salmenperä *et al.* 1991). In dogs, blood plasma levels of

dexmedetomidine and medetomidine as low as 2.0 ng/ml and 3.9 ng/ml, respectively produced significant bradycardia (< 70 bpm) without any significant changes in the respiration rate (Kuusela *et al.* 2000). The bradycardia produced by medetomidine and dexmedetomidine is believed to be dose-dependent. A dose-titration study in dogs shows that the low doses (1 and 2 μg/kg) of medetomidine produce less cardiovascular depression but these doses might not be enough to produce sedation (Pypendop and Verstegen 1998).

2.4.2.4 Interactions between opioids and alpha -2-adrenergic agonists combination

The mechanism of synergistic interaction between opioids and alpha-2-adrenoceptor agonists is not completely understood, but the involvement of several spinal and supraspinal sites have been postulated. The alpha-2-adrenoceptor agonists and opiate receptors can interact with the modulation of nociceptive transmission in the spinal cord (Sullivan *et al.* 1987). Single-unit recording techniques have shown that opiates and alpha-2-adrenoceptor agonists have action at independent binding sites and they may activate common or interrelated mechanisms (Hamburg and Tallman 1981; Browning *et al.* 1982; Sullivan *et al.* 1987). The anti-nociceptive activity of both these classes of drugs is partially mediated by descending and serotonergic pathways within the CNS (Grimm *et al.* 2000). These descending systems inhibit the spinal transmission of nociceptive messages (Yaksh 1985; Besson and Chaouch 1987).

Cardoso *et al.* (2014) reported that antinociceptive effects of dexmedetomidine were enhanced when combined with morphine and methadone, all injected intramuscularly as demonstrated by pedal withdrawal reflex test in dogs. In another study, the combination

of butorphanol (a synthetic opioid), and medetomidine showed greater reduction in the MAC and lesser response to the tail clamp than butorphanol or medetomidine alone indicating that the combination could produce superior analgesic effects (Grimm *et al.* 2000).

In a rat study, spinally (epidural and intrathecal) injected alpha-2 agonists (medetomidine, dexmedetomidine, xylazine, clonidine and detomidine) potentiated the analgesic effect of the subcutaneously injected opioid, fentanyl as shown by the tail withdrawal reaction test (Meert and De Kock 1994). Also, adrenoceptor agonists such as ST-9, clonidine and medetomidine potentiated the anti-nociceptive effect produced by several opioids analgesics in various pain models in primates (Yaksh and Reddy 1981), cats (Murata *et al.* 1989) and rats (Wang *et al.* 1980; Ossipov *et al.* 1989).

2.4.3 Neurokinin 1 (NK1) antagonists

Maropitant is a neurokinin 1 (NK1) receptor antagonist commonly used as an antiemetic in dogs and cats (Trepanier 2015). In dogs, it can prevent vomiting associated with motion sickness (Benchaoui *et al.* 2007b), cisplatin in cancer chemotherapy (Vail *et al.* 2007) and administration of a variety of emetogens acting centrally and peripherally (Sedlacek *et al.* 2008). Several studies have shown that NK1 receptor antagonists can be effective in managing visceral pain in animals (Laird *et al.* 2000; Ruggieri *et al.* 2000; Okano *et al.* 2002; Greenwood-Van Meerveld *et al.* 2003).

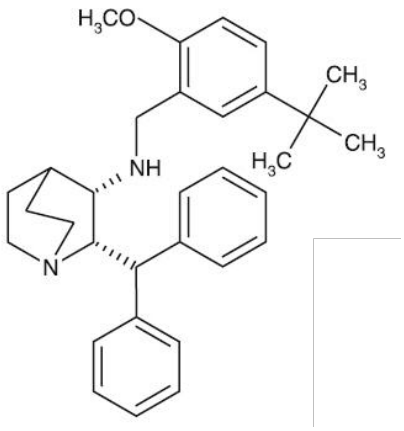


Figure 2.6 Molecular structure of maropitant

From: Krecic and Lavan (2011)

2.4.3.1 Mechanism of action

Maropitant inhibits the binding of endogenously produced substance P, which is an emetogen, to NK1 receptors and thus controls vomiting (Diemunsch and Grélot 2000). Binding of substance P to NK1 receptors has many other functions including the transmission of afferent noxious input (Gamse and Saria 1986; Go and Yaksh 1987; Yashpal and Dam 1990). Substance P is released in the peripheral sensory nerve terminals, in the spinal cord and brain in response to noxious stimulation. It functions alongside other excitatory neurotransmitters such as glutamate and facilitates afferent noxious transmission resulting in nociception (Go and Yaksh 1987).

2.4.3.2 Reports from previous studies on antinociceptive properties of maropitant

Studies in substance P or NK1 knocked out mice show that they have an important but complex role in nociception. Laird *et al.* (2000) suggest that two separate anti-nociceptive pathways may exist, one involving substance P and NK1 genes and another pathway that does not rely on substance P and NK1 (Laird *et al.* 2000). Substance P

may also have a dual action in the brain; at lower doses, it releases endorphins that produce analgesic effects but at higher doses, it directly excites neuronal activity in nociceptive pathways (Frederickson *et al.* 1978). A study in rabbits showed that NK1 receptors play an important role in visceral pain and TAK-637 (a novel NK1 receptor) inhibits the viscerosensory response produced due to colorectal distention (Okano *et al.* 2002). NK1 antagonist drugs such as aprepitant, sendide and maropitant show antinociceptive effect in the rat formalin test model (Sakurada *et al.* 1995; Aguado *et al.* 2015).

Maropitant showed an anaesthetic dose sparing effect during visceral stimulation in dogs and cat undergoing OHE (Boscan *et al.* 2011; Niyom *et al.* 2013). Only one study in dogs compared the efficacy of maropitant to morphine on intra-operative haemodynamic variables and post-OHE pain (Marquez *et al.* 2015). Maropitant group showed a significantly lower heart rate and systolic arterial pressure compared to morphine group during the surgical stimulation. Dogs in both treatment groups required rescue analgesia from 15 min after extubation but the dogs in maropitant group had a better quality of recovery and lower pain scores than the ones in morphine group, in the immediate post-operative period.

Although lots of preclinical and some clinical studies in animal models mentioned above showed that NK1 receptor antagonists can be a promising analgesic, human clinical trials have failed to show their effectiveness in managing pain as expected (Hill 2000; Rost *et al.* 2006). One of the possible explanations for this discrepancy could be that the human studies have considered mainly somatic pain models whereas several

animal studies have shown the analgesic effect of NK1 antagonists using the visceral pain models.

2.4.3.3 Pharmacokinetics of maropitant in dogs

Maropitant is commonly administered peroral and subcutaneously (SC). The absolute bioavailability of maropitant is higher when administered SC (90.7% at the dose of 1 mg/kg) compared to the oral administration (23.7% at the 2 mg/kg) (Benchaoui *et al.* 2007a). The bioavailability of orally administered maropitant increases (37% at 8 mg/kg) in a non-linear manner with the increase in the dose of maropitant. The hepatic first-pass effect is responsible for the low bioavailability of orally administered maropitant. After SC administration, the absorption is rapid. A study in a gerbil model has shown that maropitant can cross the blood-brain barrier and act on the central NK1 receptors in the brain (de la Puente-Redondo *et al.* 2007).

The drug is metabolized in the liver with the involvement of CYP2D15 and CYP3A12, two isoforms of cytochrome P-450 enzyme system (Benchaoui *et al.* 2007a). The terminal half-life of maropitant is 7.75 hours after dosing at 1 mg/kg and 4.03 and 5.46 hour after dosing at 2 mg/kg and 8 mg/kg, respectively (Benchaoui *et al.* 2007a).

Pharmacokinetics of maropitant administered orally and via SC route has been studied previously but the pharmacokinetics after intramuscular injection remains uninvestigated.

2.4.3.4 The adverse effects of maropitant

In addition to its antiemetic and potential analgesic effects, maropitant also produces some adverse effects (Trepanier 2015). The SC injection can lead to pain at the injection site, which can be reduced by refrigeration of the drug solution prior to injection. The drug can also lead to bone marrow hyperplasia in the puppies and thus is not recommended for puppies under 8 weeks of age. Finally, because the drug undergoes hepatic metabolization, it should not be used in patients with hepatic dysfunction.

2.4.3.5 Interaction of maropitant with morphine and dexmedetomidine

An additive/supra-additive interaction in analgesic effect is expected between opioids, alpha-2-adrenoceptor agonists and NK1 receptor antagonists based on the action via the widespread neurotransmitter, substance P (Nicoll *et al.* 1980; Sakurada *et al.* 1995). One of the mechanisms by which opioids and alpha-2 adrenoceptor agonists produce anti-nociception is by inhibiting the release of substance P, at primary afferents. Maropitant can block the binding of substance P to NK1 receptors.

Maropitant has been used in combination with opioids in previous studies in dogs and cats (Claude *et al.* 2014; Martin-Flores *et al.* 2016). However, these studies focussed on assessing the antiemetic effect of maropitant and their analgesic effects were not assessed. These studies show that the combination of morphine and maropitant are well tolerated and has no adverse effects in dogs and cats.

2.5 Reference:

Abbracchio MP, Reggiani AM. Pain and nociception. In: Giovanni G, Pierre-marie L (eds). *Neurosciences-From Molecule to Behavior: A University Textbook*. Pp 445-59. Springer-Verlag, Berlin, Germany, 2013

Aguado D, Abreu M, Benito J, García-Fernández J, de Segura IAG. Amitriptyline, minocycline and maropitant reduce the sevoflurane minimum alveolar concentration and potentiate remifentanil but do not prevent acute opioid tolerance and hyperalgesia in the rat: A randomised laboratory study. *European Journal of Anaesthesiology* 32, 248-54, 2015

Anil SS, Anil L, Deen J. Challenges of pain assessment in domestic animals. *Journal of the American Veterinary Medical Association* 220, 313-9, 2002

Barnhart MD, Hubbell JA, Muir WW, Sams RA, Bednarski RM. Pharmacokinetics, pharmacodynamics, and analgesic effects of morphine after rectal, intramuscular, and intravenous administration in dogs. *American Journal of Veterinary Research* 61, 24-8, 2000

Becker S, Navratilova E, Nees F, Van Damme S. Emotional and motivational pain processing: current state of knowledge and perspectives in translational research. *Pain Research and Management* 2018, 2018

Benchaoui H, Cox S, Schneider R, Boucher J, Clemence R. The pharmacokinetics of maropitant, a novel neurokinin type-1 receptor antagonist, in dogs. *Journal of Veterinary Pharmacology and Therapeutics* 30, 336-44, 2007a

Benchaoui H, Siedek E, De La Puente-Redondo V, Tilt N, Rowan T, Clemence R. Efficacy of maropitant for preventing vomiting associated with motion sickness in dogs. *Veterinary Record* 161, 444-7, 2007b

Besson J-M, Chaouch A. Peripheral and spinal mechanisms of nociception. *Physiological Reviews* 67, 67-186, 1987

Blancquaert J-P, Lefebvre RA, Willems JL. Emetic and antiemetic effects of opioids in the dog. *European Journal of Pharmacology* 128, 143-50, 1986

Borer-Weir K. Analgesia. In: Dethioux F (ed). *Veterinary Anaesthesia*. Pp 101-33. Elsevier Saunders, Oxford, United Kingdom, 2014

Boscan P, Monnet E, Mama K, Twedt DC, Congdon J, Steffey EP. Effect of maropitant, a neurokinin 1 receptor antagonist, on anesthetic requirements during noxious visceral stimulation of the ovary in dogs. *American Journal of Veterinary Research* 72, 1576-9, 2011

Brazier MAB. *Electrical Activity of the Nervous System.* 4th Edtn. Williams and Wilkins, London, United Kingdom, 1977

Brimijoin S, Lundberg JM, Brodin E, Ho T. Axonal transport of substance P in the vagus and sciatic nerves of the guinea pig. *Brain Research* 191, 443-57, 1980

Brown A, Iggo A. A quantitative study of cutaneous receptors and afferent fibres in the cat and rabbit. *The Journal of Physiology* 193, 707, 1967

Brown AG. *Organization in the Spinal Cord: The Anatomy and Physiology of Identified Neurones.* Springer Science & Business Media, Berlin, Germany, 2012

Browning S, Lawrence D, Livingston A, Morris B. Interactions of drugs active at opiate receptors and drugs active at α2-receptors on various test systems. *British Journal of Pharmacology* 77, 487-91, 1982

Bubalo V, Moens YP, Holzmann A, Coppens P. Anaesthetic sparing effect of local anaesthesia of the ovarian pedicle during ovariohysterectomy in dogs. *Veterinary Anaesthesia and Analgesia* 35, 537-42, 2008

Budai D. Neurotransmitters and receptors in the dorsal horn of the spinal cord. *Acta Biologica Szegediensis* 44, 21-38, 2000

Buerkle H, Yaksh T. Pharmacological evidence for different alpha 2-adrenergic receptor sites mediating analgesia and sedation in the rat. *British Journal of Anaesthesia* 81, 208-15, 1998

Burke A, Smyth E, FitzGerald GA. Analgesic-antipyretic agents; pharmacotherapy of gout. *The Pharmacological Basis of Therapeutics* 1, 706, 2006

Butterworth JF, Strichartz GR. Molecular mechanisms of local anesthesia: A review. *Anesthesiology* 72, 711-734, 1990

Cardoso CG, Marques DR, da Silva TH, de Mattos-Junior E. Cardiorespiratory, sedative and antinociceptive effects of dexmedetomidine alone or in combination with methadone, morphine or tramadol in dogs. *Veterinary Anaesthesia and Analgesia* 41, 636-43, 2014

Carlstead, K, Brown, JL, Strawn, W. Behavioral and physiological correlates of stress in laboratory cats. *Applied Animal Behaviour Science* 38(2), 143-158, 1993

Carpenter RE, Wilson DV, Evans AT. Evaluation of intraperitoneal and incisional lidocaine or bupivacaine for analgesia following ovariohysterectomy in the dog. *Veterinary Anaesthesia and Analgesia* 31, 46-52, 2004

Capner A, Lascelles BDX, Waterman-Pearson AE. Current British veterinary attitudes to perioperative analgesia for dogs. *Veterinary Record* 145(4), 95-99, 1999

Cechetto DF, Standaert DG, Saper CB. Spinal and trigeminal dorsal horn projections to the parabrachial nucleus in the rat. *Journal of Comparative Neurology* 240, 153-60, 1985

Cedarbaum JM, Aghajanian GK. Noradrenergic neurons of the locus coeruleus: inhibition by epinephrine and activation by the α-antagonist piperoxane. *Brain Research* 112, 413-9, 1976

Chen AC, Dworkin SF, Haug J, Gehrig J. Topographic brain measures of human pain and pain responsivity. *Pain* 37, 129-41, 1989

Clarke K, Trim C, Hall L. Anaesthesia of the dog. In: Dethioux F (ed). *Veterinary Anaesthesia.* Pp 405-98. Elsevier Saunders, Oxford, United Kingdom, 2014

Claude A, Dedeaux A, Chiavaccini L, Hinz S. Effects of maropitant citrate or acepromazine on the incidence of adverse events associated with hydromorphone premedication in dogs. *Journal of Veterinary Internal Medicine* 28, 1414-7, 2014

Cong X, Campomanes P, Kless A, Schapitz I, Wagener M, Koch T, Carloni P. Structural determinants for the binding of morphinan agonists to the μ-opioid receptor. *PloS One* 10, 2015

Connolly G. Companion animal analgesics: Assessment of pain. *FDA Veterinarian Newsletter September/October XV (V)*, 2000

Corletto F. Multimodal and balanced analgesia. *Veterinary Research Communications*, 31, 59-63, 2007

Cowen R, Stasiowska MK, Laycock H, Bantel, C. Assessing pain objectively: the use of physiological markers. *Anaesthesia*, 70(7), 828-47, 2015

Craig AD, Dostrovsky JO. Medulla to thalamus. In: Wall PD, Melzack R (eds). *Wall and Melzack's Textbook of Pain*. Pp 183-214. Elsevier Saunders, London, United Kingdom, 1999

D'Amour FL, Smith DL. A method for determining loss of pain sensation. *Journal of Pharmacology and Experimental Therapeutics* 72, 74-9, 1941

De Beer N, Van Hooff J, Cluitmans P, Korsten H, Grouls R. Haemodynamic responses to incision and sternotomy in relation to the auditory evoked potential and spontaneous EEG. *British Journal of Anaesthesia* 76, 685-93, 1996

de la Puente-Redondo V, Tingley III F, Schneider R, Hickman M. The neurokinin-1 antagonist activity of maropitant, an antiemetic drug for dogs, in a gerbil model. *Journal of Veterinary Pharmacology and Therapeutics* 30, 281-87, 2007

Diemunsch P, Grélot L. Potential of substance P antagonists as antiemetics. *Drugs* 60, 533-46, 2000

Dohoo S, Tasker RA, Donald A. Pharmacokinetics of parenteral and oral sustained-release morphine sulphate in dogs. *Journal of Veterinary Pharmacology and Therapeutics* 17, 426-33, 1994

Dohoo SE, Tasker R. Pharmacokinetics of oral morphine sulfate in dogs: a comparison of sustained release and conventional formulations. *Canadian Journal of Veterinary Research* 61, 251, 1997

Donica CL, Awwad HO, Thakker DR, Standifer KM. Cellular mechanisms of nociceptin/orphanin FQ (N/OFQ) peptide (NOP) receptor regulation and heterologous regulation by N/OFQ. *Molecular Pharmacology* 83, 907-18, 2013

Doubell TP, Mannion RJ, Woolf CJ. The dorsal horn: state-dependent sensory processing, plasticity and the generation of pain. In: Wall PD, Melzack R (eds). *Wall and Melzack's Textbook of Pain.* Pp 165-82. Elsevier Saunders, London, United Kingdom, 1999

Eddy NB, Leimbach D. Synthetic analgesics. II. Dithienylbutenyl-and dithienylbutylamines. *Journal of Pharmacology and Experimental Therapeutics* 107, 385-93, 1953

England A, Jones R. Inhaled anaesthetic agents: from halothane to the present day. *British Journal of Hospital Medicine* 48, 254-57, 1992

Epstein ME, Rodan I, Griffenhagen G, Kadrlik J, Petty MC, Robertson SA, Simpson W. 2015 AAHA/AAFP pain management guidelines for dogs and cats. *Journal of Feline Medicine and Surgery* 17, 251-72, 2015

Fitzpatrick CL, Weir HL, Monnet E. Effects of infiltration of the incision site with bupivacaine on postoperative pain and incisional healing in dogs undergoing ovariohysterectomy. *Journal of the American Veterinary Medical Association* 237, 395-401, 2010

Frederickson RC, Burgis V, Harrell C, Edwards J. Dual actions of substance P on nociception: possible role of endogenous opioids. *Science* 199, 1359-62, 1978

Gamse R, Saria A. Nociceptive behavior after intrathecal injections of substance P, neurokinin A and calcitonin gene-related peptide in mice. *Neuroscience Letters* 70, 143-47, 1986

Gates MC, Littlewood KE, Kongara K, Odom TF, Sawicki RK. Cross-sectional survey of anaesthesia and analgesia protocols used to perform routine canine and feline ovariohysterectomies. *Veterinary Anaesthesia and Analgesia* 47(1):38-46, 2020

Gaynor JS, Muir W. Acute pain management: a case-based approach. In: *Handbook of Veterinary Pain Management*. Pp 444-46. Elsevier, USA, 2014a

Gaynor JS, Muir WW. *Handbook of Veterinary Pain Management-E-Book.* 3rd Edtn. Elsevier Health Sciences, 2014b

Gibson T, Johnson C, Stafford K, Mitchinson S, Mellor D. Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning. *New Zealand Veterinary Journal* 55, 152-57, 2007

Giesler GJ, Menétrey D, Basbaum AI. Differential origins of spinothalamic tract projections to medial and lateral thalamus in the rat. *Journal of Comparative Neurology* 184, 107-25, 1979

Glare P, Walsh T. Clinical pharmacokinetics of morphine. *Therapeutic Drug Monitoring* 13, 1-23, 1991

Go VL, Yaksh T. Release of substance P from the cat spinal cord. *The Journal of Physiology* 391, 141, 1987

Greenwood-Van Meerveld B, Gibson MS, Johnson AC, Venkova K, Sutkowski-Markmann D. NK1 receptor-mediated mechanisms regulate colonic hypersensitivity in the guinea pig. *Pharmacology Biochemistry and Behavior* 74, 1005-13, 2003

Grimm KA, Tranquilli WJ, Thurmon JC, Benson GJ. Duration of nonresponse to noxious stimulation after intramuscular administration of butorphanol, medetomidine, or a butorphanol-medetomidine combination during isoflurane

administration in dogs. *American Journal of Veterinary Research* 61, 42-47, 2000

Grint N, Johnson C, Clutton R, Whay H, Murrell J. Spontaneous electroencephalographic changes in a castration model as an indicator of nociception: a comparison between donkeys and ponies. *Equine Veterinary Journal* 47, 36-42, 2015

Guedes AG, Rudé EP, Rider MA. Evaluation of histamine release during constant rate infusion of morphine in dogs. *Veterinary Anaesthesia and Analgesia* 33, 28-35, 2006

Guerrero KSK, Campagna I, Bruhl-Day R, Hegamin-Younger C, Guerrero TG. Intraperitoneal bupivacaine with or without incisional bupivacaine for postoperative analgesia in dogs undergoing ovariohysterectomy. *Veterinary Anaesthesia and Analgesia* 43, 571-78, 2016

Gutstein HB, Akil H. Opioid analgesics. In: Hardman JG, Limbird LE (eds). *Goodman & Gillman's The Pharmacological Basis of Therapeutics*. McGraw Hill, New York, USA, 2001

Haga HA, Ranheim B. Castration of piglets: the analgesic effects of intratesticular and intrafunicular lidocaine injection. *Veterinary Anaesthesia and Analgesia* 32, 1-9, 2005

Hamburg M, Tallman JF. Chronic morphine administration increases the apparent number of α2-adrenergic receptors in rat brain. *Nature* 291, 493-95, 1981

Hansen BD, Hardie EM, Carroll GS. Physiological measurements after ovariohysterectomy in dogs: what's normal? *Applied Animal Behaviour Science* 51, 101-09, 1997

Hansen BD. Assessment of pain in dogs: veterinary clinical studies. *Institute of Laboratory Animal Research Journal* 44, 197-205, 2003

Hardy JD, Wolff HG, Goodell H. Studies on pain. A new method for measuring pain threshold: observations on spatial summation of pain. *Journal of Clinical Investigation* 19, 649, 1940

Hasselström J, Säwe J. Morphine pharmacokinetics and metabolism in humans. *Clinical Pharmacokinetics* 24, 344-54, 1993

Heinricher MM, Fields H. Central nervous system mechanisms of pain modulation. In: *Wall & Melzack's Textbook of Pain*. Pp 129-42. Elsevier Health Sciences, Philadelphia, USA, 2013

Hellyer PW, Robertson SA, Fails A. Pain and its management.In. Grimm KA, Lamont LA, Tranquilli WJ, Greene SA, Robertson SA (eds). *Lumb & Jones' Veterinary Anesthesia & Analgesia*. Pp 31-57. Wiley-Blackwell, New Jersey, USA, 2007

Hernandez-Avalos I, Mota-Rojas D, Mora-Medina P, Martínez-Burnes J, Casas Alvarado A, Verduzco-Mendoza A, Lezama-García K, & Olmos-Hernandez A. Review of different methods used for clinical recognition and assessment of pain in dogs and cats. *International Journal of Veterinary Science and Medicine* 7(1), 43–54, 2019

Hewson CJ, Dohoo IR, Lemke KA. Perioperative use of analgesics in dogs and cats by Canadian veterinarians in 2001. *The Canadian Veterinary Journal = La revue veterinaire canadienne* 47(4), 352–59, 2006

Hill R. NK 1 (substance P) receptor antagonists–why are they not analgesic in humans? *Trends in Pharmacological Sciences* 21, 244-46, 2000

Hökfelt T, Kellerth J-O, Nilsson G, Pernow B. Experimental immunohistochemical studies on the localization and distribution of substance P in cat primary sensory neurons. *Brain Research* 100, 235-52, 1975

Holton L, Scott E, Nolan A, Reid J, Welsh E. Relationship between physiological factors and clinical pain in dogs scored using a numerical rating scale. *Journal of Small Animal Practice* 39, 469-74, 1998a

Holton L, Reid J, Scott E, Pawson P, Nolan A. Development of a behaviour-based scale to measure acute pain in dogs. *The Veterinary Record* 148, 525-31, 2001

Holton LL, Scott E, Nolan AM, Reid J, Welsh E, Flaherty D. Comparison of three methods used for assessment of pain in dogs. *Journal of the American Veterinary Medical Association* 212, 61-66, 1998b

Hug C, Murphy M, Rigel E, Olson W. Pharmacokinetics of morphine injected intravenously into the anesthetized dog. *Anesthesiology* 54, 38-47, 1981

Hugonnard M, Leblond A, Keroack S, Cadoré JL, Troncy E. Attitudes and concerns of French veterinarians towards pain and analgesia in dogs and cats. *Veterinary Anaesthesia and Analgesia* 31(3), 154-163, 2004

Ikeda R, Cha M, Ling J, Jia Z, Coyle D, Gu JG. Merkel cells transduce and encode tactile stimuli to drive Aβ-afferent impulses. *Cell* 157, 664-75, 2014

Inturrisi CE. Clinical pharmacology of opioids for pain. *The Clinical Journal of Pain* 18, S3-S13, 2002

Jacqz E, Ward S, Johnson R, Schenker S, Gerkens J, Branch RA. Extrahepatic glucuronidation of morphine in the dog. *Drug Metabolism and Disposition* 14, 627-30, 1986

Johnson C, Taylor P. Comparison of the effects of halothane, isoflurane and methoxyflurane on the electroencephalogram of the horse. *British Journal of Anaesthesia* 81, 748-53, 1998

Johnson C, Bloomfield M, Taylor P. Effects of ketamine on the equine electroencephalogram during anesthesia with halothane in oxygen. *Veterinary Surgery* 28, 380-85, 1999

Johnson CB, Stafford K, Sylvester S, Ward R, Mitchinson S, Mellor D. Effects of age on the electroencephalographic response to castration in lambs anaesthetised using halothane in oxygen. *New Zealand Veterinary Journal* 53, 433-37, 2005a

Johnson CB, Wilson PR, Woodbury MR, Caulkett NA. Comparison of analgesic techniques for antler removal in halothane-anaesthetized red deer (Cervus elaphus): electroencephalographic responses. *Veterinary Anaesthesia and Analgesia* 32, 61-71, 2005b

Johnson CB, Wilson PR, Woodbury MR, Caulkett NA. Comparison of analgesic techniques for antler removal in halothane-anaesthetized red deer (Cervus elaphus): electroencephalographic responses. *Veterinary Anaesthesia and Analgesia* 32, 61-71, 2005c

Kehlet H. Effect of postoperative pain treatment on outcome—current status and future strategies. *Langenbeck's Archives of Surgery* 389(4), 244-49, 2004

Kieffer BL. Opioids: first lessons from knockout mice. *Trends in Pharmacological Sciences* 20, 19-26, 1999

Kolesnikov YA, Chereshnev I, Pasternak GW. Analgesic synergy between topical lidocaine and topical opioids. *Journal of Pharmacology and Experimental Therapeutics* 295(2), 546-55, 2000

Kongara K, Chambers JP, Johnson CB. Electroencephalographic responses of tramadol, parecoxib and morphine to acute noxious electrical stimulation in anaesthetised dogs. *Research in Veterinary Science* 88, 127-33, 2010

Kongara K, Chambers J, Johnson C. Effects of tramadol, morphine or their combination in dogs undergoing ovariohysterectomy on peri-operative electroencephalographic responses and post-operative pain. *New Zealand Veterinary Journal* 60, 129-35, 2012

Kongara K, Johnson L, Kells N, Johnson C, Dukkipati V, Mitchinson SL. Alteration of electroencephalographic responses to castration in cats by administration of opioids. *GSTF Journal of Veterinary Science* 1, 38-42, 2014a

Kongara K, Johnson L, Kells NJ, Johnson CB, Dukkipati VS, Mitchinson SL. Alteration of Electroencephalographic Responses to Castration in Cats by Administration of Opioids. *GSTF Journal of Veterinary Science* 1, 2014b

Kongara K, Mcllhone A, Kells N, Johnson C. Electroencephalographic evaluation of decapitation of the anaesthetized rat. *Laboratory Animals* 48, 15-19, 2014c

Krecic M, Lavan R. Value of CERENIA® (maropitant citrate) in the treatment of acute vomiting in dogs. Pfizer Animal Health Technical Bulletin. 2011

KuKanich B, Lascelles B, Papich M. Pharmacokinetics of morphine and plasma concentrations of morphine-6-glucuronide following morphine administration to dogs. *Journal of Veterinary Pharmacology and Therapeutics* 28, 371-76, 2005

Kuusela E, Raekallio M, Anttila M, Falck I, Molsa S, Vainio O. Clinical effects and pharmacokinetics of medetomidine and its enantiomers in dogs. *Journal of Veterinary Pharmacology and Therapeutics* 23, 15-20, 2000

Laird J, Olivar T, Roza C, De Felipe C, Hunt S, Cervero F. Deficits in visceral pain and hyperalgesia of mice with a disruption of the tachykinin NK1 receptor gene. *Neuroscience* 98, 345-52, 2000

Lamont L, Mathews K. Opioids, nonsteroidal anti-inflammatories, and analgesic adjuvants.In: Grimm KA, Lamont LA, Tranquilli WJ, Greene SA, Robertson SA (eds). *Lumb & Jones' Veterinary Anesthesia & Analgesia*. Pp 241-71. Wiley-Blackwell, New Jersey, USA, 2007

Lamont LA, Tranquilli WJ, Grimm KA. Physiology of pain. *Veterinary Clinics of North America Small Animal Practice* 30, 703-28, 2000

Le Bars D, Gozariu M, Cadden SW. Animal models of nociception. *Pharmacological Reviews* 53, 597-652, 2001

Livingston A. Pain and analgesia in domestic animals. In: Cunningham F, Elliot J, Lees P (eds). *Comparative and Veterinary Pharmacology*. Pp 159-89. Springer-Verlag, Berlin, Germany, 2010

Loeser JD, Treede R-D. The Kyoto protocol of IASP basic pain terminology. *Pain* 137, 473-77, 2008

Long CW, Shah NK, Loughlin C, Spydell J, Bedford RF. A comparison of EEG determinants of near-awakening from isoflurane and fentanyl anesthesia. Spectral edge, median power frequency, and delta ratio. *Anesthesia & Analgesia* 69, 169-73, 1989

Lord JA, Waterfield AA, Hughes J, Kosterlitz HW. Endogenous opioid peptides: multiple agonists and receptors. *Nature* 267, 495-99, 1977

Lueptow LM, Fakira AK, Bobeck EN. The contribution of the descending pain modulatory pathway in opioid tolerance. *Frontiers in Neuroscience* 12, 886, 2018

Maiante A, Teixeira Neto FJ, Beier S, Corrente J, Pedroso C. Comparison of the cardio-respiratory effects of methadone and morphine in conscious dogs. *Journal of Veterinary Pharmacology and Therapeutics* 32, 317-28, 2009

Mantyh PW, Rogers SD, Honore P, Allen BJ, Ghilardi JR, Li J, Daughters RS, Lappi DA, Wiley RG, Simone DA. Inhibition of hyperalgesia by ablation of lamina I spinal neurons expressing the substance P receptor. *Science* 278, 275-79, 1997

Marquez M, Boscan P, Weir H, Vogel P, Twedt DC. Comparison of NK-1 receptor antagonist (maropitant) to morphine as a pre-anaesthetic agent for canine ovariohysterectomy. *PloS One* 10, e0140734, 2015

Martin-Flores M, Mastrocco A, Lorenzutti AM, Campoy L, Kirch P, Stone M, Learn MM, Boesch JM. Maropitant administered orally 2–2.5 h prior to morphine and dexmedetomidine reduces the incidence of emesis in cats. *Journal of Feline Medicine and Surgery*, 1098612X16663595, 2016

Mastrocinque S, Fantoni DT. A comparison of preoperative tramadol and morphine for the control of early postoperative pain in canine ovariohysterectomy. *Veterinary Anaesthesia and Analgesia* 30, 220-8, 2003

Matthews EA, Dickenson, AH. A combination of gabapentin and morphine mediates enhanced inhibitory effects on dorsal horn neuronal responses in a rat model of neuropathy. *Anesthesiology* 96(3), 633-40, 2002

Maze M, Tranquilli W. Alpha-2 Adrenoceptor AgonistsDefining the Role in Clinical Anesthesia. *The Journal of the American Society of Anesthesiologists* 74, 581-605, 1991

McGrath PA. An assessment of children's pain: a review of behavioral, physiological and direct scaling techniques. *Pain* 31, 147-76, 1987

McGreevy K, Bottros MM, Raja SN. Preventing chronic pain following acute pain: risk factors, preventive strategies, and their efficacy. *European Journal of Pain Supplements* 5, 365-76, 2011

McMahon SB, Koltzenburg M, Tracey I, Turk D. *Wall & Melzack's Textbook of Pain: Expert Consult-Online and Print.* 6th Edtn. Elsevier Health Sciences, Philadelphia, USA, 2013

Meert TF, De Kock M. Potentiation of the analgesic properties of fentanyl-like opioids with alpha 2-adrenoceptor agonists in rats. *Anesthesiology* 81, 677-88, 1994

Mellor DJ, Cook CJ, Stafford KJ. Quantifying some responses to pain as a stressor. *In*: Moberg GP and Mench JA (Ed), The Biology of Animal Stress: Basic Principles and Implications for Welfare, 171-198, 2000

Melzack R, Casey KL. Sensory, motivational, and central control determinants of pain: a new conceptual model. *The Skin Senses* 1, 423-43, 1968

Menétrey D, Chaouch A, Binder D, Besson JM. The origin of the spinomesencephalic tract in the rat: an anatomical study using the retrograde transport of horseradish peroxidase. *Journal of Comparative Neurology* 206, 193-207, 1982

Merskey H. Pain terms: a list with definitions and notes on usage. Recommended by the IASP Subcommittee on Taxonomy. *Pain* 6, 249-52, 1979

Milne RW, Nation RL, Somogyi AA. The disposition of morphine and its 3-and 6-glucuronide metabolites in humans and animals, and the importance of the metabolites to the pharmacological effects of morphine. *Drug Metabolism Reviews* 28, 345-472, 1996

Miranda HF, Prieto JC, Puig MM, Pinardi G. Isobolographic analysis of multimodal analgesia in an animal model of visceral acute pain. *Pharmacology Biochemistry and Behavior* 88, 481-6, 2008

Mizobe T, Maghsoudi K, Sitwala K, Tianzhi G, Ou J, Maze M. Antisense technology reveals the alpha2A adrenoceptor to be the subtype mediating the hypnotic response to the highly selective agonist, dexmedetomidine, in the locus coeruleus of the rat. *The Journal of Clinical Investigation* 98, 1076-80, 1996

Muir III WW, Wiese AJ, March PA. Effects of morphine, lidocaine, ketamine, and morphine-lidocaine-ketamine drug combination on minimum alveolar concentration in dogs anesthetized with isoflurane. *American Journal of Veterinary Research* 64, 1155-60, 2003

Murata K, Nakagawa I, Kumeta Y, Kitahata LM, Collins J. Intrathecal clonidine suppresses noxiously evoked activity of spinal wide dynamic range neurons in cats. *Anesthesia & Analgesia* 69, 185-91, 1989

Murrell J, Johnson C. Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapeutics* 29, 325-35, 2006

Murrell JC, Johnson CB, White KL, Taylor PM, Haberham ZL, Waterman–Pearson AE. Changes in the EEG during castration in horses and ponies anaesthetized with halothane. *Veterinary Anaesthesia and Analgesia* 30, 138-46, 2003

Murrell JC, Hellebrekers LJ. Medetomidine and dexmedetomidine: a review of cardiovascular effects and antinociceptive properties in the dog. *Veterinary Anaesthesia and Analgesia* 32, 117-27, 2005

Murthy BP, Pollack GM, Brouwer KL. Contribution of morphine-6-glucuronide to antinociception following intravenous administration of morphine to healthy volunteers. *The Journal of Clinical Pharmacology* 42, 569-76, 2002

National Research Council. Recognition and alleviation of pain in laboratory animals. *National Academies Press*, 2010

National Center for Biotechnology Information. PubChem Database. Dexmedetomidine, CID=5311068, https://pubchem.ncbi.nlm.nih.gov/compound/Dexmedetomidine (accessed on Feb. 28, 2020)

Nicoll R, Schenker C, Leeman S. Substance P as a transmitter candidate. *Annual Review of Neuroscience* 3, 227-68, 1980

Niedermeyer E, da Silva FL. *Electroencephalography: Basic Principles, Clinical Applications, and Related Fields.* Lippincott Williams & Wilkins, Philadelphia, USA, 2005

Niyom S, Boscan P, Twedt DC, Monnet E, Eickhoff JC. Effect of maropitant, a neurokinin-1 receptor antagonist, on the minimum alveolar concentration of sevoflurane during stimulation of the ovarian ligament in cats. *Veterinary Anaesthesia and Analgesia* 40, 425-31, 2013

North RA, Williams JT, Surprenant A, Christie MJ. Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proceedings of the National Academy of Sciences* 84, 5487-91, 1987

Okano S, Ikeura Y, Inatomi N. Effects of tachykinin NK1 receptor antagonists on the viscerosensory response caused by colorectal distention in rabbits. *Journal of Pharmacology and Experimental Therapeutics* 300, 925-31, 2002

Ong R, Morris J, O'dwyer J, Barnett J, Hemsworth P, Clarke I. Behavioural and EEG changes in sheep in response to painful acute electrical stimuli. *Australian Veterinary Journal* 75, 189-93, 1997

Ossipov MH, Suarez LJ, Spaulding TC. Antinociceptive interactions between alpha-2-adrenergic and opiate agonists at the spinal level in rodents. *Anesthesia & Analgesia* 68, 194-200, 1989

Ossipov MH, Harris S, Lloyd P, Messineo E, Lin B, Bagley J. Antinociceptive interaction between opioids and medetomidine: systemic additivity and spinal synergy. *Anesthesiology* 73, 1227-35, 1990

Purves D, Augustine G, Fitzpatrick D, Katz L, LaMantia A, McNamara J, Williams S. The major afferent pathway for mechanosensory information: the dorsal column-medial lemniscus system. *The Somatic Sensory System*, 199-202, 2001

Pypendop BH, Verstegen JP. Hemodynamic effects of medetomidine in the dog: a dose titration study. *Veterinary Surgery* 27, 612-22, 1998

Raffa RB, Pergolizzi Jr JV, Tallarida RJ. The determination and application of fixed-dose analgesic combinations for treating multimodal pain. *The Journal of Pain* 11, 701-9, 2010

Ralston HJ, Ralston DD. The distribution of dorsal root axons in laminae I, II and III of the macaque spinal cord: a quantitative electron microscope study. *Journal of Comparative Neurology* 184, 643-83, 1979

Rampil IJ, Matteo RS. Changes in EEG spectral edge frequency correlate with the hemodynamic response to laryngoscopy and intubation. *Anesthesiology* 67, 139-42, 1987

Reid J, Nolan A, Hughes J, Lascelles D, Pawson P, Scott E. Development of the short-form Glasgow Composite Measure Pain Scale (CMPS-SF) and derivation of an analgesic intervention score. *Animal Welfare-Potters Bar Then Wheathampstead-* 16, 97, 2007

Reid J, Nolan AM, Scott EM. Measuring pain in dogs and cats using structured behavioural observation. *The Veterinary Journal* 236, 72-79, 2018.

Rexed B. The cytoarchitectonic organization of the spinal cord in the cat. *Journal of Comparative Neurology* 96, 415-95, 1952

Rice F, Albrecht P. Cutaneous mechanisms of tactile perception: morphological and chemical organization of the innervation to the skin. *The Senses: A Comprehensive Reference* 6, 1-32, 2008

Robinson E, Faggella A, Henry D, Russell W. Comparison of histamine release induced by morphine and oxymorphone administration in dogs. *American Journal of Veterinary Research* 49, 1699-701, 1988

Rost K, Fleischer F, Nieber K. [Neurokinin 1 receptor antagonists--between hope and disappointment]. *Medizinische Monatsschrift fur Pharmazeuten* 29, 200-5, 2006

Ruel HL, Steagall PV. Adjuvant analgesics in acute pain management. The *Veterinary Clinics of North America: Small Animal Practice* 49, 1127-41, 2019

Ruggieri MR, Filer-Maerten S, Hieble JP, Hay DW. Role of neurokinin receptors in the behavioral effect of intravesical antigen infusion in guinea pig bladder. *The Journal of Urology* 164, 197-202, 2000

Sakurada T, Katsumata K, Yogo H, Tan-No K, Sakurada S, Ohba M, Kisara K. The neurokinin-1 receptor antagonist, sendide, exhibits antinociceptive activity in the formalin test. *Pain* 60, 175-80, 1995

Salmenperä M, Szlam F, Hug CC. Anesthetic and hemodynamic interactions of dexmedetomidine and fentanyl in dogs. *The Journal of the American Society of Anesthesiologists* 75, A307-A, 1991

Salonen J. Pharmacokinetics of medetomidine. *Acta veterinaria Scandinavica. Supplementum* 85, 49-54, 1989

Salonen J. Chemistry and pharmacokinetics of the alpha-2-adrenoreceptor agonists. In: Short CE, Poznack AV (eds). *Animal Pain*. Pp 191-200. Churchill Livingstone,Edinburgh, Scotland, 1992

Schaible H-G. Peripheral and central mechanisms of pain generation. In: Stein C (ed). *Analgesia*. Pp 3-28. Springer-Verlag, Berlin, Germany, 2006

Schnitzler A, Ploner M. Neurophysiology and functional neuroanatomy of pain perception. *Journal of Clinical Neurophysiology* 17, 592-603, 2000

Schraag S, Mohl U, Bothner U, Georgieff M. Clinical utility of EEG parameters to predict loss of consciousness and response to skin incision during total intravenous anaesthesia. *Anaesthesia* 53, 320-25, 1998

Schwender D, Daunderer M, Mulzer S, Klasing S, Finsterer U, Peter K. Spectral edge frequency of the electroencephalogram to monitor "depth" of anaesthesia with isoflurane or propofol. *British Journal of Anaesthesia* 77, 179-84, 1996

Scott J, Huskisson E. Graphic representation of pain. *Pain* 2, 175-84, 1976

Sedlacek H, Ramsey D, Boucher J, Eagleson J, Conder G, Clemence R. Comparative efficacy of maropitant and selected drugs in preventing emesis induced by centrally or peripherally acting emetogens in dogs. *Journal of Veterinary Pharmacology and Therapeutics* 31, 533-37, 2008

Sherrington CS. *The integrative action of the nervous system.* Yale University Press, London, United Kingdom, 1906

Shilo Y, Pascoe PJ. Anatomy, Physiology, and Pathophysiology of Pain. *Pain Management in Veterinary Practice*, 9-27, 2014

Simons A, Boezeman E, Pronk R. Automatic EEG monitoring of anaesthesia. *Baillière's Clinical Anaesthesiology* 3, 623-46, 1989

Singh P, Kongara K, Harding D, Ward N, Dukkipati VSR, Johnson C, Chambers P. Comparison of electroencephalographic changes in response to acute electrical and thermal stimuli with the tail flick and hot plate test in rats administered with opiorphin. *BMC Neurology* 18, 43, 2018

Smith ESJ, Lewin GR. Nociceptors: a phylogenetic view. *Journal of Comparative Physiology A* 195, 1089-106, 2009

Stein C. The control of pain in peripheral tissue by opioids. *New England Journal of Medicine* 332, 1685-90, 1995

Stein C. Opioid Receptors on Peripheral Sensory Neurons. *Advances in Experimental Medicine and Biology* 521, 69-76, 2013

Stucky CL, Gold MS, Zhang X. Mechanisms of pain. *Proceedings of the National Academy of Sciences* 98, 11845-46, 2001

Sugiura Y, Lee C, Perl E. Central projections of identified, unmyelinated (C) afferent fibers innervating mammalian skin. *Science* 234, 358-61, 1986

Sullivan AF, Dashwood MR, Dickenson AH. α 2-Adrenoceptor modulation of nociception in rat spinal cord: location, effects and interactions with morphine. *European Journal of Pharmacology* 138, 169-77, 1987

Svensson T, Bunney B, Aghajanian G. Inhibition of both noradrenergic and serotonergic neurons in brain by the α-adrenergic agonist clonidine. *Brain Research* 92, 291-06, 1975

Tallarida R. J. Quantitative methods for assessing drug synergism. *Genes & Cancer* 2(11), 1003–1008, 2011

Taylor PM, Houlton JE. Post-operative analgesia in the dog: a comparison of morphine, buprenorphine and pentazocine. *Journal of Small Animal Practice* 25, 437-51, 1984

Todd A, Spike R. The localization of classical transmitters and neuropeptides within neurons in laminae I–III of the mammalian spinal dorsal horn. *Progress in Neurobiology* 41, 609-45, 1993

Todd A. Anatomy of primary afferents and projection neurones in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor. *Experimental physiology* 87, 245-49, 2002

Tracey I, Mantyh PW. The cerebral signature for pain perception and its modulation. *Neuron* 55, 377-91, 2007

Tranquilli W, Grimm K, Lamont L. *Pain Management for the Small Animal Practitioner.* 2nd Edtn. Teton NewMedia, Wyoming, USA, 2004

Trepanier LA. Maropitant: Novel Antiemetic. *Clinicians Brief University of Wisconsin-Madison*, 75-77, 2015

Vail D, Rodabaugh H, Conder G, Boucher J, Mathur S. Efficacy of injectable maropitant (Cerenia™) in a randomized clinical trial for prevention and treatment of cisplatin-induced emesis in dogs presented as veterinary patients. *Veterinary and Comparative Oncology* 5, 38-46, 2007

Van Crugten JT, Somogyi AA, Nation RL, Reynolds G. Concentration-effect relationships of morphine and morphine-6β-glucuronide in the rat. *Clinical and Experimental Pharmacology and Physiology* 24, 359-64, 1997

Vandam LD. Some aspects of the history of local anesthesia. In: Strichartz GR (ed). *Local Anesthetics*. Pp 1-19. Springer-Verlag, Berlin, Germany, 1987

Vane JR, Botting RM. The mechanism of action of aspirin. *Thrombosis Research* 110, 255-58, 2003

Vane JR, Botting RM. Mechanism of action of nonsteroidal anti-inflammatory drugs. *The American Journal of Medicine* 104, 2S-8S, 1998

Virtanen R, Savola J-M, Saano V, Nyman L. Characterization of the selectivity, specificity and potency of medetomidine as an α2-adrenoceptor agonist. *European Journal of Pharmacology* 150, 9-14, 1988

Waldhoer M, Bartlett SE, Whistler JL. Opioid receptors. *Annual Review of Biochemistry* 73, 953-90, 2004

Wang J, Yasuoka S, Yaksh T. Studies on the analgetic effect of intrathecal ST-91 (2-[2, 6-diethyl-phenylamino]-2-imidazoline): antagonism, tolerance and interaction with morphine. *Pharmacologist* 22, 302, 1980

Waterman A, Kalthum W. Pharmacokinetics of intramuscularly administered pethidine in dogs and the influence of anaesthesia and surgery. *The Veterinary Record* 124, 293-96, 1989

White PF, Boyle WA. Relationship between hemodynamic and electroencephalographic changes during general anesthesia. *Anesthesia & Analgesia* 68, 177-81, 1989

Wickremaratchi M, Llewelyn J. Effects of ageing on touch. *Postgraduate Medical Journal* 82, 301-04, 2006

Wilder-Smith O, Hagon O, Tassonyi E. EEG arousal during laryngoscopy and intubation: comparison of thiopentone or propofol supplemented with nitrous oxide. *British Journal of Anaesthesia* 75, 441-46, 1995

Williams V, Lascelles B, Robson M. Current attitudes to, and use of, peri-operative analgesia in dogs and cats by veterinarians in New Zealand. *New Zealand Veterinary Journal* 53, 193-02, 2005

Yaksh TL, Reddy S. Studies in the primate on the analgetic effects associated with intrathecal actions of opiates, alpha-adrenergic agonists and baclofen. *Anesthesiology* 54, 451-67, 1981

Yaksh TL. Pharmacology of spinal adrenergic systems which modulate spinal nociceptive processing. *Pharmacology Biochemistry and Behavior* 22, 845-58, 1985

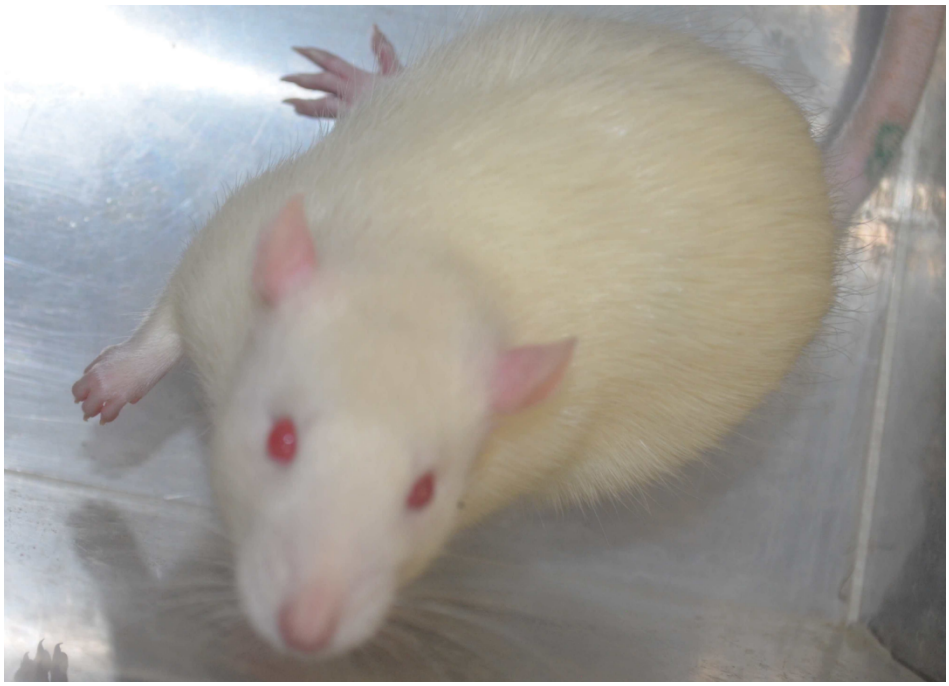
Yashpal K, Dam T-V. Quantitative autoradiographic distribution of multiple neurokinin binding sites in rat spinal cord. *Brain Research* 506, 259-66, 1990

Yeh S, Gorodetzky C, Krebs H. Isolation and identification of morphine 3-and 6-glucuronides, morphine 3, 6-diglucuronide, morphine 3-ethereal sulfate, normorphine, and normorphine 6-glucuronide as morphine metabolites in humans. *Journal of Pharmaceutical Sciences* 66, 1288-93, 1977

Young A, Buvanendran A. Recent advances in multimodal analgesia. *Anesthesiology Clinics* 30, 91-100, 2012

CHAPTER 3

Evaluation of analgesic interaction between morphine, dexmedetomidine and maropitant using hot-plate and tail-flick tests in rats



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3.1 Abstract

Objective To determine if the combinations of morphine, dexmedetomidine, and maropitant enhance the analgesic effect and decrease the dose of individual drugs in rats subjected to noxious thermal stimulation with hot plate and tail flick tests.

Study design Randomized, blinded, prospective experimental study.

Animals 96 male Sprague-Dawley rats.

Methods The rats were randomly assigned to the following groups: 1) morphine (3 mg kg^{-1}); 2) dexmedetomidine (10 μg kg^{-1}); 3) maropitant (20 mg kg^{-1}); 4) morphine (1.5 mg kg^{-1}) + dexmedetomidine (5 μg kg^{-1}); 5) dexmedetomidine (5 μg kg^{-1}) + maropitant (10 mg kg^{-1}); 6) morphine (1.5 mg kg^{-1}) + maropitant (10 mg kg^{-1}); 7) morphine (1mg kg^{-1}) + dexmedetomidine (3.5 μg kg^{-1}) + maropitant (6.5 mg kg^{-1}); and 8) normal saline (0.5 mL), all injected intravenously. The tail flick and hot plate tests were performed before and 5, 15, 30, 45, 60, 90 and 120 minutes after the injection of the drugs. These variables were analysed with the effect-time area under curve (AUC) analysis and a mixed linear model.

Results: Data were analyzed in 94 rats. The rank order of the total analgesic effects of the treatment groups shown by AUC analysis was found to be Mor > Maro+Mor > Dex+Mor > Dex > Maro > Dex+Maro+Mor > Dex+Maro > saline for the hot plate test, and Maro+Mor > Mor > Dex+Mor > Dex+Maro+Mor > Maro > Dex > Dex+Maro > saline for the tail flick test. The mixed model analysis showed a significant difference between latencies of the group morphine+maropitant *versus* all other treatment groups in the tail flick test ($p < 0.0001$) and morphine *versus* saline in the hot plate test ($p < 0.05$).

Conclusions and clinical relevance Morphine and maropitant appeared to show a supra-additive effect for analgesia shown by the tail flick test. Clinical trials should be conducted to establish its use in treating pain.

Keywords Morphine, Dexmedetomidine, Maropitant, Combination, Analgesia, Rat model

3.2 Introduction

There is no single analgesic that can control pain effectively and is free of side effects. Opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) remain the major analgesics to treat moderate-to-severe pain in veterinary patients (Gaynor 1999). These drugs, when used singly, are not always able to provide effective analgesia and are associated with many adverse effects. Opioids can cause respiratory depression, nausea, and prolonged sedation (Pascoe 2000), and NSAIDS are associated with gastric bleeding, ulcers and potential renal damage (Lascelles et al. 2005). These adverse effects limit the use of these drugs at higher dosages. Therefore, drug combination therapy (multimodal analgesia) is recommended for management of pain. Multimodal analgesia involves administration of two or more classes of drugs (e.g. opioids, NSAIDs, alpha-2 agonists etc.) with different modes of action: it is postulated that this approach may result in an enhanced analgesic effect and require lower doses of each drug, reducing adverse effects (Jin & Chung 2001; Young & Buvanendran 2012).

Morphine is the prototypical opiate analgesic and one of the most commonly used perioperative analgesics, but a single dose may not provide an adequate level of postoperative analgesia and further dosing may be associated with increased incidence of clinically significant side effects (Pascoe 2000). Dexmedetomidine is a highly

selective alpha-2 adrenoceptor agonist (Vainio 1989). It can produce analgesia at both the spinal (Kalso et al. 1991) and supraspinal level (Guo et al. 1996). However, its potential side effects such as sedation, bradycardia and decrease in cardiac output limit its use as a potent analgesic (Kuusela et al. 2001). Studies in rodents combining morphine with alpha-2 agonists showed a decrease in the dose of the individual drugs required, and better analgesia with little to no cardiovascular effects (Ossipov et al. 1990; Stone et al. 2014).

Maropitant is a neurokinin-1 (NK1) receptor antagonist which may be able to block the substance P mediated transmission of noxious stimuli. Substance P is released in the peripheral sensory nerve terminals, in the spinal cord and brain in response to noxious stimulation (Gamse & Saria 1986; Go & Yaksh 1987; Yashpal & Dam 1990). NK1 receptor antagonist have been shown to block noxious stimuli in the rat formalin test model (Sakurada et al. 1995; Aguado et al. 2015) and to have an anaesthetic dose sparing effect in the dog and cat ovariohysterectomy (OHE) model (Boscan et al. 2011; Niyom et al. 2013). One study in dogs has compared the efficacy of maropitant to morphine on intra-operative cardiovascular changes and post-OHE pain (Marquez et al. 2015).

There is a lack of data regarding analgesic interaction between morphine, dexmedetomidine and maropitant. In this study, we examined the analgesic interactions using a hotplate and tail flick test in a rat model. Hot plate and tail flick tests are simple, sensitive and commonly used tests to study analgesic efficacy of drugs in rats and mice (D'Amour & Smith 1941; Eddy & Leimbach 1953). They are easy to perform, require minimal instrumentation, and have well-defined endpoints. This was a pilot study to test the efficacy, suitability and safety of the combination of these drugs to be used for

clinical trials in small animals. Our hypothesis was that each combination would be additive, i.e., a combination of two drugs at half of the full dose or three drugs at one third of the full dose would produce the same degree of analgesia as each drug alone at the full dose.

3.3 Materials and methods

3.3.1 Study design

After approval from Massey University Animal Ethics Committee (Protocol 16/115), male Sprague Dawley rats (n = 96) weighing 250- 450g (average 354 g) were used. Their age at the time of arrival was 6-9 weeks. The animals were obtained from the Small Animal Production Unit, Massey University, New Zealand.

The rats were randomly selected by coin toss from a population of 384. The cage pairs that achieved heads were included in the study until we received 96 rats in total. This study is reported in accordance with the ARRIVE guidelines for experiments involving animals (Kilkenny et al. 2010). The sample size was calculated with the power of 80% and the probability of alpha (α) error of 5%.The effect size was estimated as 1.2 based on previous studies (Hunskaar et al. 1986; Keyhanfar et al. 2013) using similar methods.

The rats were housed in pairs in plastic cages placed on cage racks in an environmentally controlled room and standard food and water were available *ad libitum* at all times. For identification, the rats were marked on the tail using a non-toxic permanent marker. The room was kept at a controlled temperature (22 ± 1 °C) and humidity (40-60%) and maintained on a 12-hour light/dark cycle. All testing was

carried out during the light phase. The rats were allowed to acclimatize in the new environment for 7 days, and they were picked up and handled gently once a day during that period. The rats were also adapted to the non-functional hot-plate for 1 minute per day during the handling period. The health of the rats was checked daily by the small animal production unit veterinary staff.

The trials in all 96 rats were performed in a one-week period. A tail flick followed by a hotplate test for one rat was performed on the same day. Rats were separated from each other only when tests were being performed on one of them. Rats were returned to the home cage immediately after completion of the tests.

3.3.2 Hot plate and tail flick test

The hot plate and tail flick tests were performed at a station in the same room the rats were housed. The rats were randomly allocated into eight experimental groups of 12 using a randomization plan generated by an online software QuickCalcs (GraphPad Software, CA, USA) and the nociceptive response was measured using tail-flick and hot-plate tests. The treatment groups were as follows:

Group 1 (Mor): morphine (3 mg kg^{-1})

Group 2 (Dex): dexmedetomidine (10 μg kg^{-1})

Group 3 (Maro): maropitant (20 mg kg^{-1})

Group 4 (Dex+Mor): dexmedetomidine (5 μg kg^{-1}) + morphine (1.5 mg kg^{-1})

Group 5 (Dex+Maro): dexmedetomidine (5 μg kg^{-1}) + maropitant (10 mg kg^{-1})

Group 6 (Maro+Mor): maropitant (10 mg kg^{-1}) + morphine (1.5 mg kg^{-1})

Group 7 (Dex+Maro+Mor): dexmedetomidine (3.5 μg kg^{-1}) + maropitant (6.5) mg kg^{-1}

+ morphine (1mg kg^{-1})

Group 8 (control): normal saline (0.5 mL)

The drugs used were: morphine sulphate (DBL Morphine sulphate injection BP; Hospira Australia Pty. Ltd., Australia) 5 mg mL^{-1}, dexmedetomidine hydrochloride (Dexdomitor; Zoetis, New Zealand) 0.5 mg mL^{-1}, and maropitant citrate (Cerenia; Zoetis, New Zealand), 10 mg mL^{-1} (0.9% Sodium Chloride; Baxter, Australia). The rats were manually restrained using a towel and a perforated perspex tube and the drugs were injected into the tail vein using a 1 mL syringe and 26-gauge 1/2-inch needle. Morphine and dexmedetomidine were administered after being mixed together in a syringe; maropitant was administered separately. For each animal, a baseline response latency for both tests was determined by two consecutive responses measured at 5 minutes intervals before the administration of the drugs, that were averaged for statistical analysis. The person performing tests and recording latencies was unaware of the treatment groups.

A tail flick followed by a hotplate test was performed at 5, 15, 30, 45, 90 and 120 minutes after administration of the drugs. For the tail flick test, a water bath was maintained at 55 ± 0.5 °C, the rats restrained manually in a soft towel and the distal two thirds of the tail submerged in the water bath. The tail was quickly removed after the flick. The time the rat kept its tail in the water was recorded. To protect against tissue injury, the test was terminated after 10 seconds if the animal did not flick its tail.

A metal hot-plate with a rim was placed in a water bath at 55 ± 0.5 °C so that the bottom of the plate was in contact with the water, but the top was dry. A transparent acrylic cylinder was placed on the hot-plate to prevent escape. Rats were placed on the

hot plate and the response time for behavioural changes like paw licking, stamping or jumping was recorded. The plate was wiped with a wet cloth to remove traces of urine and faeces and dried after each measurement. A cut-off latency of 15 seconds was used to avoid tissue damage.

Rats were returned to their home cages and replaced on the cage rack immediately after completion of the tests. Each animal received only one drug treatment and was euthanized using carbon dioxide after the tests were performed in the both rats in a cage. For euthanasia, rats in their home cages were placed into a chamber containing the room air. Carbon dioxide was gradually delivered at a filling rate of 20% of the chamber volume per minute until 1 minute after respiratory arrest.

3.3.3 Data Analysis

All data are expressed as mean ± standard deviation (SD). The overall antinociceptive effect of the treatment drugs in the hotplate test and tail flick tests were expressed as a) mean time-response (latencies) data of all animals in a group at each time point, and b) the mean area under the effect-time curve (AUC) over the entire time period using GraphPad Prism version 7.00 for Mac OS X (GraphPad Software, CA, USA).

The dependent variables (tail flick and hot plate latencies) were analysed using a linear mixed model using the MIXED procedure of the Statistical Analysis System software, university edition 9.4 (SAS Institute Inc., NC, USA). The linear model included the fixed effect of time, treatment and interaction between time and treatment and the random effect of animal to account for repeated measures in the same animal. Least squares (LS Means) and standard errors (SE) for treatment time and combinations between treatments and times were obtained and used for multiple mean comparisons

using the Least Significant Difference as implemented in the MIXED procedure. A *p*-value of less than 0.05 was considered statistically significant. There was no significant difference between the weights of the rats and baseline values, hence those parameters were not included in the model.

3.4 Results

One rat from group Maro and another from group Mor+Maro groups died during the study. Data analysis was performed in 94 rats only (n=11 for group Maro and Mor + Maro, and n=12 for all other treatment groups).

3.4.1 Hot plate test

3.4.1.1 AUC

The mean area under the effect-time curve (AUC) is shown in Figure 3.1a. The rank order of the AUC which shows the total effect of the drugs was found to be Mor (41.6 ± 15.8 seconds) > Maro + Mor (39.9 ± 21.0 seconds) > Dex + Mor (38.9 ± 21.5 seconds) > Dex (38.4 ± 10.3 seconds) > Maro (33.4 ± 13.9 seconds) > Dex + Maro + Mor (31.2 ± 10.1 seconds) > Dex + Maro (30.9 ± 12.7 seconds) > Saline (28.5 ± 8.2 seconds).

3.4.1.2 Mean time-response latencies

After administration of the drugs there was a significant increase in latency times compared to their baselines in groups Maro ($p < 0.05$), Maro + Mor ($p < 0.0001$), Mor + Dex ($p < 0.05$), and Mor ($p < 0.001$). The increase in latency lasted for 30 minutes in Mor + Dex, 60 minutes in Maro and Mor and 90 minutes in Maro + Mor. Other treatment groups showed no statistically significant increase in the latency times

compared to their baselines. Only rats in the treatment group Mor + Maro reached the cut off time of 10 seconds. The mean time-response latencies are shown in Table 3.1a. Although there appeared to be some numerical differences in the time-response values at some time points and the effect-time AUC between treatment groups, the overall difference between the Least Squares means of the latencies for the treatment groups was only significant for morphine *versus* saline ($p < 0.05$).

3.4.2 Tail flick test

3.4.2.1 AUC

The mean area under the effect-time curve (AUC) is shown in Figure 3.1b. The rank order of the AUC for the treatment groups was found to be Maro + Mor (630.8 ± 530.0 seconds) > Mor (383.0 ± 249.3 seconds) > Dex + Mor (351.3 ± 143.1 seconds) > Dex + Maro + Mor (322.8 ± 135.5 seconds) > Maro (282.8 ± 206.5 seconds) > Dex (267.8 ± 96.2 seconds) > Dex + Maro (253.5 ± 12.7 seconds)> saline (212.0 ± 71.4 seconds).

3.4.2.2 Mean time-response latencies

The mean time-response latencies and the time of peak effect are shown in Table 3.1b. The treatment groups Mor + Maro ($p < 0.0001$), Mor ($p < 0.05$), Mor + Dex ($p < 0.05$) and Maro ($p < 0.05$) showed increased latency times compared to their baselines 15 minutes after administration of the drugs. The increase in latency was observed only at 15 minutes in the group Maro, and till 30, 60, and 90 minutes in the groups Mor + Dex, Mor and Mor + Maro respectively. Some rats in treatment group Mor + Maro reached the cut-off of 10 seconds from 15 up to 120 minutes after injection. The proc mixed procedure showed a significant difference ($p < 0.0001$) between means of the latencies between Mor + Maro and all other treatment groups in the tail flick test. This significant

difference was observed until 60 minutes after drug administration.

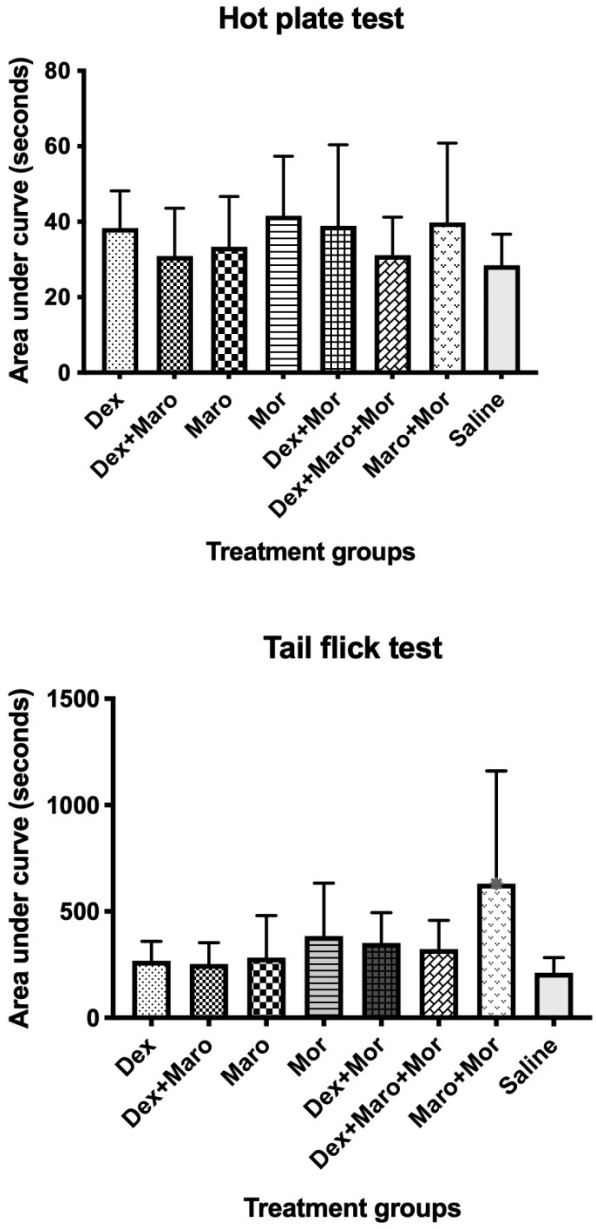

Figure 3.1 Comparison of the analgesic effects in rats of eight drug combinations using the mean area under the effect-time curve (AUC) of the latencies in a) hot plate test and b) tail flick test.

Mor, morphine 3 mg kg^{-1}; Dex, dexmedetomidine 10 μg kg^{-1}; Maro, maropitant 20 mg kg^{-1}; Dex+Mor, dexmedetomidine 5 μg kg^{-1} + morphine: 1.5 mg kg^{-1}; Dex+ Maro, dexmedetomidine 5 μg kg^{-1} + maropitant 10 mg kg^{-1}; Maro+Mor, maropitant 10 mg kg^{-1} + morphine 1.5 mg kg^{-1}; Maro+Mor+Dex, maropitant 6.5 mg kg^{-1} + morphine 1 mg kg^{-1} + dexmedetomidine 3.5 μg kg^{-1}; normal saline 0.5 mL.

Table 3.1 The mean time-response latencies of rats during (a) hot plate test and (b) tail flick test after treatment with eight different analgesic combinations.

Mor, morphine 3 mg kg^{-1}; Dex, dexmedetomidine 10 µg kg^{-1}; Maro, maropitant 20 mg kg^{-1}; Dex+Mor, dexmedetomidine 5 µg kg^{-1} + morphine: 1.5 mg kg^{-1}; Dex+ Maro, dexmedetomidine 5 µg kg^{-1} + maropitant 10 mg kg^{-1}; Maro+Mor, maropitant 10 mg kg^{-1} + morphine 1.5 mg kg^{-1}; Maro+Mor+Dex, maropitant 6.5 mg kg^{-1} + morphine 1 mg kg^{-1} + dexmedetomidine 3.5 µg kg^{-1}; normal saline 0.5 mL.

a)

	Mean time-response latency (seconds)							
Time after injection (minutes)	**Dex** (*n* = 12)	**Dex+Maro** (*n* = 12)	**Dex+Maro+ Mor** (*n*=12)	**Dex+Mor** (*n*=12)	**Maro** (*n*=11)	**Maro+Mor** (*n*=11)	**Mor** (*n*=12)	**Saline** (*n*=12)
0	2.1 ± 0.4	2.2 ± 0.6	2.2 ± 0.5	2.3 ± 0.6	2.0 ± 0.3	2.3 ± 0.5	2.2 ± 0.5	2.0 ± 0.3
5	2.5 ± 1.5	2.7 ± 1.9	2.6 ± 1.3	3.1 ± 1.6	2.3 ± 2.3	4.4 ± 4.3	2.9 ± 1.0$^{\#}$	1.9 ± 0.7
15	2.4 ± 0.9	1.9 ± 0.5	3.3 ± 1.5	3.7 ± 1.8$^{*\#}$	3.2 ± 4.2*	7.4 ± 5.2$^{\dagger\#}$	4.1 ± 1.6$^{*\#}$	1.8 ± 0.7
30	2.4 ± 0.9	2.3 ± 0.9	3.3 ± 1.5	3.8 ± 1.4$^{*\#}$	2.2 ± 1.5	7.8 ± 5.8$^{\dagger\#}$	4.3 ± 2.4$^{*\#}$	1.7 ± 0.4
45	2.5 ± 0.9	2.4 ± 0.6	2.9 ± 1.0	3.4 ± 1.3	2.9 ± 2.7$^{*\#}$	6.1 ± 4.7$^{\dagger\#}$	4 ± 3.0$^{*\#}$	1.8 ± 0.4
60	2.3 ± 0.8	2.0 ± 0.5	2.8 ± 1.1	3.0 ± 1.2	2.2 ±1.0*	6 ± 5.1$^{\dagger\#}$	3.8 ± 3.2$^{*\#}$	1.9 ± 0.5
90	2.0 ± 0.6	2.0 ± 0.8	2.3 ± 1.0	2.3 ± 1.0	2.1 ± 1.1	3.8 ± 3.2$^{*\#}$	2.2 ± 0.9	1.6 ± 0.7
120	1.9 ± 0.7	1.9 ± 1.0	2.1 ± 1.2	2.1 ± 0.7	2.1 ± 0.6	2.8 ± 2.2	1.9 ± 0.5	1.8 ± 0.5

b)

	Mean time-response latency (seconds)							
Time after injection (seconds)	**Dex** (*n*=12)	**Dex+Maro** (*n*=12)	**Dex+Maro+Mor** (*n*=12)	**Dex+Mor** (*n*=12)	**Maro** (*n*=11)	**Maro+Mor** (*n*=11)	**Mor** (*n*=12)	**Saline** (*n*=12)
0	4.7 ± 1.2	4.4 ± 1.3	4.1 ± 1.1	4.4 ± 1.3	4.6 ± 1.4	4.2 ± 1.5	4.8 ± 1.2	4.0 ± 0.7
5	5.2 ± 1.2	5.0 ± 2.9	4.5 ± 1.5	6 ± 4.4	4.7 ± 2.4	6.4 ± 3.6*	6 ± 2.2	4.1 ± 0.9
15	5.5 ± 1.4	4.4 ± 1.7	4.9 ± 2.3	6.3 ± 4.1$^{*\#}$	5.4 ± 2.8*	6.9 ± 4.4*†	7.1 ± 3.2$^{*\#}$	4.3 ± 1.4
30	4.9 ± 1	4.0 ± 1.2	4.8 ± 1.3	6.6 ± 4.0$^{*\#}$	4.9 ± 1.7	5.2 ± 2.3*†	5.9 ± 1.7$^{*\#}$	3.9 ± 1.1
45	5.8 ± 1.4	4.9 ± 2.7	4.7 ± 1.9	6.0 ± 3.3	4.3 ± 1.9$^{\#}$	6.0 ± 3.6*†	6.6 ± 3.2$^{*\#}$	4.0 ± 1.4
60	6.5 ± 2.6	4.6 ± 1.9	4.5 ± 1.2	5.2 ± 2.8	4.9 ± 2.1	5.8 ± 3.6*†	6.2 ± 3$^{*\#}$	4.2 ± 1.5
90	5.5 ± 1.6	3.9 ± 1.1	3.8 ± 1.5	4.3 ± 1.9	4.5 ± 2.0	5.4 ± 3.4*	4.9 ± 1.4	4.1 ± 1.3
120	5.2 ± 1.6	3.8 ± 1.5	3.8 ± 1.7	4.6 ± 2.0	4.8 ± 2.3	4.1 ± 2.8	5.0 ± 1.8	3.8 ± 1.5

***** The least square (LS) means statistically different ($p < 0.05$) from baseline (T0) within the treatment group.

† The LS means statistically significant ($p < 0.05$) compared to morphine

$^{\#}$ The LS means statistically significant compared to **s**aline

3.5 Discussion

We found that the combination of morphine and maropitant provides a potent anti-nociceptive effect at doses that are below the individual drug doses in a thermal-induced pain model. There appears to be a supra-additive effect between morphine and maropitant that could be due to their action on inhibiting the effects of substance P at the spinal level. Substance P is one of the neurotransmitters released by nociceptive primary afferents of the C fibres after noxious thermal stimulus (Duggan et al. 1987) which binds to neurokinin (NK1) receptors (IUPHAR/BPS 2018). Depletion of primary afferent substance P resulted in substantial thermal anti-nociception in Guinea pigs (Buck et al. 1981) and increased tail flick and hot-plate thresholds in adult rats (Nagy et al. 1980). The synergy between morphine and maropitant is biologically plausible as opioids can inhibit the release of substance P at primary afferents (Nicoll et al. 1980) and maropitant is an NK1 antagonist (Benchaoui et al. 2007).

Our results are consistent with findings by (De Felipe et al. (1998) who examined the analgesic response to morphine in NK1 R knockout (NK1−/−) and normal (NK1+/+) mice using hotplate and tail flick tests. Morphine increased the hot plate latencies (ie, provided analgesia) in both NK1−/− and NK1+/+ but the effect in NK1 −/− mice was significantly lower ($p < 0.05$) compared to NK1 +/+ mice. This, along with our results, indicates that the greater-than-additive effect between morphine and maropitant is more observable at the spinal level than supraspinal level. This could also be the reason why the significant difference between Mor + Maro and other treatment groups was exhibited in the tail flick test (spinally mediated) but not in the hotplate test (supraspinally mediated) in our study. Thus, it is expected that the anti-nociceptive

effect of a drug combination acting at the spinal level would be more obvious in a tail flick test.

Dexmedetomidine and maropitant when used singly produced little to no anti-nociception in both the tests. To our knowledge, there are no studies evaluating the analgesic efficacy of maropitant using hotplate and tail flick tests. Dexmedetomidine has been shown to be a very potent anti-nociceptive when administered intrathecally to rats but the dose required to produce anti-nociception by other parental routes is much higher (20-30 μg kg^{-1}) (Guneli et al. 2007; Gursoy et al. 2011). We chose the lowest doses of drugs we considered likely to be effective when used singly. These doses were chosen out of concern that high doses of individual drugs might overshadow any interactions. It is possible that the doses were too low to show a significant increase in latency times in either test. Dexmedetomidine at higher dose would also have caused sedation, therefore affecting the observations.

Although the hotplate and tail flick tests are simple to execute, they have some limitations. They are both thermal stimuli which have been developed and used mainly to test opioid analgesics (Le Bars et al. 2001) and they have been shown to be less sensitive for non-opioid analgesics. In our hotplate tests, we relied upon behaviours such as forepaw or hind paw licking and/or jumping as the endpoint. The paw licking behaviour is more stereotyped for mice and is complex in rats. These behavioural endpoints may be affected differently by different drugs and this could affect the test sensitivity (Carter 1991).

A limitation of our study was that the number of experiments was too low to show synergy between these drugs. One approach to demonstrate such synergy is isobolographic analysis which involves a series of experiments with combinations of drugs at different dose ratios. This was a pilot study which aimed to produce some initial indicative data about the interactions between morphine, dexmedetomidine and maropitant to guide studies in the future.

One rat from group Maro and another from group Mor + Maro died during the study. Both rats died immediately after injection of the treatment drugs. Postmortem examination of the Maro rat revealed a large amount of fluid within the airways. This is a rather non-specific finding and can be attributed to a large number of causes, however, given the temporal association with the IV administration of maropitant, a non-cardiogenic cause for the pulmonary oedema secondary to the administration of this drug cannot be ruled out. Postmortem of another rat from group Mor + Maro did not show any gross nor histological lesions which could explain the cause of death. Because the intravenous dose of maropitant in rats has not been reported in the literature, the dose was extrapolated from that in a dog study (Boscan et al. 2011) using allometric scaling (Boxenbaum 1982). It is possible that the dose of maropitant used was toxic, although Aguado et al. (2015) used 30 mg kg-1 intraperitoneally in rats. A dose effect and toxicological study of maropitant in rats is recommended. Tails of some rats in each treatment group showed multifocal to coalescing, orange red, crusty exudative cutaneous excoriative lesions. The histopathology revealed the presence of a vasculitis in all the tail lesions which is not specific for an aetiology. To minimize the volume of injection, we used concentrated solutions. Thus, we cannot rule out a drug-induced irritation leading to self-mutilation in these cases.

This is the first study we are aware of evaluating the analgesic efficacy of the combination of these three drugs. The brief noxious stimuli involved in these studies in normal rats cannot be equated to the pain originating from the tissue damage. These results offer promise for clinical trials testing analgesic efficacy of these drug combination during various painful procedures in different veterinary patients.

3.6 References

Aguado D, Abreu M, Benito J et al. (2015) Amitriptyline, minocycline and maropitant reduce the sevoflurane minimum alveolar concentration and potentiate remifentanil but do not prevent acute opioid tolerance and hyperalgesia in the rat: A randomised laboratory study. European Journal of Anaesthesiology 32, 248-254.

Benchaoui H, Cox S, Schneider R et al. (2007) The pharmacokinetics of maropitant, a novel neurokinin type-1 receptor antagonist, in dogs. Journal of Veterinary Pharmacology and Therapeutics 30, 336-344.

Boscan P, Monnet E, Mama K et al. (2011) Effect of maropitant, a neurokinin 1 receptor antagonist, on anesthetic requirements during noxious visceral stimulation of the ovary in dogs. American Journal of Veterinary Research 72, 1576-1579.

Boxenbaum H (1982) Interspecies scaling, allometry, physiological time, and the ground plan of pharmacokinetics. Journal of Pharmacokinetics and Biopharmaceutics 10, 201-227.

Buck S, Deshmukh P, Yamamura H et al. (1981) Thermal analgesia and substance P depletion induced by capsaicin in guinea-pigs. Neuroscience 6, 2217-2222.

Carter RB (1991) Differentiating analgesic and non-analgesic drug activities on rat hot plate: effect of behavioral endpoint. Pain 47, 211-220.

D'Amour FL, Smith DL (1941) A method for determining loss of pain sensation. Journal of Pharmacology and Experimental Therapeutics 72, 74-79.

De Felipe C, Herrero JF, O'Brien JA et al. (1998) Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. Nature 392, 394-397.

Duggan A, Morton C, Zhao Z et al. (1987) Noxious heating of the skin releases immunoreactive substance P in the substantia gelatinosa of the cat: a study with antibody microprobes. Brain Research 403, 345-349.

Eddy NB, Leimbach D (1953) Synthetic analgesics. II. Dithienylbutenyl-and dithienylbutylamines. Journal of Pharmacology and Experimental Therapeutics 107, 385-393.

Gamse R, Saria A (1986) Nociceptive behavior after intrathecal injections of substance P, neurokinin A and calcitonin gene-related peptide in mice. Neuroscience Letters 70, 143-147.

Gaynor J (1999) Is postoperative pain management important in dogs and cats? Veterinary Medicine 94, 254-258.

Go V, Yaksh T (1987) Release of substance P from the cat spinal cord. The Journal of Physiology 391, 141.

Guneli E, Yavasoglu NUK, Apaydin S et al. (2007) Analysis of the antinociceptive effect of systemic administration of tramadol and dexmedetomidine combination on rat models of acute and neuropathic pain. Pharmacology Biochemistry and Behavior 88, 9-17.

Guo T-Z, Jiang J-Y, Buttermann AE et al. (1996) Dexmedetomidine injection into the locus ceruleus produces antinociception. The Journal of the American Society of Anesthesiologists 84, 873-881.

Gursoy S, Ozdemir E, Bagcivan I et al. (2011) Effects of alpha 2-adrenoceptor agonists dexmedetomidine and guanfacine on morphine analgesia and tolerance in rats. Uppsala Journal of Medical Sciences 116, 238-246.

Hunskaar S, Berge O-G, Hole K (1986) A modified hot-plate test sensitivie to mild analgesics. Behavioural Brain Research 21, 101-108.

IUPHAR/BPS (2018). http://www.guidetopharmacology.org/

Jin F, Chung F (2001) Multimodal analgesia for postoperative pain control. Journal of Clinical Anaesthesia 13, 524-539.

Kalso EA, Pöyhiä R, Rosenberg PH (1991) Spinal antinociception by dexmedetomidine, a highly selective α2-adrenergic agonist. Basic & Clinical Pharmacology & Toxicology 68, 140-143.

Keyhanfar F, Meymandi MS, Sepehri G et al. (2013) Evaluation of antinociceptive effect of pregabalin in mice and its combination with tramadol using tail flick test. Iranian Journal of Pharmaceutical Research 12, 483.

Kilkenny C, Browne WJ, Cuthill IC et al. (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biology 8, e1000412.

Kuusela E, Vainio O, Kaistinen A et al. (2001) Sedative, analgesic, and cardiovascular effects of levomedetomidine alone and in combination with dexmedetomidine in dogs. American Journal of Veterinary Research 62, 616-621.

Lascelles BDX, McFarland JM, Swann H (2005) Guidelines for safe and effective use of NSAIDs in dogs. Veterinary Therapeutics 6, 237.

Le Bars D, Gozariu M, Cadden SW (2001) Animal models of nociception. Pharmacological Reviews 53, 597-652.

Marquez M, Boscan P, Weir H et al. (2015) Comparison of NK-1 receptor antagonist (maropitant) to morphine as a pre-anaesthetic agent for canine ovariohysterectomy. PloS One 10, e0140734.

Nagy J, Vincent S, Staines W et al. (1980) Neurotoxic action of capsaicin on spinal substance P neurons. Brain Research 186, 435-444.

Nicoll R, Schenker C, Leeman S (1980) Substance P as a transmitter candidate. Annual Review of Neuroscience 3, 227-268.

Niyom S, Boscan P, Twedt DC et al. (2013) Effect of maropitant, a neurokinin-1 receptor antagonist, on the minimum alveolar concentration of sevoflurane during stimulation of the ovarian ligament in cats. Veterinary Anaesthesia and Analgesia 40, 425-431.

Ossipov MH, Harris S, Lloyd P et al. (1990) Antinociceptive interaction between opioids and medetomidine: systemic additivity and spinal synergy. Anesthesiology 73, 1227-1235.

Pascoe PJ (2000) Opioid analgesics. Veterinary Clinics: Small Animal Practice 30, 757-772.

Sakurada T, Katsumata K, Yogo H et al. (1995) The neurokinin-1 receptor antagonist, sendide, exhibits antinociceptive activity in the formalin test. Pain 60, 175-180.

Stone LS, German JP, Kitto KF et al. (2014) Morphine and clonidine combination therapy improves therapeutic window in mice: synergy in antinociceptive but not in sedative or cardiovascular effects. PloS One 9, e109903.

Vainio O (1989) Introduction to the clinical pharmacology of medetomidine. Acta Veterinaria Scandinavica Supplementum 85, 85-88.

Yashpal K, Dam T-V (1990) Quantitative autoradiographic distribution of multiple neurokinin binding sites in rat spinal cord. Brain Research 506, 259-266.

Young A, Buvanendran A (2012) Recent advances in multimodal analgesia. Anesthesiology Clinics 30, 91-100.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Sandeep Raj Karna
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Name of Research Output and full reference:	
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• The percentage of the manuscript/Published Work that was contributed by the candidate:	80
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Sandeep had a primary role in study design, conducting experiments, data collection, statistical analysis, interpretation and preparation of the manuscript, with guidance from supervisors.	
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Primary Supervisor's Signature:	Kavitha Kongara Digitally signed by Kavitha Kongara Date: 2020.02.25 08:44:32 +13'00'
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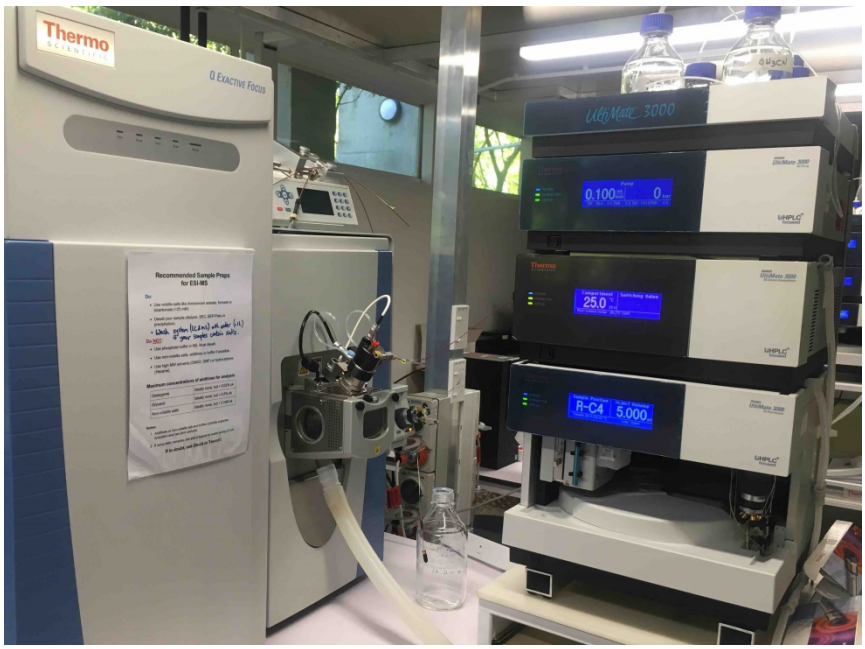
CHAPTER 4

Pharmacokinetics of morphine in combination with dexmedetomidine and maropitant following intramuscular injection in dogs anaesthetized with halothane



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4.1 Abstract

The purpose of this study was to evaluate the pharmacokinetics of morphine in combination with dexmedetomidine and maropitant injected intramuscularly in dogs under general anaesthesia. Eight healthy dogs weighing 25.76 ± 3.16 Kg and 3.87 ± 1.64 years of age were used in a crossover study. Dogs were randomly allocated to four groups: 1) morphine 0.6 mg/kg; 2) morphine 0.3 mg/kg + dexmedetomidine 5 μg/kg; 3) morphine 0.3 mg/kg + maropitant 1 mg/kg; 4) morphine 0.2 mg/kg + dexmedetomidine 3 μg/kg + maropitant 0.7 mg/kg. Blood samples were collected before, 15 and 30 min, and 1, 2, 3 4, 6 and 8 h after injection of the test drugs. Plasma concentration of the drugs was determined by liquid chromatography-mass spectrometry. The elimination half-life ($T_{1/2}$) of morphine was higher and the clearance rate (CL) was lower when combined with dexmedetomidine ($T_{1/2}$ = 77.72 ± 20.27 min, CL= 119.41 ± 23.34 mL/kg/min) compared to maropitant ($T_{1/2}$ = 52.73 min ± 13.823 mL/kg/min, CL= 178.57 ± 70.55) or morphine alone at higher doses ($T_{1/2}$ = 50.53 ± 12.55 min, CL= 187.24 ± 34.45 mL/kg/min). Combining morphine with dexmedetomidine may increase the dosing interval of morphine and may have a clinical advantage.

Keywords: alpha-2 adrenoceptor agonist, dogs, halothane, intramuscular, neurokinin -1 receptor antagonist, opioid

4.2 Introduction

Morphine is a prototypical opiate and one of the most commonly used analgesics in perioperative and post-operative procedures as well as other situations in dogs. The

intravenous (IV) or intramuscular (IM) administration of morphine is characterized by a rapid increase in the plasma concentration and fast decline due to a rapid clearance (Hug, Murphy, Murphy, Rigel, & Olson, 1981). The high clearance rate may lead to the requirement of frequent dosing or administration of a higher dose in the postoperative period.

Morphine is associated with several adverse effects in dogs. Studies have shown that IV and IM dose of morphine at or below 1 mg/kg can cause emesis (Barnhart, Hubbell, Muir, Sams, & Bednarski, 2000; Blancquaert, Lefebvre, & Willems, 1986; Kongara, Chambers, & Johnson, 2012). Morphine can also cause respiratory depression (Taylor & Houlton, 1984; Mastrocinque & Fantoni, 2003) and release of histamines leading to cardiovascular changes when used intravascularly (Guedes, Rudé, & Rider, 2006; Maiante, Teixeira Neto, Beier, Corrente, & Pedroso, 2009; Robinson, Faggella, Henry, & Russell, 1988). To lower the required doses of morphine and decrease the potential adverse effects, multimodal analgesia protocols are recommended for pain management in dogs and cats (Epstein et al., 2015). This is achieved by combining morphine with other analgesics that act by different mechanisms resulting in synergistic or additive analgesia (Corletto, 2007). However, pharmacokinetic interaction may occur between the co-administered drugs which may alter the individual pharmacokinetics and have clinical relevance (Maurer & Bartkowski, 1993).

A multimodal analgesia protocol combining low doses of morphine with dexmedetomidine and maropitant has been proposed by the authors for providing peri- and post-operative analgesia in dogs. Dexmedetomidine is a highly selective alpha-2-adrenoceptor agonist (Vainio, 1989) and a commonly used sedative and analgesic in dogs and cats (Murrell & Hellebrekers, 2005). Rat studies have shown anti-nociceptive

synergism between opioids and alpha-2 adrenoceptor agonist (Ossipov et al., 1990). Maropitant is a neurokinin -1 (NK1) receptor antagonist used to control emesis of different aetiologies in dogs and cats (Diemunsch & Grélot, 2000; De la Puente-Redondo et al., 2007). Maropitant also has an anaesthetic dose sparing effect in dogs and cats undergoing ovariohysterectomy (OHE) (Boscan et al., 2011; Niyom, Boscan, Twedt, Monnet, & Eickhoff, 2013) and a supra-additive effect for analgesia in combination with morphine in a rat model (Karna, Kongara, Singh, Chambers, & Lopez-Villalobos, 2019).

Pharmacokinetics of IM administered morphine has been studied previously, (Barnhart et al., 2000; Dohoo, Tasker, & Donald, 1994) but not in combination with other two drugs dexmedetomidine and maropitant, and not in anaesthetised dogs. The recommended dose of morphine for sedation and premedication in dogs is 0.2-1 mg/kg IM (Hall & Clarke, 1983). However, the pharmacokinetics of morphine below 0.5 mg/kg dose has not been studied. The primary aim of the present study was to describe the pharmacokinetics of three different doses of IM administered morphine (at 0.6 mg/kg when used singly, and 0.3 and 0.2 mg/kg in combination with maropitant and dexmedetomidine) under anaesthesia. The secondary aim was to investigate the pharmacokinetics of dexmedetomidine and maropitant in combination with morphine administered intramuscularly in dogs under anaesthesia.

4.3 Materials and methods

4.3.1 Study design and dogs

The study was approved by Massey University Animal Ethics Committee (protocol no 17/57) and is reported in accordance with the ARRIVE guidelines for experiments

involving animals (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010). The sample size calculation was based on the expected difference in pharmacokinetic parameters of morphine derived from previous studies (Dohoo et al., 1994; Barnhart et al., 2000). The sample size was estimated to achieve the power of experiment of 80% with an alpha error of 0.05.

Eight healthy dogs (5 Harrier Hounds; 3 Shetland Sheepdogs) were used in a randomised crossover study with four treatments for each dog separated by a three-week washout period. The blood plasma concentration level of the test drugs was analysed at the end of the three-week washout period and no detectable level of drugs was present. The randomisation schedule of the treatment was generated using PLAN procedure in SAS for Windows v 9.4 (SAS Institute Inc Cary NC, USA).

The dogs were judged to be in good health by physical examination and history. The mean age and body weight of dogs were 3.87 ± 1.64 years and 25.76 ± 3.16 kg respectively.

The dogs were obtained from the Centre for Canine Nutrition (CCN) at Massey University, Palmerston North, New Zealand. They were housed in a larger outer pen during the day and indoor pens in pairs in the night. Dogs were fed once a day and had unlimited access to water all the time. The dogs were brought to the laboratory on the day of the experiment and were kept in the laboratory until the final blood collection time point. After that, they were housed at the MU Veterinary Hospital overnight and returned to the CCN facility, the next day.

The treatment groups were as follows: group 1 (Mor): morphine 0.6 mg/kg; group 2 (Dex+Mor): morphine 0.3 mg/kg + dexmedetomidine 5 μg/kg; group 3 (Maro+Mor):

morphine 0.3 mg/kg + maropitant 1 mg/kg; group 4 (Dex+Maro+Mor): morphine 0.2 mg/kg + dexmedetomidine 3 µg/kg + maropitant 0.7 mg/kg, all injected intramuscularly. The dosages of the drugs were based on clinical opinion of the authors.

4.3.2 Drug administration

The dogs were fasted for 12 hr before each treatment and had unlimited access to water until premedication. They were premedicated with acepromazine (Acezine 2; Ethical Agents Ltd, New Zealand) 0.02 mg/kg IM 40 min prior to the anaesthetic induction. Right saphenous vein was catheterised percutaneously using a 22 gauge 1.00-inch catheter for anaesthetic induction, blood sampling and administration of Hartmann's solution (Baxter Healthcare Ltd, Australia). Anaesthesia was induced with IV Propofol (Propofol Injection; Mayne Pharma Pty Ltd, Australia) to effect.

After induction of anaesthesia, a cuffed endotracheal tube was used to intubate the trachea. Anaesthesia was maintained by halothane (HalothaneVet; Merial NZ Limited, New Zealand) in oxygen delivered via a circle breathing system for 35 min. The End-tidal halothane (EtHal) was maintained between 0.85% and 0.95% and dogs breathed spontaneously. The dogs were given Hartmann's solution IV to maintain systolic arterial blood pressure above 100 mmHg throughout the anaesthetic period and their systolic arterial blood pressure was monitored using a Doppler ultrasound pressure transducer with cuff (Doppler flow detector, Parks Medical Electronics Inc., USA). Heart rate (HR), respiratory rate (RR), end-tidal halothane tension (EtHal) and end-tidal CO_2 tension ($EtCO_2$) of dogs were monitored using an anaesthetic agent monitor (Hewlett Packard M1025B; Hewlett Packard, Germany). Arterial haemoglobin oxygen saturation (SpO_2) and the temperature was monitored using a pulse oximeter (Pulse Ox-

Fisher & Paykel Healthcare Ltd, Auckland, NZ), and a digital veterinary thermometer (Kamsay, USA), respectively. All of these parameters were monitored continuously and recorded every 5 min.

Dogs were positioned into left lateral recumbency. Immediately after the target EtHal was achieved, treatment drugs were injected into lumbar epaxial muscles using a 2 ml syringe and 22 gauge, 1-inch needle. All drugs were injected separately within a minute starting with morphine followed by maropitant and dexmedetomidine. The drugs used were morphine sulphate (DBL morphine sulphate injection BP; Hospira Australia Pty. Ltd., Australia) 5 mg/ml, dexmedetomidine hydrochloride (Dexdomitor; Zoetis, New Zealand) 0.5 mg/ml and maropitant citrate (Cerenia; Zoetis, New Zealand) 10 mg/ml.

This study was performed in combination with another study measuring the electroencephalographic (EEG) responses of the treatment drugs after noxious electric stimulus which has not been described here in detail. Dogs received a brief electrical stimulus after 30 min of achieving the target EtHal. After 5 min, the halothane was discontinued and the endotracheal tube was removed when the dogs were able to maintain their airway. Dogs were monitored for any adverse effects throughout the study period.

Blood samples (2 ml) were collected before and 15 and 30 min and 1, 2, 3 4, 6 and 8 hr after injection of the test drugs. Blood collection before injection of the test drugs and at 15 and 30 min was performed under anaesthesia, at 1 and 2 hr were performed during recovery and the dogs were fully awake at the remaining blood collection time points. Blood collection was performed using a modified push-pull method (Barr, Gianotti et al., 2017): before collecting the blood sample for analysis, 2 ml blood was drawn in 5 ml syringe already filled with 2 ml heparinised saline and reinfused after the blood

sample was collected. Blood samples were collected in heparin tubes which were refrigerated. Plasma was collected by centrifuging at 1,000 *g* for 10 min and stored at −80 °C until analysis.

4.3.3 Analytical methodology

4.3.3.1 Liquid Chromatography-Mass Spectrometry (LCMS)

The assay of maropitant was based on published guidelines by Kenward, Elliott, Elliott, Lee and Pelligand (2017) with some modifications whereas a common method for assay of morphine and dexmedetomidine was developed in the investigators' laboratory.

4.3.3.1.1 Instrumentation

Chromatic separation of dexmedetomidine, maropitant and morphine was determined from plasma samples using the Ultra High Performance Liquid Chromatography system (Dionex UltiMate 3000 System; Thermo Scientific, Germany) consisting of a vacuum degasser, a tertiary loading pump, a column oven and an autosampler. A 2.6 µm particle size 100 mm x 2.1 mm column (Accucore 150 C18 Column; Thermo Scientific, Germany) fitted with an identically packed guard column (Accucore Defender Guard Column; Thermo Scientific, Germany) was used for all analyses. Mass spectrometry detection was performed with a hybrid quadrupole orbitrap mass spectrometer (Q Exactive Focus; Thermo Scientific, Germany).

4.3.3.1.2 Sample preparation

The standards and plasma samples were prepared by precipitating 120 µl of plasma with 300 µl of acetonitrile and vortexing for 30 s. The mixture was centrifuged at 14,000 *g* for 10 min and the clear supernatant was passed through phospholipid removal tubes

(Phree Phospholipid removal; Phenomenex, USA). The filtrate was centrifuged again at 14,000 *g* for 10 min. The supernatant was transferred to the autosampler vials, 5 µl of which was injected into the column.

4.3.3.1.3 LCMS Conditions for morphine and dexmedetomidine

For morphine and dexmedetomidine, the mobile phase consisted of 0.1% formic acid in H_20 and & 0.1% formic acid in acetonitrile (ratio of 90:10). An isocratic flow was maintained with a flow rate of 0.3 ml/min and a run time of 6 min. The injection volume was 5 µl and the temperature of the column was 25 °C. The heated electrospray ionization probe was maintained at 3.30 KV and all analyses were performed in the positive ionization mode. Nitrogen was used and the sheath, auxiliary and ion sweep gas flow was 30, 5, and 1 arbitrary units respectively. The capillary tube was kept at 320 °C.

4.3.3.1.4 LCMS conditions for maropitant

For maropitant, the mobile phase consisted of 0.3% formic acid in 5 mM ammonium formate solution and 0.3% formic acid in acetonitrile. A gradient flow was maintained with the flow rate of 0.3 ml/min and the run time was of 10 min. The injection volume was 5 µl and the temperature of the column was 55 °C. The heated electrospray ionization probe was maintained at 3.30 KV and all analyses were performed in the positive ionization mode. Nitrogen was used at the sheath, auxiliary and ion sweep gas flow at 15, 7, and 1 arbitrary units, respectively. The capillary tube was kept at 320 °C.

4.3.3.2 LCMS method validation

The validation for each drug was performed separately. A blank plasma obtained from an untreated healthy dog was fortified with the stock solution of drugs. The stock solutions of the drugs were the same as used for the treatment of dogs mentioned above.

Dexmedetomidine and morphine were dissolved in water and maropitant were dissolved in methanol. Six standards for the calibration curve ranged from 0.52 to 166 ng/ml for morphine, 0.25 to 16.6 ng/ml for dexmedetomidine and 5.2 to 1666.67 ng/ml for maropitant, were used. The lower limits of quantification (LOQ) of the compounds were determined by the signal-to-noise ratio of 10:1. Relative recoveries at each concentration were calculated by comparing the mean area response of unextracted samples (spiked after extraction) with that of control standards following the same sample preparation. Intra-day and inter-day variation was calculated at all five concentrations of independently prepared spiked plasma on the same day and three consecutive days, respectively.

4.3.4 Pharmacokinetic analysis

The pharmacokinetic analysis of concentration-time data was performed using a non-compartmental model. The maximum plasma concentration of the drug ($C_{max,}$ ng/ml) and time to reach C_{max} ($T_{max,}$ min) were determined as direct observation from the plasma drug concentration results. Other pharmacokinetic parameters including half-life of terminal elimination phase ($T_{1/2}$, min), area under the plasma concentration-time curve (AUC_{0-t}, ng*min/ml), area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$, ng*min/ml), volume of distribution based on the terminal slope (V_z, mL/kg), clearance (CL, mL/min/kg) and mean resident time (MRT, min) were calculated using the PKSolver add-in program for Excel 2010 (Zhang, Huo, Huo, Zhou, & Xie, 2010).

4.3.5 Statistical analysis

All data are presented as mean ± SD unless otherwise stated. All statistical analyses were performed using Prism 8 for Macintosh (GraphPad Software, Inc., San Diego, CA, USA). Normality of the data was checked by the D' Agostino and Pearson normality test. For the pharmacokinetic parameters for morphine, which was compared in four treatment groups, the normally distributed data were analysed by one-way ANOVA (analysis of variance) with post-hoc Tukey's multiple comparison test and the non-normally distributed data were analysed using Kruskal–Wallis test with Dunn's multiple comparisons as a post-hoc test. For dexmedetomidine and maropitant, which were used in two treatment groups, the normally distributed data were analysed using a paired t-test and non-normally distributed data were analysed using Wilcoxon matched-pairs signed rank test. The association of body weight, breed, and age with PK parameters were also compared in a similar way as of PK parameters mentioned above. The value of $p < 0.05$ was considered significant.

4.4 Results

4.4.1 LCMS method validation

The retention time of morphine, dexmedetomidine and maropitant was 0.8 min, 1.4 min and 6.65 min and the LOQ was 0.52 ng/ml, 0.25 ng/ml and 5.21 ng/ml respectively. The calibration curve was linear with correlation coefficient (R^2) 0.997 for morphine, and 0.998 for dexmedetomidine and maropitant. The relative standard deviations for intra-day assay of morphine, dexmedetomidine and maropitant were ≤ 4.62%, ≤ 4.90% and ≤ 2.90% and for inter-day assay were ≤ 10.95%, ≤ 11.18% and ≤ 8.15%, respectively. All

sample analyses were performed in duplicate and analyses were repeated if duplicates varied more than 5%.

4.4.2 Pharmacokinetics of morphine, dexmedetomidine and maropitant

No adverse effects were seen except bradycardia. The heart rate decreased by 20.00-61.81% in group Dex+Mor and by 16.66-38.46% in group Dex+Maro+Mor 1-2 min after administration of dexmedetomidine and remained stable at the decreased level until recovery from anaesthesia. For all treatment drugs, there was no association of body weight, age and breed with the clearance of the drugs and other pharmacokinetic parameters. The semi-log concentration-time curve of different doses of morphine, dexmedetomidine and maropitant have been shown in Figure 1 and the non-compartmental model pharmacokinetics of the drugs with statistical analyses are shown in Table 4.1.

4.4.2.1 Morphine

The C_{max} of morphine was 45.92 ± 15.26, 25.7 ± 9.64, 21.79 ± 7.33, and 17.06 ± 4.90 ng/ml in treatment groups Mor, Dex+Mor, Maro+Mor, and Dex+Maro+Mor respectively. The C_{max} was observed at 15 (77% of dogs) and 30 (33% of dogs) min after administration and its concentration decreased below the LOQ 4h after its administration in the majority of dogs in all the treatment groups. The C_{max} and $AUC_{0-\infty}$ also showed a decrease with the dose. Although groups Dex+Mor and Maro+Mor had the same dose of morphine (0.3 mg/kg), the group Dex+Mor had higher $AUC_{0-\infty}$, $T_{1/2}$, and MRT and lower CL than the group Maro+Mor.

4.4.2.2 Dexmedetomidine

The C_{max} of dexmedetomidine was 5.44 ± 3.07 and 1.83 ± 0.49 ng/ml in treatment groups Dex+Mor and Dex+Maro+Mor respectively. The $C_{max,}$ was observed at 15 (50% of dogs) and 30 (50% of dogs) min after administration and its concentration decreased below the LOQ after 4 h in the majority of dogs in all the treatment groups. The C_{max} and $AUC_{0-\infty}$ decreased significantly with a decrease in the dose.

4.4.2.3 Maropitant

The C_{max} of maropitant was 1507.62 ± 499.55 and 1269.67 ± 668.04 ng/ml in treatment groups Maro+Mor and Dex+Maro+Mor respectively. The C_{max} of maropitant was observed at 15 (43.75% of dogs) and 30 (56.25% of dogs) min after administration and the concentration was detected above the LOQ throughout the study period (up to 8 hr after administration of the drugs). The AUC and C_{max} showed a decrease in the mean values but was not significantly different.

Table 4.1 Pharmacokinetic variables after IM administration of different combinations of a) morphine, b) dexmedetomidine, and c) maropitant.

Values are reported as mean ± SD.

a)

Parameter	Unit	Mor	Dex+Mor	Maro+Mor	Dex+Maro+Mor
$T_{1/2}$	min	50.53 ± 12.55	77.72 ± 20.27	52.73 ± 13.823	58.58 ± 10.71
C_{max}	ng/ml	45.92 ± 15.26	25.7 ± 9.64	21.79 ± 7.33*	17.06 ± 4.90*
T_{max}	min	16.87 ± 5.30	20.62 ± 7.76	18.75 ± 6.94	16.87 ± 5.30
$AUC_{0-\infty}$	ng/ml*min	3313.55 ± 685.43	2614.67 ± 534.38	1749.23187 ± 817.92*	1289.26 ± 226.63*a
$MRT_{0-\infty}$	min	75.32 ± 7.48	118.68 ± 28.70*	83.618947 ± 18.87	87.03 ± 15.94
V_z	L/Kg	13.66 ± 4.32	13.51 ± 4.98	16.07545 ± 10.35	13.76 ± 4.34
CL	mL/kg/min	187.24 ± 34.45	119.41 ± 23.34*	178.57 ± 70.55	161.01 ± 33.94

*= significantly different with Morphine (0.6 mg/kg), a= significantly different with Morphine (0.3 mg/kg) + Dexmedetomidine

b)

Parameters	Units	Dex+Mor	Dex+Maro+Mor
$T_{1/2}$	min	94.46 ± 24.83	102.24 ± 46.75
C_{max}	ng/ml	5.44 ± 3.07	1.83 ± 0.49*
T_{max}	min	31.9 ± 23.3	41.25 ± 20.83
$AUC_{0-\infty}$	ng/ml*min	769.33 ± 327.26	304.06 ± 113.86*
MRT0–∞	min	146.80 ± 21.51	162.63 ± 65.36
V_z	L/kg	1.17 ± 0.68	1.52 ± 0.72
CL	mL/kg/min	8.56 ± 3.89	11.01 ± 3.96

*= significantly different with Morphine (0.6 mg/kg), a= significantly different with Morphine (0.3 mg/kg) + Dexmedetomidine

c)

Parameter	Unit	Maro+Mor	Dex+Maro+Mor
$T_{1/2}$	min	255.81 ± 127.01	177.41 ± 86.86
C_{max}	ng/ml	1507.62 ± 499.55	1269.67 ± 668.04
T_{max}	min	22.5 ± 8.02	26.25 ± 15.63
$AUC_{0-\infty}$	ng/ml*min	227629.49 ± 70610.39	172683.24 ± 74781.54
$MRT_{0-\infty}$	min	323.36 ± 116.01	221.86 ± 70.58
V_z	L/kg	1.61 ± 0.59	1.35 ± 0.82
CL	mL/kg/min	4.77 ± 1.45	4.15 ± 1.64

Treatment groups: 1) **Mor**: morphine 0.6 mg/kg; 2) **Dex+Mor**: morphine 0.3 mg/kg + dexmedetomidine 5 µg/kg; 3) **Maro+Mor**: morphine 0.3 mg/kg + maropitant 1 mg/kg; 4) **Dex+Maro+Mor**: morphine 0.2 mg/kg + dexmedetomidine 3 µg/kg + maropitant 0.7 mg/kg

Abbreviation: $T_{1/2,}$ half-life of terminal elimination phase; $C_{max,}$ maximum plasma concentration of the drug; $T_{max,}$ time to reach $C_{max,}$ $AUC_{0-\infty,}$ area under the plasma concentration-time curve from time zero to infinity; $MRT_{0-\infty}$, mean resident time from time zero to infinity, $V_{z,}$ volume of distribution based on the terminal slope; CL, Plasma clearance

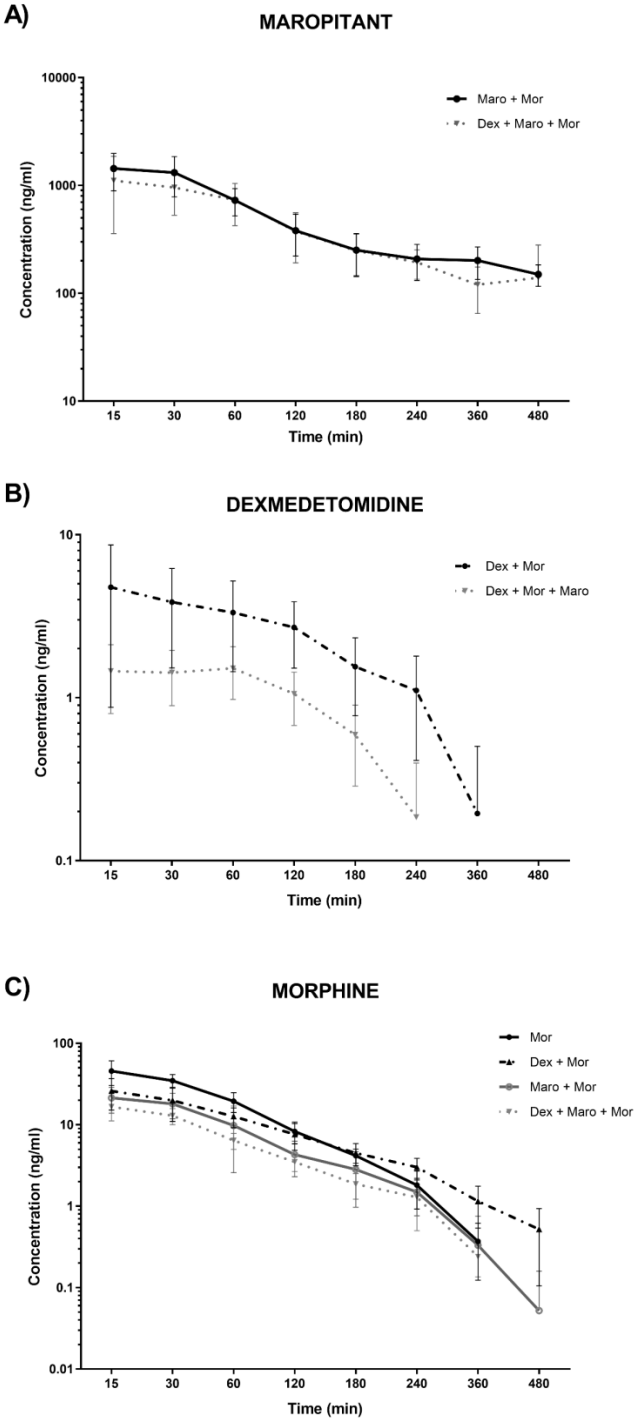

Figure 4.1 Concentration-time curves for pharmacokinetic variables after IM. administration of different combinations of (a) morphine, (b) dexmedetomidine and (c) maropitant in dogs.

4.5 Discussion

The results of morphine pharmacokinetics in the present study are different from previous studies (Dohooet al., 1994; Barnhart et al., 2000) that investigated the disposition of morphine after IM injection in conscious dogs. In the present study, the single treatment group which received 0.6 mg/kg of morphine, had lower C_{max}, $AUC_{0-\infty}$, CL, higher V_z, and similar $T_{1/2}$ as in the study conducted by Dohooet al. (1994) after administering 0.5 mg/kg of morphine.

Multiple factors are responsible for the disposition of the drugs after IM injection, including perfusion at the site of injection and physiological state of the animal (Zuidema, Pieters, & Duchateau, 1988). In addition, anaesthesia including premedication with acepromazine can influence the pharmacokinetics of the drugs by altering perfusion at the site of injection due to decrease in muscle blood flow, extraction by target organs, and protein binding (Nimmo & Peacock, 1988; Sinclair & Dyson, 2012). A study in the sheep model showed that halothane anaesthesia can decrease regional blood flow and extraction of the test drugs by the liver, lung and kidneys (Mather, Runciman, &Ilsley, 1982). Thus, premedication with acepromazine and general anaesthesia for 35 min could be the reason for lower C_{max} of morphine in this study. In addition, the positioning of dogs during anaesthesia (Queckenberg & Fuhr, 2009) and possible differences in IM injection site compared to the published studies could also have contributed to the lower concentration of morphine. This study showed lower C_{max} and similar $T_{1/2}$ of dexmedetomidine compared to a study by Di Cesare et al. (2019) who investigated the pharmacokinetic profile of 10 µg/kg dexmedetomidine administered IM in combination with methadone in dogs under anaesthesia (Di Cesare et al., 2019). The lower C_{max} of dexmedetomidine in the present

study was potentially due to the lower dose of the drug used. Also, a high inter-animal variation in the plasma concentration of dexmedetomidine observed in the present study might be resulting from dexmedetomidine administration after the induction of anaesthesia. Di Cesare et al. (2019) has also reported higher variability in plasma drug concentration of dexmedetomidine during the anaesthetic period which supports our explanation. The pharmacokinetics of IM maropitant in dogs has not been studied previously and thus, the comparison could not be made.

Higher $T_{1/2}$ and MRT and lower clearance rate of morphine were seen in group Dex+Mor compared to Maro+Mor, which used the same doses of morphine (0.3mg/kg). Morphine is primarily metabolized by the liver and the rate of blood flow to the liver is an important factor for the clearance of morphine (Garrett & Jackson, 1979). Dexmedetomidine can decrease the cardiac output and the total flow of blood through the liver in dogs (Lawrence, Prinzen, & Lange, 1996) and humans (Dutta, Lal, Karol, Cohen, & Ebert, 2000). A decrease in the flow of blood to the liver could be a reason for the difference in the pharmacokinetics of morphine when combined with dexmedetomidine. Having a lower clearance rate has a clinical benefit as this can increase the dosing interval of morphine and decrease the risk of potential adverse effects where multiple dosing is required (Toutain & Bousquet-Melou, 2004).

The T_{max} of morphine and maropitant was observed at 15 min for the majority of dogs. Previous studies have shown T_{max} at 5.75 - 15.48 min after IM injection of 0.5 and 1 mg/kg morphine (Dohoo et al., 1994; Barnhart et al., 2000). In the present study, the first blood sampling point was at 15 min and it could be possible that the actual T_{max} and C_{max} was missed. However, T_{max} was delayed up to 60 min for dexmedetomidine. This might be due to the interaction of dexmedetomidine with α-2b-receptors in the

peripheral vascular beds (Murrell & Hellebrekers, 2005) leading to vasoconstriction and decreased absorption of itself and other co-administered drugs (Kallio-Kujala et al., 2018).

Significant inter-animal variability was seen in the pharmacokinetics of all the three drugs. IM injection can have large inter-animal variation compared to IV route of administration because of the variation in the regional blood flow and unintentional administration into peri-muscular fat (Nordberg, Borg, Hedner, & Mellstrand, 1985; Autefage, Fayolle, & Toutain, 1990). Some drugs including morphine have been reported to bind to human muscle and intersubjective variability in the binding could cause variation in their pharmacokinetics (Fichtl & Kurz, 1978). Human studies have also shown a wide variation in absorption and distribution of morphine in surgical patients under anaesthesia (Rigg, Browne, Davis, Khandelwal, & Goldsmith, 1978).

The result of the present study should be interpreted with some limitations. First, disposition of the drugs was studied in dogs premedicated with acepromazine and maintained under halothane anaesthesia which has been shown to alter the drug disposition. Thus, these results apply only to dogs premedicated with acepromazine, anaesthetized with propofol and maintained under halothane anaesthesia for the initial 35 min. Second, the disposition of drugs can be altered by other co-administered drugs and different doses of the same drugs were used in the study. This study was a part of a project that aims to evaluate a multimodal analgesia protocol consisting of a lower dose of morphine (0.3 or 0.2 mg/kg) combined with dexmedetomidine and maropitant and compare it with a higher dose of morphine (0.6 mg/kg). Therefore, the results are applicable only for studies using a combination of morphine, dexmedetomidine and maropitant at the dose rates used in this study.

4.6 Conclusion

This study showed that combining morphine with dexmedetomidine and maropitant can lead to alteration in the pharmacokinetics of the drugs. Combining morphine with dexmedetomidine can lead to prolonged half-life and decreased clearance rate which may have a clinical advantage of an increase in the dosing interval.

4.7 References:

Autefage, A., Fayolle, P. & Toutain, P. (1990) Distribution of material injected intramuscularly in dogs. *American Journal of Veterinary Research*, **51**(6), 901-904.

Barnhart, M.D., Hubbell, J.A., Muir, W.W., Sams, R.A. & Bednarski, R.M. (2000) Pharmacokinetics, pharmacodynamics, and analgesic effects of morphine after rectal, intramuscular, and intravenous administration in dogs. *American Journal of Veterinary Research*, **61**(1), 24-28.

Barr, C.A., Gianotti, G., Graffeo, C.E., Drobatz, K.J. & Silverstein, D.C. (2017) Effect of blood collection by the push-pull technique from an indwelling catheter versus direct venipuncture on venous blood gas values before and after administration of alfaxalone or propofol in dogs. *Journal of the American Veterinary Medical Association*, **251**(10), 1166-1174.

Blancquaert, J.-P., Lefebvre, R.A. & Willems, J.L. (1986) Emetic and antiemetic effects of opioids in the dog. *European Journal of Pharmacology*, **128**(3), 143-150.

Boscan, P., Monnet, E., Mama, K., Twedt, D.C., Congdon, J. & Steffey, E.P. (2011) Effect of maropitant, a neurokinin 1 receptor antagonist, on anesthetic requirements during noxious visceral stimulation of the ovary in dogs. *American Journal of Veterinary Research*, **72**(12), 1576-1579.

Corletto, F. (2007) Multimodal and balanced analgesia. *Veterinary Research Communications*, **31**, 59-63.

De la Puente-Redondo, V., Siedek, E., Benchaoui, H., Tilt, N., Rowan, T. & Clemence, R. (2007) The anti-emetic efficacy of maropitant (Cerenia™) in the treatment of ongoing emesis caused by a wide range of underlying clinical aetiologies in canine patients in Europe. *Journal of Small Animal Practice*, **48**(2), 93-98.

Di Cesare, F., Gioeni, D., Ravasio, G., Pellegrini, A., Lucatello, L., Bisutti, V., Villa, R. & Cagnardi, P. (2019) Clinical pharmacokinetics of a dexmedetomidine–methadone combination in dogs undergoing routine anaesthesia after buccal or intramuscular administration. *Journal of Veterinary Pharmacology and Therapeutics*.

Diemunsch, P. & Grélot, L. (2000) Potential of substance P antagonists as antiemetics. *Drugs*, **60**(3), 533-546.

Dohoo, S., Tasker, R.A.R. & Donald, A. (1994) Pharmacokinetics of parenteral and oral sustained-release morphine sulphate in dogs. *Journal of Veterinary Pharmacology and Therapeutics*, **17**(6), 426-433.

Dutta, S., Lal, R., Karol, M.D., Cohen, T. & Ebert, T. (2000) Influence of cardiac output on dexmedetomidine pharmacokinetics. *Journal of Pharmaceutical Sciences*, **89**(4), 519-527.

Epstein, M.E., Rodan, I., Griffenhagen, G., Kadrlik, J., Petty, M.C., Robertson, S.A. & Simpson, W. (2015) 2015 AAHA/AAFP pain management guidelines for dogs and cats. *Journal of Feline Medicine And Surgery*, **17**(3), 251-272.

Fichtl, B. & Kurz, H. (1978) Binding of drugs to human muscle. *European Journal of Clinical Pharmacology*, **14**(5), 335-340.

Garrett, E.R. & Jackson, A.J. (1979) Pharmacokinetics of morphine and its surrogates III: morphine and morphine 3-monoglucuronide pharmacokinetics in the dog as a function of dose. *Journal of Pharmaceutical Sciences*, **68**(6), 753-771.

Guedes, A.G., Rudé, E.P. & Rider, M.A. (2006) Evaluation of histamine release during constant rate infusion of morphine in dogs. *Veterinary Anaesthesia and Analgesia*, **33**(1), 28-35.

Hall, L.W. & Clarke, K.W. (1983). Veterinary Anaesthesia. Bailliere Tindall.

Hug, C., Murphy, M., Rigel, E. & Olson, W. (1981) Pharmacokinetics of morphine injected intravenously into the anesthetized dog. *Anesthesiology*, **54**(1), 38-47.

Kallio-Kujala, I.J., Raekallio, M.R., Honkavaara, J., Bennett, R.C., Turunen, H., Scheinin, M., Hautajärvi, H. & Vainio, O. (2018) Peripheral α2-adrenoceptor antagonism affects the absorption of intramuscularly coadministered drugs. *Veterinary Anaesthesia and Analgesia*, **45**(4), 405-413.

Karna, S.R., Kongara, K., Singh, P.M., Chambers, P. & Lopez-Villalobos, N. (2019) Evaluation of analgesic interaction between morphine, dexmedetomidine and maropitant using hot-plate and tail-flick tests in rats. *Veterinary Anaesthesia and Analgesia*.

Kenward, H., Elliott, J., Lee, T. & Pelligand, L. (2017) Anti-nausea effects and pharmacokinetics of ondansetron, maropitant and metoclopramide in a low-dose cisplatin model of nausea and vomiting in the dog: a blinded crossover study. *BMC Veterinary Research*, **13**(1), 244.

Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M. & Altman, D.G. (2010) Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biology*, **8**(6), e1000412.

Kongara, K., Chambers, J. & Johnson, C. (2012) Effects of tramadol, morphine or their combination in dogs undergoing ovariohysterectomy on peri-operative electroencephalographic responses and post-operative pain. *New Zealand Veterinary Journal*, **60**(2), 129-135.

Lawrence, C., Prinzen, F. & De Lange, S. (1996) The effect of dexmedetomidine on nutrient organ blood flow. *Anesthesia & Analgesia*, **83**(6), 1160-1165.

Maiante, A., Teixeira Neto, F.J., Beier, S., Corrente, J. & Pedroso, C. (2009) Comparison of the cardio-respiratory effects of methadone and morphine in conscious dogs. *Journal of Veterinary Pharmacology and Therapeutics*, **32**(4), 317-328.

Mastrocinque, S. & Fantoni, D.T. (2003) A comparison of preoperative tramadol and morphine for the control of early postoperative pain in canine ovariohysterectomy. *Veterinary Anaesthesia and Analgesia*, **30**(4), 220-228.

Mather, L.E., Runciman, W.B. & Ilsley, A.H. (1982) Anesthesia-induced changes in regional blood flow: implications for drug disposition. *Regional Anesthesia and Pain Medicine*, **7**(Suppl 4), S24-S33.

Maurer, P.M. & Bartkowski, R.R. (1993) Drug interactions of clinical significance with opioid analgesics. *Drug Safety*, **8**(1), 30-48.

Murrell, J.C. & Hellebrekers, L.J. (2005) Medetomidine and dexmedetomidine: a review of cardiovascular effects and antinociceptive properties in the dog. *Veterinary Anaesthesia and Analgesia*, **32**(3), 117-127.

Nimmo, W.S. & Peacock, J.E. (1988) Effect of anaesthesia and surgery on pharmacokinetics and pharmacodvamics. *British Medical Bulletin*, **44**(2), 286-301.

Niyom, S., Boscan, P., Twedt, D.C., Monnet, E. & Eickhoff, J.C. (2013) Effect of maropitant, a neurokinin-1 receptor antagonist, on the minimum alveolar concentration of sevoflurane during stimulation of the ovarian ligament in cats. *Veterinary Anaesthesia and Analgesia*, **40**(4), 425-431.

Nordberg, G., Borg, L., Hedner, T. & Mellstrand, T. (1985) CSF and plasma pharmacokinetics of intramuscular morphine. *European Journal of Clinical Pharmacology*, **27**(6), 677-681.

Ossipov, M.H., Harris, S., Lloyd, P., Messineo, E., Lin, B. & Bagley, J. (1990) Antinociceptive interaction between opioids and medetomidine: systemic additivity and spinal synergy. *Anesthesiology*, **73**(6), 1227-1235.

Queckenberg, C. & Fuhr, U. (2009) Influence of posture on pharmacokinetics. *European Journal of Clinical Pharmacology*, **65**(2), 109.

Rigg, J., Browne, R., Davis, C., Khandelwal, J. & Goldsmith, C. (1978) Variation in the disposition of morphine after im administration in surgical patients. *British Journal of Anaesthesia*, **50**(11), 1125-1130.

Robinson, E., Faggella, A., Henry, D. & Russell, W. (1988) Comparison of histamine release induced by morphine and oxymorphone administration in dogs. *American Journal of Veterinary Research*, **49**(10), 1699-1701.

Sinclair, M.D. & Dyson, D.H. (2012) The impact of acepromazine on the efficacy of crystalloid, dextran or ephedrine treatment in hypotensive dogs under isoflurane anesthesia. *Veterinary Anaesthesia and Analgesia*, **39**(6), 563-573.

Taylor, P. & Houlton, J.E. (1984) Post-operative analgesia in the dog: a comparison of morphine, buprenorphine and pentazocine. *Journal of Small Animal Practice*, **25**(7), 437-451.

Toutain, P.-L. & Bousquet-Melou, A. (2004) Plasma terminal half-life. *Journal of Veterinary Pharmacology and Therapeutics*, **27**(6), 427-439.

Vainio, O. (1989) Introduction to the clinical pharmacology of medetomidine. *Acta Veterinaria Scandinavica. Supplementum*, **85**, 85-88.

Zhang, Y., Huo, M., Zhou, J. & Xie, S. (2010) PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Computer Methods and Programs in Biomedicine*, **99**(3), 306-314.

Zuidema, J., Pieters, F. & Duchateau, G. (1988) Release and absorption rate aspects of intramuscularly injected pharmaceuticals. *International Journal of Pharmaceutics*, **47**(1-3), 1-12.

4.8 Appendices

Table 4.2 a) Intra-day and b) inter-day variation of LCMS method for analysis of morphine in the plasma.

Six different concentrations of maropitant were made in the plasma. Run 1, 2 and 3 represent separate runs in the same day whereas Day 1, 2 and 3 represent separate runs in the three different days. All samples were run in duplicates. The area for each peak was used to calculate the mean, percent relative standard deviation (RSD) and standard deviation (SD).

a)

Concentration (ng)	**0.25**	**0.52**	**1.04**	**2.08**	**4.16**	**8.33**	**16.67**
			Area				
Run1	144308.81	291902.24	508719.26	998990.13	1967296.52	3920263.60	7232942.71
	141438.23	286909.17	517746.99	1035300.74	2049871.59	3839211.48	6933678.24
Run 2	154203.63	292498.00	547845.07	982958.21	1976668.59	3676472.52	7002147.86
	159086.45	271667.48	523597.74	982250.91	1881582.85	3712350.62	7135113.04
Run 3	153401.95	282635.90	553999.72	935529.30	1913911.04	3513552.67	7056087.44
	156286.66	303137.48	524226.65	957719.16	1958788.04	3755902.94	7144411.21
Mean	151454.29	288125.04	529355.90	982124.74	1958019.77	3736292.30	7084063.42
SD	6990.47	10594.56	17715.93	34291.71	57722.38	140418.61	108186.39
RSD%	4.62	3.68	3.35	3.49	2.95	3.76	1.53

b)

Concentration (ng)	**0.25**	**0.52**	**1.04**	**2.08**	**4.16**	**8.33**	**16.67**
				Area			
Day 1	134203.63	292498.00	508719.26	998990.13	1967296.52	3920263.60	7232942.71
	120786.45	271667.48	517746.99	1035300.74	2049871.59	3839211.48	6933678.24
Day 2	131909.80	262449.46	486413.88	883119.82	2075474.92	3646712.88	7977620.17
	124365.00	231902.24	470572.81	884929.19	2010185.29	3697788.65	8153927.22
Day 3	155221.48	241175.63	601024.14	959924.30	2111502.17	4019376.10	7242108.42
	156680.13	232338.80	601527.91	1056663.79	1963855.96	4068394.25	7066712.71
Mean	137194.41	255338.60	531000.83	969821.33	2029697.74	3865291.16	7434498.24
SD	15332.03	24400.25	56901.68	74164.37	59672.92	170016.89	505229.62
RSD%	11.18	9.56	10.72	7.65	2.94	4.40	6.80

Table 4.3 a) Intra-day and b) inter-day variation of LCMS method for analysis of dexmedetomidine in the plasma.

Six different concentrations of maropitant were made in the plasma. Run 1, 2 and 3 represent separate runs in the same day whereas Day 1, 2 and 3 represent separate runs in the three different days. All samples were run in duplicates. The area for each peak was used to calculate the mean, percent relative standard deviation (RSD) and standard deviation (SD).

a)

Concentration (ng)	**0.25**	**0.52**	**1.04**	**2.08**	**4.16**	**8.33**	**16.67**
			Area				
Run1	144308.81	291902.24	508719.26	998990.13	1967296.52	3920263.60	7232942.71
	141238.23	286909.17	517746.99	1035300.74	2049871.59	3839211.48	6933678.24
Run 2	155203.63	292498.00	547845.07	982958.21	1976668.59	3676472.52	7002147.86
	159086.45	271667.48	523597.74	982250.91	1881582.85	3712350.62	7135113.04
Run 3	153401.95	282635.90	553999.72	935529.30	1913911.04	3513552.67	7056087.44
	158286.66	303137.48	524226.65	957719.16	1958788.04	3755902.94	7144411.21
Mean	151920.96	288125.04	529355.90	982124.74	1958019.77	3736292.30	7084063.42
SD	7441.79	10594.56	17715.93	34291.71	57722.38	140418.61	108186.39
RSD %	4.90	3.68	3.35	3.49	2.95	3.76	1.53

b)

Concentration (ng)	**0.25**	**0.52**	**1.04**	**2.08**	**4.16**	**8.33**	**16.67**
			Area				
Day 1	134203.63	292498.00	508719.26	998990.13	1967296.52	3920263.60	7232942.71
	120786.45	271667.48	517746.99	1035300.74	2049871.59	3839211.48	6933678.24
Day 2	131909.80	262449.46	486413.88	883119.82	2075474.92	3646712.88	7977620.17
	124365.00	231902.24	470572.81	884929.19	2010185.29	3697788.65	8153927.22
Day 3	155221.48	241175.63	601024.14	959924.30	2111502.17	4019376.10	7242108.42
	156680.13	232338.80	601527.91	1056663.79	1963855.96	4068394.25	7066712.71
Mean	137194.41	255338.60	531000.83	969821.33	2029697.74	3865291.16	7434498.24
SD	15332.03	24400.25	56901.68	74164.37	59672.92	170016.89	505229.62
RSD%	11.18	9.56	10.72	7.65	2.94	4.40	6.80

Table 4.4 a) Intra-day and b) inter-day variation of LCMS method for analysis of maropitant in the plasma.

Six different concentrations of maropitant were made in the plasma. Run 1, 2 and 3 represent separate runs in the same day whereas Day 1, 2 and 3 represent separate runs in the three different days. All samples were run in duplicates. The area for each peak was used to calculate the mean, percent relative standard deviation (RSD) and standard deviation (SD).

a)

Concentration (ng)	**5.21**	**10.42**	**20.83**	**41.67**	**83.33**	**1666.67**
			Area			
Run 1	267025.71	689666.70	1359472.48	2640513.63	5654598.82	101772976.14
	269151.02	694885.82	1338913.59	2772220.11	5530387.97	101055137.65
Run 2	252033.26	684073.92	1355473.99	2670896.51	5530387.97	105960114.03
	257267.75	675375.39	1328746.11	2777500.30	5314277.85	107278662.10
Run 3	264913.87	663957.67	1367854.12	2704111.55	5410428.59	106332708.49
	272179.35	682191.46	1328746.11	2638050.58	5321773.33	101650306.35
Mean	263761.83	681691.83	1346534.40	2700548.78	5460309.09	104008317.46
SD	7636.89	10936.19	16689.90	62386.71	134592.68	2799489.09
RSD%	2.90	1.60	1.24	2.31	2.46	2.69

b)

Concentration (ng)	**5.21**	**10.42**	**20.83**	**41.67**	**83.33**	**1666.67**
			Area			
Day 1	297025.71	689666.70	1238913.59	3034720.79	5854598.82	101772976.14
	249151.02	694885.82	1279633.50	2956483.02	5027136.43	111055137.65
Day 2	240249.75	597913.38	1438557.72	3010351.56	5530387.97	102960114.03
	241353.14	581331.72	1439408.59	2772220.11	5580754.37	119278662.10
Day 3	255808.56	672516.25	1387889.05	2540513.63	4970554.78	112332708.49
	257267.75	684992.39	1455473.99	2977500.30	4870128.48	105650306.35
Mean	256809.32	653551.04	1373312.74	2881964.90	5305593.47	108841650.79
SD	20930.04	50343.47	92116.71	191379.39	401764.10	6644720.13
RSD%	8.15	7.70	6.71	6.64	7.57	6.10

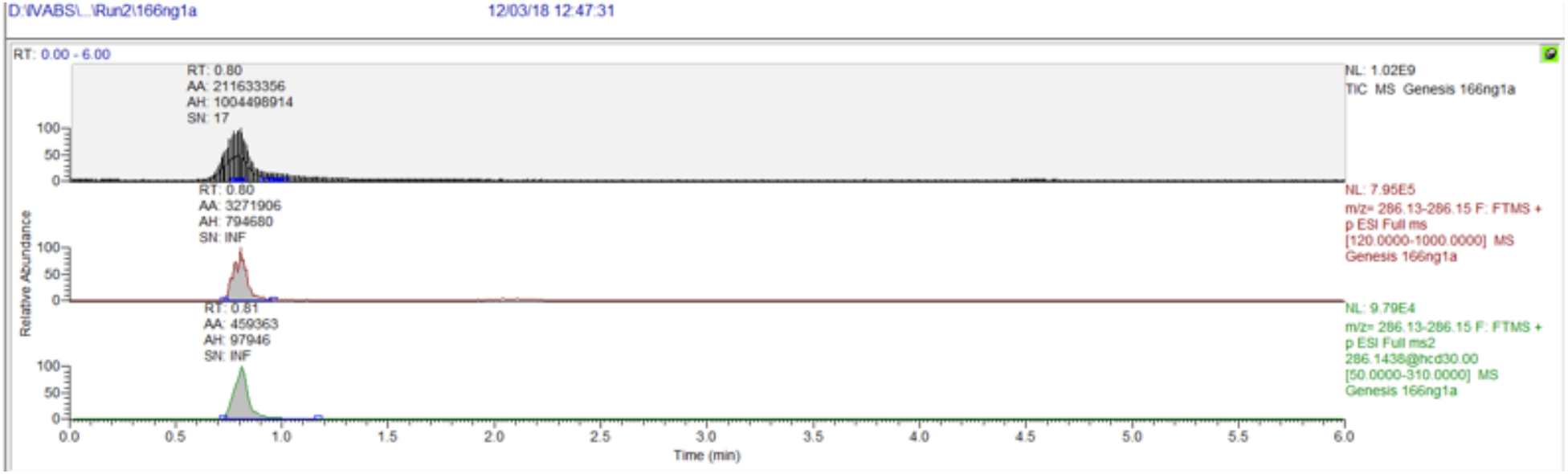

Figure 4.2 Chromatogram showing morphine standard solution 166 ng in mobile phase.

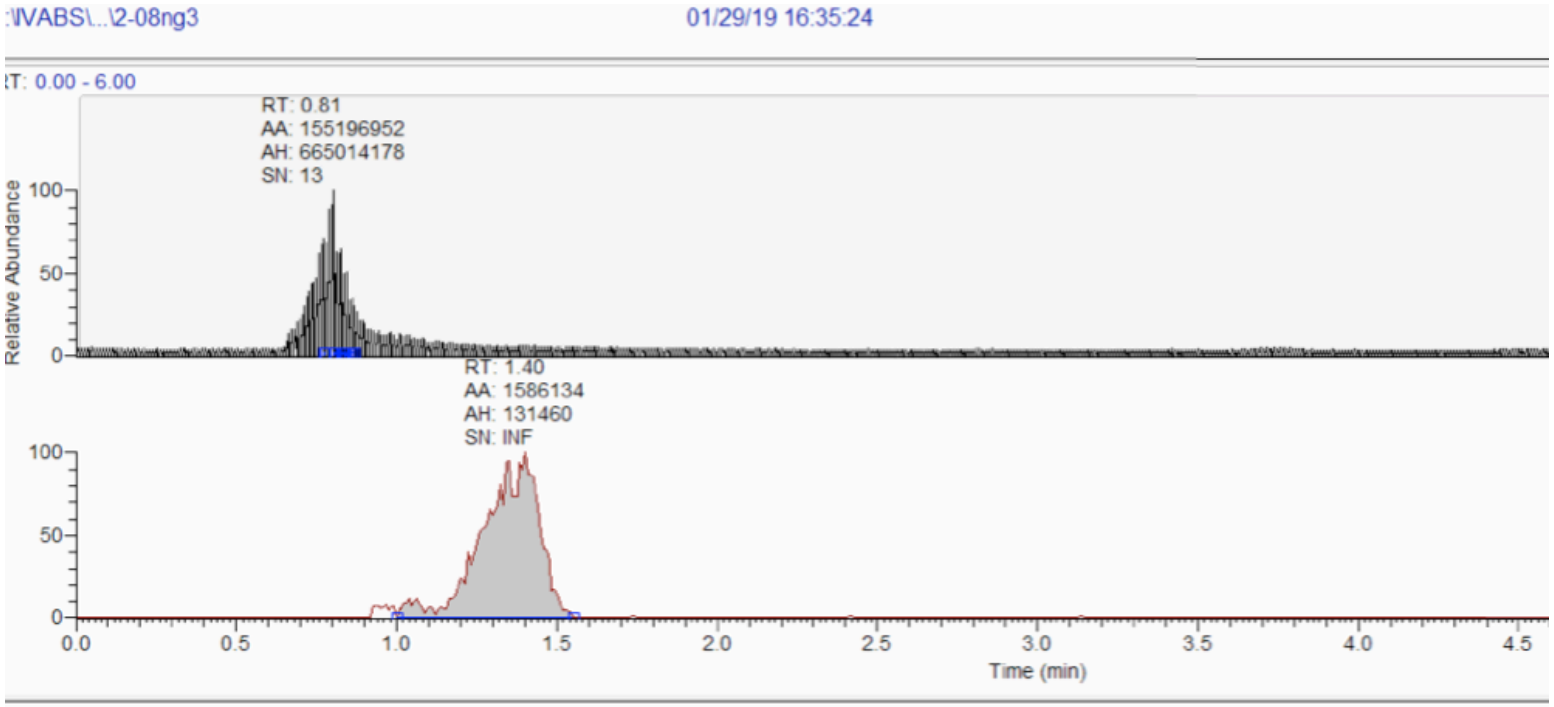

Figure 4.3 Chromatogram showing dexmedetomidine standard solution 2.08 ng, in mobile phase.

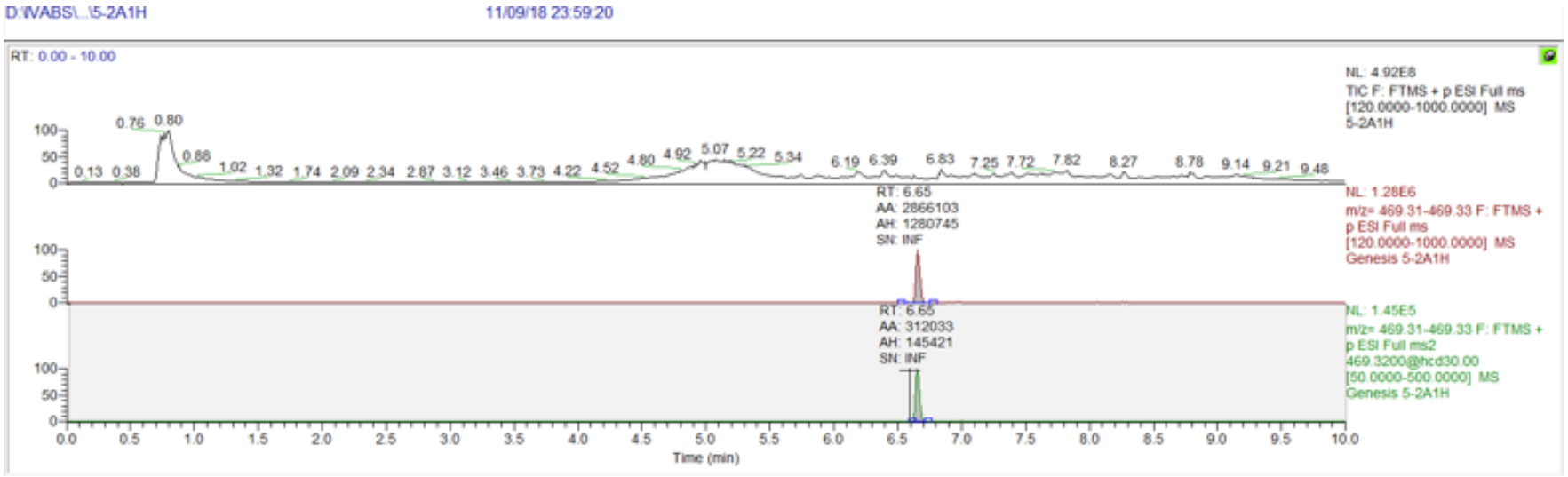

Figure 4.4 Chromatogram showing maropitant standard solution 5.2 ng, in mobile phase.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

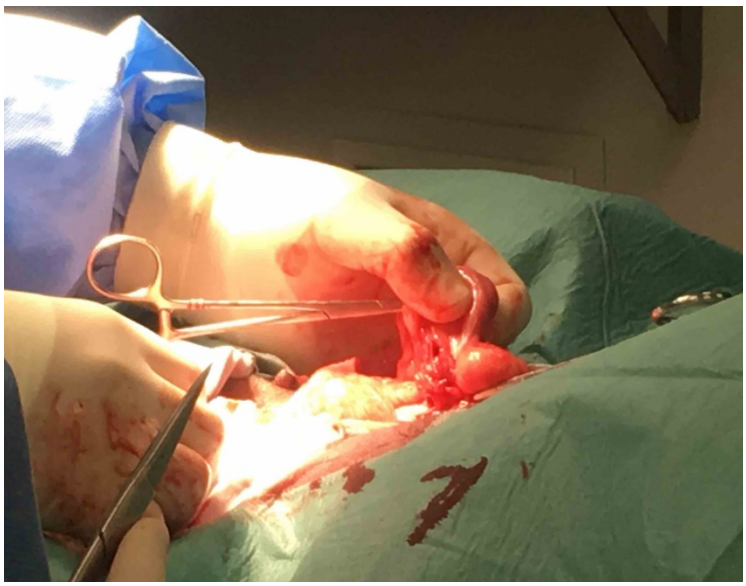
We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Sandeep Raj Karna
Name/title of Primary Supervisor:	Dr Kavitha Kongara
Name of Research Output and full reference:	
Karna, S. R., Singh, P., Chambers, P., & Kongara, K. (2019). Pharmacokinetics of morphine in combination with dexmedetomidine and maropitant following intramuscular injection in dogs anaesthetized with halothane. Journal of Veterinary Pharmacology and Therapeutics.	
In which Chapter is the Manuscript /Published work:	Chapter 4
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Sandeep had a primary role in study design, conducting experiments, data collection, statistical analysis, interpretation and preparation of the manuscript, with guidance from supervisors.	
For manuscripts intended for publication please indicate target journal:	
Published in the Journal of Veterinary Pharmacology and Therapeutics.	
Candidate's Signature:	
Date:	24/02/2020
Primary Supervisor's Signature:	Kavitha Kongara Digitally signed by Kavitha Kongara Date: 2020.02.25 11:33:13 +13'00'
Date:	25.02.2020

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

CHAPTER 5

Evaluation of analgesic interaction between morphine, dexmedetomidine and maropitant in dogs undergoing ovariohysterectomy



Author's note: Chapter 5 is presented in the style of the *New Zealand Veterinary Journal* where it is intended to be submitted for publication as:

Karna, S. R., Chambers, P., Singh, P. & Kongara, K. (2020). Evaluation of analgesic interaction between morphine, dexmedetomidine and maropitant in dogs undergoing ovariohysterectomy. *New Zealand Veterinary Journal*.

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5.1 Abstract

Objective To assess if the combination of morphine with maropitant and dexmedetomidine produces better analgesia than morphine alone in dogs undergoing ovariohysterectomy (OHE).

Study design Randomized, blinded, prospective experimental study.

Animals Forty client owned-dogs.

Methods The dogs were randomised into four treatment groups (n=10/group); morphine 0.6 mg kg^{-1} (Mor); morphine 0.3 mg kg^{-1} + maropitant 1 mg kg^{-1} (Maro+Mor); morphine 0.3 mg kg^{-1} + dexmedetomidine 10 μg kg^{-1} (Dex+Mor); morphine 0. 2 mg kg^{-1} + dexmedetomidine 7 μg kg^{-1} + maropitant 1 mg kg^{-1} (Dex+Maro+Mor), all injected intramuscularly (IM). Dogs were premedicated with acepromazine (0.02 mg kg^{-1} IM) and the test drugs were administered after induction of anaesthesia and thirty minutes before the expected start time of surgery. The short form of the Glasgow composite measure pain scale (CMPS-SF) and visual analogue scale (VAS) were used for pain assessment at 15 and 30 minutes and 1, 2, 3, 6, 9 and 24 hours after extubation. Dogs with CMPS-SF pain score ≥ 6 received rescue analgesia. Blood samples were collected before, 15, 30, 60 and 120 minutes after injection of the test drugs and plasma concentration of the drugs was determined by liquid chromatography-mass spectrometry.

Results The group Dex+Mor had significantly lower ($p < 0.05$) CMPS-SF and VAS scores compared to the group Mor. The number of dogs requiring rescue analgesia was 0 %, 1 (11%), 1 (11%) and 3 (33%), in the groups Dex+Mor, Dex+Maro+Mor, Maro+Mor and Mor, respectively. All dogs that received dexmedetomidine showed arrhythmia and second-degree heart block. The C_{max} *of* morphine was 6.80 ±4.56, 9.56

±8.29, 9.30 ±3.35 and 18.99 ±9.41 ng/ml in the groups Dex+Mor, Dex+Maro+Mor, Maro+Mor and Mor respectively.

Conclusion and clinical relevance Lower doses of morphine (0.2 and 0.3 mg kg^{-1}) combined with dexmedetomidine or maropitant can provide analgesia similar to or better than morphine alone at a higher dose (0.6 mg kg^{-1}).

Keywords

analgesia, dexmedetomidine, maropitant, morphine, dogs

5.2 Introduction

Ovariohysterectomy (OHE) is a common surgical procedure known to cause pain in dogs. Somatic pain originating from the surgical incision, and visceral pain due to manipulation of ovaries and uterus and stretching of the associated ligaments are the types of pain involved during this procedure (Gaynor and Muir, 2014). OHE is a relatively standardized and commonly used surgical model to study soft tissue pain and the efficacy of analgesics (Hansen, 2003).

Opioids such as morphine are commonly used analgesics in dogs undergoing OHE. Opioids are associated with adverse effects such as prolonged sedation, respiratory depression, vomiting, defecation and dysphoria (Barnhart *et al.*, 2000; Maiante *et al.*, 2009), which limit their use as sole analgesic agents, especially at higher doses. An approach to minimise the adverse effects of a drug is to combine it with other classes of drugs; this approach should allow reduction in the dose of each drug resulting in an enhanced analgesic effect with lower adverse effects (Young and Buvanendran, 2012).

Dexmedetomidine is a highly selective alpha-2-adrenoceptor agonist and a commonly used sedative and analgesic in dogs which can produce analgesia at both spinal (Kalso *et al.*, 1991) and supraspinal (Guo *et al.*, 1996) levels. Rat studies have shown systemic additivity and spinal synergy in analgesia between opioids and medetomidine (Ossipov *et al.*, 1990). Maropitant is a neurokinin -1 (NK1) receptor antagonist used as an antiemetic in dogs and cats. Maropitant decreased the anaesthetic requirements during visceral stimulation of the ovaries and ovarian ligaments during ovariectomy in dogs (Boscan *et al.*, 2011) and cats (Niyom *et al.*, 2013). Dogs premedicated with maropitant had lower pain scores at extubation compared to the dogs premedicated with morphine during OHE (Marquez *et al.*, 2015). Our previous study has demonstrated a supra-additive effect of analgesia between morphine and maropitant in rats undergoing tail flick tests (Karna *et al.*, 2019a).

This study aimed to evaluate a multimodal analgesia protocol comparing a lower dose of morphine (0.3 or 0.2 mg kg^{-1}) combined with dexmedetomidine and maropitant to a single higher dose of morphine (0.6 mg kg^{-1}). Our null hypothesis was that a combination of three drugs at one-third of the full dose or two drugs at half dose would produce the same degree of analgesia as morphine alone at the full dose.

5.3 Materials and methods

The study was approved by the Animal Ethics Committee (protocol no 17/33) and is reported following the ARRIVE guidelines for experiments involving animals (Kilkenny *et al.*, 2010). The sample was estimated to achieve the power of experiment of 80% with an alpha error of 0.05 to detect a difference of 10% between treatment

means. The standard deviation (SD) to perform this power analysis was assumed 0.6 for CMPS-SF based on data from a previous study using similar design and methods (Kim *et al.*, 2012).

5.3.1 Animals and test groups

Forty client-owned, healthy and intact female dogs arriving at the Massey University Veterinary Teaching Hospital for elective OHE were recruited in the trial after an informed and written owner's consent. Inclusion criteria were; dogs more than 6 months of age and weighing more than 10 kg, non-brachiocephalic breed, and fits in American Society of Anaesthesiologist category (ASA) = 1. Aggressive dogs and those showing any other behavioural problems were not included in the study. The dogs were determined to be healthy based on history, physical examination and stable body weight (< 1% change for a period of 14 days). Packed cell volume (PCV) and the total plasma protein (PP) were determined before anaesthetic premedication and the dogs with these parameters within the normal range (PCV= 35% – 57%, PP= 60 – 75 g L^{-1}) (Fielder 2016) were included in the study.

The dogs were randomised into four groups (n=10/group): 1) morphine 0.6 mg kg^{-1} (Mor); 2) morphine 0.3 mg kg^{-1} + maropitant 1 mg kg^{-1} (Maro+Mor); 3) morphine 0.3 mg kg^{-1} + dexmedetomidine 10 µg kg^{-1} (Dex+Mor); 4) morphine 0.2 mg kg^{-1} + dexmedetomidine 7 µg kg^{-1} + maropitant 1 mg kg^{-1} (Dex+Maro+Mor), all injected intramuscularly. Because the drug combinations have not been tested before, the dosages of the drugs were based on clinical opinion of the authors based on previous studies. The drugs were assumed to contribute equally to the total analgesia.

A chit-pull system was used to randomise the dogs into the treatment groups. For this, forty chits, each labelled with a treatment group were placed in a container and one was drawn randomly for each dog on the day of surgery.

5.3.2 Premedication

All dogs were fasted for at least 12 hours before the induction of anaesthesia and had unlimited access to water until premedication. Thirty minutes prior to the induction of anaesthesia, dogs were premedicated with acepromazine, 0.02 mg kg^{-1} (Acezine 2; Ethical Agents Ltd, New Zealand) injected into the lumbar epaxial muscles.

5.3.3 Anaesthesia and administration of test drugs

Two 18- or 20-gauge catheters (Optiva I.V. Catheter Radiopaque, Smiths Medical International Ltd, United Kingdom) were placed into cephalic veins on different legs, one for induction of anaesthesia and another for blood collection. Anaesthesia was induced with propofol (Repose; Norbrook NZ Ltd, New Zealand) to effect.

After induction of anaesthesia, a cuffed endotracheal tube was placed. Anaesthesia was maintained with isoflurane (Attane; Bayer Animal Health, New Zealand) in 100% oxygen delivered via a circle system and dogs breathed spontaneously. The concentration of isoflurane was adjusted to keep the dog under the surgical plane of anaesthesia. The dogs were given Hartmann's solution IV @ 5 mL kg^{-1} $hour^{-1}$ to maintain systolic arterial blood pressure above 100 mmHg throughout the anaesthetic period. The heart rate (HR), respiratory rate (f_R), blood pressure (indirect oscillometric method), haemoglobin oxygen saturation (SpO_2) and End-tidal partial pressure of

carbon dioxide ($P_E'CO_2$) were monitored during anaesthesia using a multipurpose bedside monitor (Life Scope BSM-3763; Nihon Kohden, Japan) and recorded every 5 minutes. Electrocardiogram (ECG) was also monitored using the same monitor and any clinically significant changes were recorded. Rectal temperature was also measured and recorded before induction and at every 5 minutes during anaesthesia using a digital thermometer. Body temperature was maintained using forced-air warming system (Bair Hugger; 3M Company, USA).

The test drugs were administered after induction of anaesthesia and thirty minutes before the expected start time of surgery. Treatment drugs were injected into lumbar epaxial muscles using a 2 ml syringe and 22 gauge, 1-inch needles. All drugs were injected separately within a minute starting with morphine followed by maropitant and dexmedetomidine. OHE was performed via a mid-ventral abdominal incision by a final-year veterinary student under the direct supervision of a veterinary surgeon.

5.3.4 Blood sampling for pharmacokinetic data

At 15, 30, 60 and 120 minutes after administration of the drugs, 2 mL blood samples were collected from a separate previously placed intravenous (IV) catheter in the cephalic vein using a modified push-pull method (Barr *et al.*, 2017). Before collecting the blood sample for analysis, 2 mL blood was drawn in 5 ml syringe already filled with 2 mL heparinised saline and reinfused after the blood sample was collected. Blood samples were transferred to heparin tubes and refrigerated immediately. Plasma was collected by centrifuging at 1000 *g* for 10 minutes and stored at −80 °C until analysis.

5.3.5 Postoperative pain and sedation assessment

Dogs were moved to a cage before the recovery and the endotracheal tube was removed after the restoration of laryngeal reflexes. After the recovery from anaesthesia, an experienced investigator (SK) unaware of the treatment groups performed the pain assessment using the CMPS-SF and VAS at 15 and 30 minutes and 1, 2, 3, 6, 9 and 24 hours after extubation. The CMPS-SF is a composite scale to be completed by an observer in the form of a structured questionnaire that involves assessment of animal's behaviour and its interactions with the observer (Reid *et al.*, 2007). The sum of each behavioural component had weight assigned whose sum is the pain score assigned to the animal out of a maximum score of 24. A high score indicates pain. For VAS, a mark was made in a 100 mm line where 0 (extreme left) corresponds to no pain and 100 (extreme right) corresponds to worst possible pain. The animals were initially observed from outside of the cage. They were approached later, encouraged to walk outside and the wound area was palpated afterwards.

Dogs with CMPS-SF pain score ≥ 6 received rescue analgesia with buprenorphine (0.02 mg kg^{-1}) injected subcutaneously. Data from dogs that received rescue analgesia were only included up to the time of rescue. They were still assessed for pain up to 20-24 hours (depending on time of their discharge) and were given a second dose of buprenorphine if required. All dogs received meloxicam (0.2 mg kg^{-1} IM) after 9 hours.

5.3.6 Sedation scoring

Sedation scoring was performed using a numerical descriptive scale (NDS) which ranged from 0-3, defined as follows: 0, no sedation; 1, mild sedation (less alert but still

active); 2, moderate sedation (drowsy, recumbent but can walk); and 3, severe sedation (very drowsy, unable to walk) (Valverde *et al.*, 2004; Bitti *et al.*, 2017). The sedation scoring was performed at 15 and 30 minutes and 1, 2 and 3 hours after extubation or until the sedation score was 0.

5.3.7 Pharmacokinetic assessment

Plasma samples were analysed for morphine, dexmedetomidine and maropitant using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The LC-MS/MS method was previously validated in our laboratory for linearity, precision, accuracy, recovery and specificity over a calibration range of 0.52 to166 ng mL^{-1} for morphine, 0.25 to 16.6 ng mL^{-1} for dexmedetomidine and 5.2 to 1666.67 ng mL^{-1} for maropitant.

Pharmacokinetic (PK) parameters estimated included: the maximum plasma concentration of the drug ($C_{max,}$ ng mL^{-1}), time to reach C_{max} ($T_{max,}$ minutes), half-life of terminal elimination phase ($T_{1/2}$, minutes), area under the plasma concentration-time curve (AUC_{0-t}, ng*minute mL^{-1}), area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$, ng* minute mL^{-1}), volume of distribution based on the terminal slope (V_z, L kg^{-1}), clearance (CL, mL $minute^{-1}$ kg^{-1}) and mean resident time (MRT, minute). The C_{max} was determined directly from the concentration-time data and other parameters were calculated using the PKSolver add-in program for Excel 2010 (Zhang *et al.*, 2010).

5.3.8 Statistical analysis

All statistical analyses were performed using SAS for Windows v 9.4 (SAS Institute Inc Cary NC, USA). Prism 8 for Macintosh (GraphPad Software Inc., San Diego, CA, USA) was used for figures.

The distribution of data was tested for normality by using the Kolmogorov-Smirnov test. CMPS-SF and VAS pain score data did not pass the normality test and hence logarithmic (log) transformed values were used for analysis of the variables. Dependent variables (duration of surgery and the time between medication and the onset of surgery) were analysed using with a linear model that included the fixed effect of treatment. Least squares means and standard errors for each treatment were obtained and used for multiple mean comparison using the least significant difference as implemented in the LSMEANS (least squares means) of the GLM (general linear model) procedure.

The relationship between CMPS-SF and VAS pain scores was assessed using the Pearson correlation coefficients. The effects of duration of surgery and the time between medication and the onset of surgery on CMPS-SF and VAS pain scores were assessed with the GLM procedure.

Some dogs remained sedated up to two hours after the extubation, and thus only pain scores from 2 to 9 hours were analysed. To avoid complications in the analysis due to missing values for the dogs that required rescue analgesia, “last observation carried forward” as described by Verbeke and Molenberghs (Verbeke and Molenberghs, 1997) and used by Slingsby et al. (Slingsby *et al.*, 2006) and Kongara et al. (Kongara *et al.*, 2012) in a similar study was used. In absence of this method, the statistical analysis

would have wrongly treated all the high pain scores as true missing values which would lead to underestimation of the treatment groups with high pain scores.

Two types of techniques were used for the analysis of the post-operative pain scores: a) summary statistics using the area under the curve (AUC) calculated from the pain scores, and b) a longitudinal technique using repeated measure analysis of the pain scores. The AUC of pain scores of each dog were analysed with a linear model that included the fixed effect of treatment. Least squares means and standard errors were obtained and used for multiple mean comparisons. The repeated measures of pain scores were analysed with the MIXED procedure with a mixed model that included the fixed effect of time, treatment and interaction between time and treatment and the random effect of the animal to account for repeated measures in the same animal. Least square means and standard errors for treatment time and combinations between treatments and times were obtained and used for multiple mean comparisons using the least significant difference as implemented in the MIXED procedure. Preoperative pain score was zero for all dogs and not considered for the statistical analysis. There was no significant difference between the age and weights of the dogs, hence not included in the model.

The sedation scores and physiological intraoperative variables were analysed in a similar way as that of repeated measures analysis of the pain scores.

To analyse the data rescue analgesia, GLIMMIX procedure was used with treatment group as the fixed effect. The variable was assumed to have a binomial distribution and therefore analysis of variance were performed after a logit transformation.

PK parameters were analysed using a one-way ANOVA followed with the GLM procedure and multiple mean comparisons were performed with the Tukey test. The

relationship between plasma concentration and PK parameters of morphine with the pain scores was assessed using the Pearson correlation coefficients only in group Mor.

Significant effects and differences between means were declared when p-value were less than 0.05.

5.4 Results

Data were analysed only in 36 dogs (n=9 in each group) because two dogs showed aggressive behaviour and other two received additional analgesia in the post-operative period for other reasons than high pain scores.

The demographic data, duration of surgery and time from medication to onset of surgery for each treatment group has been shown in Table 5.1. There was no significant difference between treatment groups in body weight, age, duration of surgery and time from medication to onset of surgery. There was no association of the pain scores with age and body weight demonstrated by one-way ANOVA.

The relationship between CMPS-SF and VAS pain scores with the duration of surgery, and the time between medication and the onset of surgery is shown in Table 5.2. There was a positive linear relationship between the CMPS-SF and VAS pain scores (Pearson correlation coefficient = 0.8, $p < 0.0001$).

5.4.1 AUC analysis

The rank order of the AUC between different treatment group was Mor (1497.00 ± 695.60) > Dex+Maro+Mor (1160.00 ± 629.70) > Maro+Mor (1060.00 ± 467.50) > Dex+Mor (806.70 ± 258.50) for CMPS-SF and Mor (818.40 ± 263.80) >

Dex+Maro+Mor (654.30 ± 259.80) > Maro+Mor (622.30 ± 209.50) > Dex+Mor (511.20 ± 211.10) for VAS scores. The group Dex+Mor had significantly smaller AUC ($p < 0.05$) value of AUC compared to the group Mor for both pain scores.

5.4.2 Repeated measures analysis

Differences of the least squares means of CMPS-SF and the VAS between the treatment groups shown by a mixed model analysis is shown in Table 3. The mixed model analysis showed that group Dex+Mor had significantly lower ($p < 0.05$) CMPS-SF and VAS pain score compared to the group Mor. A graphical representation of the pain scores at different time points is shown in Figure 5.1.

5.4.3 Rescue analgesia

The number of dogs requiring rescue analgesia were 0 (0%), 1 (11%), 1 (11%) and 3 (33%) in the groups Dex+Mor, Dex+Maro+Mor, Maro+Mor and Mor, respectively. There was no significant difference between the treatment groups.

5.4.4 Sedation scores

There was no significant difference in the postoperative sedation score between the treatment groups demonstrated by the mixed model analysis.

5.4.5 Adverse effects

The intraoperative physiological variables after administration of the test drugs are shown in Table 5.4. All dogs in groups Dex+Mor and Dex+Maro+Mor showed a significant ($p < 0.05$) initial increase in the blood pressure followed by a decrease but stable heart rate throughout the observation period (entire duration of surgery). All dogs

in these treatment groups showed a second-degree heart block 1-2 minutes after administration of the drug.

5.4.6 Drug plasma concentration

Plasma concentrations are shown only for 36 dogs (Mor = 10, Dex+Mor = 8, Maro+Mor = 10, Dex+Maro+Mor = 8) because the adequate number of blood samples could not be collected from the remaining dogs. The concentration-time graph shown in Figure 2 and the PK parameters are shown in Table 5.5. The plasma concentration of morphine decreased significantly with a decrease in the dose. Morphine showed longer average $T_{1/2}$ when combined with dexmedetomidine compared to when used singly or in combination with maropitant. There was no correlation between plasma concentration and PK parameters of morphine with the pain scores in the group Mor.

Table 5.1 Demographic data, duration of surgery and time from medication to onset of surgery.

Values are reported as mean ± SD. Treatment groups: 1) **Mor**: morphine 0.6 mg kg^{-1}; 2) **Dex+Mor**: morphine 0.3 mg kg^{-1}+ dexmedetomidine 10 µg kg^{-1}; 3) **Maro+Mor**: morphine 0.3 mg kg^{-1}+ maropitant 1 mg kg^{-1}; 4) **Dex+Maro+Mor**: morphine 0.2 mg kg^{-1}+ dexmedetomidine 7 µg kg^{-1}+ maropitant 0.7 mg kg^{-1} .

	Mor	**Dex+Mor**	**Maro+Mor**	**Dex+Maro+Mor**
Number of dogs	9	9	9	9
Breed	• American bulldog=1 • American Staffy=1 • Cattle dog=1 • Crossbreed=3 • Doberman=1 • Labrador=1 • Sheep dog=1	• American Staffy=1 • Border Collie=1 • Crossbreed=2 • German Shepard=1 • Husky=2 • Labrador=2	• Border Collie=1 • Crossbreed=4 • Huntaway=1 • Labrador=2 • Sheep dog=1	• American bulldog=1 • Crossbreed=5 • Husky=1 • Labrador=1 • Sheep dog=1
Age (months)	17.40 ± 16.31	18.66 ± 17.86	16.66 ± 11.12	16.66 ± 13.74
Body weight (kg)	23.63 ± 7.44	23.11 ± 6.65	23.39 ± 3.96	23.50 ± 8.34
Duration of surgery (minutes)	83.50 ± 34.24	81.67 ± 38.32	91.25 ± 26.69	71.11 ± 27.59
Time from medication to onset of surgery (minutes)	36.00 ± 11.25	37.22 ± 11.75	31.5 ± 10.46	39.67 ± 23.71

Table 5.2 Relationship of Glasgow composite measure pain scale (CMPS-SF) and the visual analogue scale (VAS) with the duration of surgery, the time between administration of drugs and onset of surgery, and their interaction shown by a linear model analysis.

Parameters	**Estimate**	**Standard Error**	***p*-value**
CMPS-SF vs			
Duration of surgery	-0.04	0.01	< 0.001
Time between administration of drugs to onset of surgery	-0.07	0.03	< 0.001
Duration of surgery * Time between administration of drugs to onset of surgery	0.00	0.00	< 0.001
VAS vs			
Duration of surgery	-0.01	0.01	0.24
Time between administration of drugs to onset of surgery	-0.03	0.01	< 0.05
Duration of surgery * Time between administration of drugs to onset of surgery	0.00	0.00	0.05

Table 5.3 Differences of the least squares means of Glasgow composite measure pain scale (CMPS-SF) and the visual analogue scale (VAS) between the treatment groups shown by a mixed model analysis.

Values are reported as means and standard error (SE). Treatment groups: 1) **Mor**: morphine 0.6 mg kg^{-1}; 2) **Dex+Mor**: morphine 0.3 mg kg^{-1}+ dexmedetomidine 10 µg kg^{-1}; 3) **Maro+Mor**: morphine 0.3 mg kg^{-1}+ maropitant 1 mg kg^{-1}; 4) **Dex+Maro+Mor**: morphine 0.2 mg kg^{-1}+ dexmedetomidine 7 µg kg^{-1}+ maropitant 0.7 mg kg^{-1}

		Time (minutes)				
		120	**180**	**240**	**360**	**540**
CMPS-SF						
Treatment groups						
Dex+Mor *vs* Mor	Estimate	-0.97	-1.61	-1.83	-1.87	-1.39
	SE	0.63	0.63	0.63	0.63	0.63
	p-value	0.13	0.01	< 0.01	< 0.01	0.03
Dex+Mor *vs* Maro+Mor	Estimate	0.06	-0.83	-0.83	-0.83	-0.11
	SE	0.65	0.65	0.65	0.65	0.65
	p-value	0.93	0.20	0.20	0.20	0.86
Dex+Mor *vs* Dex+Maro+Mor	Estimate	-0.33	-0.89	-1.06	-0.89	-0.67
	SE	0.65	0.65	0.65	0.65	0.65
	p-value	0.61	0.17	0.10	0.17	0.30
Dex+Maro+Mor *vs* Mor	Estimate	-0.63	-0.72	-0.77	-0.98	-0.73
	SE	0.63	0.63	0.63	0.63	0.63
	p-value	0.32	0.26	0.22	0.12	0.25
Dex+Maro+Mor *vs* Maro+Mor	Estimate	0.39	0.06	0.22	0.06	0.56
	SE	0.65	0.65	0.65	0.65	0.65
	p-value	0.55	0.93	0.73	0.93	0.39
Maro+Mor *vs* Mor	Estimate	-1.02	-0.77	-0.99	-1.03	-1.28
	SE	0.63	0.63	0.63	0.63	0.63
	p-value	0.11	0.22	0.12	0.10	0.04
VAS pain scores						

Treatment groups						
Dex+Mor *vs* Mor	Estimate	-0.40	-0.62	-0.77	-0.79	-0.77
	SE	0.28	0.28	0.28	0.28	0.28
	p-value	0.16	0.03	< 0.01	< 0.01	< 0.01
Dex+Mor *vs* Maro+Mor	Estimate	0.14	-0.16	-0.37	-0.33	-0.26
	SE	0.28	0.28	0.28	0.28	0.29
	p-value	0.61	0.59	0.20	0.25	0.37
Dex+Mor *vs* Dex+Maro+Mor	Estimate	-0.11	-0.38	-0.44	-0.39	-0.37
	SE	0.28	0.28	0.28	0.28	0.29
	p-value	0.70	0.19	0.12	0.17	0.20
Dex+Maro+Mor *vs* Mor	Estimate	-0.29	-0.24	-0.33	-0.39	-0.40
	SE	0.28	0.28	0.28	0.28	0.28
	p-value	0.31	0.39	0.24	0.16	0.15
Dex+Maro+Mor *vs* Maro+Mor	Estimate	0.26	0.22	0.08	0.07	0.11
	SE	0.28	0.28	0.28	0.28	0.28
	p-value	0.37	0.44	0.79	0.82	0.70
Maro+Mor *vs* Mor	Estimate	-0.54	-0.46	-0.41	-0.46	-0.52
	SE	0.28	0.28	0.28	0.28	0.28
	p-value	0.05	0.10	0.14	0.10	0.07

Table 5.4 Intraoperative physiological variables after administration of the test drugs over time (0-120 minutes).

Values are reported as mean ± SD. Treatment groups: 1) **Mor**: morphine 0.6 mg kg^{-1}; 2) **Dex+Mor**: morphine 0.3 mg kg^{-1}+ dexmedetomidine 10 µg kg^{-1}; 3) **Maro+Mor**: morphine 0.3 mg kg^{-1}+ maropitant 1 mg kg^{-1}; 4) **Dex+Maro+Mor**: morphine 0.2 mg kg^{-1}+ dexmedetomidine 7 µg kg^{-1}+ maropitant 0.7 mg kg^{-1}.

		Time after administration of drugs (minutes)								
Variables	**Treatment group**	**0**	**15**	**30**	**45**	**60**	**75**	**90**	**105**	**120**
Heart rate	Mor	113.80 ± 15.77	98.31 ± 15.81*	95.11 ± 15.55*	95.20 ± 10.62*	98.10 ± 11.49*	102.80.± 11.46*	106.30 ± 11.61*	105.60 ± 10.50*	108.20 ± 14.81
(beats minute^{-1})	Dex + Mor	99.77 ± 17.70	53.88 ± 16.35*†#	56.00 ± 17.61*†#	63.77 ± 20.77*†#	68.77 ± 17.19*†#	71.00 ± 15.91*†#	73.22 ± 14.57*†#	76.00 ± 13.60*†#	77.11 ± 16.28*†#
	Dex + Maro + Mor	103.57 ± 15.47	61.28 ± 16.35*†#	61.57 ± 17.54*†#	66.00 ± 15.96*†#	67.57 ± 12.88*†#	68.14 ± 14.01*†#	69.00 ± 8.96*†#	72.43 ± 6.05*†#	74.57 ± 5.88*†#
	Maro + Mor	95.14 ± 10.21	94.29 ± 9.25	91.29 ± 9.46	94.00 ± 9.85	93.86 ± 10.53	96.57 ± 9.38	102.00 ± 10.71	104.29 ± 10.36	109.00 ± 11.7
Non-invasive	Mor	65.26 ± 16.4	63.87 ± 14.4	64.54 ± 13.71	68.93 ± 12.83	71.13 ± 12.03	70.03 ± 12.21	69.30 ± 6.39	69.30 ± 9.04	69.67 ± 9.54
mean arterial	Dex + Mor	71.76 ± 11.76	87.05 ± 22.85*†#	78.47 ± 21.12*†#	75.91 ± 21.01	74.43 ± 17.15	76.63 ± 20.19	78.83 ± 20.57	77.02 ± 17.99	78.47 ± 20.26
pressure	Dex + Maro +									
(mmHg)	Mor	71.52 ± 13.81	84.68 ± 10.35*†#	83.75 ± 6.72*†#	79.76 ± 10.56*#	80.61 ± 12.55*	78.09 ± 10.45	78.37 ± 10.63	78.46 ± 10.02	79.39 ± 11.25

	Maro + Mor	65.56 ± 15.14	71.88 ± 10.67	69.29 ± 9.18	66.14 ± 6.86	68.82 ± 9.29	68.92 ± 10.89	71.21 ± 10.85	69.78 ± 11.18	74.18 ± 10.98
Respiration	Mor	16.00 ± 5.93	14.10 ± 3.73	13.60 ± 4.81	13.50 ± 3.44	13.20 ± 3.77	14.00 ± 3.65	14.20 ± 3.43	13.90 ± 3.38	13.20 ± 3.26
rate (no	Dex + Mor	13.89 ± 8.39	12.44 ± 3.68	12.22 ± 2.86	12.56 ± 2.51	12.22 ± 3.03	13.67 ± 40	12.78 ± 3.07	14.10 ± 2.18	13.89 ± 2.76
minute-1)	Dex + Maro + Mor	14.71 ± 5.74	13.29 ± 4.75	14.86 ± 5.61	14.14 ± 6.23	15.86 ± 6.74	15.00 ± 5.42	15.10 ± 6.68	14.57 ± 6.16	15.43 ± 6.27
	Maro + Mor	15.43 ± 7.09	16.20 ± 4.86	15.14 ± 4.18	14.14 ± 3.29	14.71 ± 4.54	14.29 ± 4.39	14.43 ± 3.36	15.14 ± 4.18	15.57 ± 4.69
P'E' CO_2	Mor	43.63 ± 4.96	45.25 ± 3.33	45.13 ± 1.89	43.75 ± 4.17	45.52 ± 3.93	44.63 ± 6.12	44.38 ± 5.80	44.51 ± 6.19	44.25 ± 6.39
	Dex + Mor	41.29 ± 8.32	45.10 ± 8.72	46.14 ± 8.25	45.86 ± 6.57	47.71 ± 4.64	46.86 ± 4.49	48.00 ± 4.55	47.29 ± 5.91	48.14 ± 4.67
	Dex + Maro + Mor	43.43 ± 3.69	43.57 ± 3.78	44.29 ± 4.07	44.43 ± 3.91	43.71 ± 3.59	42.71 ± 4.82	42.16 ± 3.29	43.57 ± 4.54	44.43 ± 4.61
	Maro + Mor	38.14 ± 3.34	40.14 ± 5.34	40.86 ± 3.67	40.00 ± 5.83	40.71 ± 7.25	40.29 ± 5.09	40.29 ± 6.18	39.71 ± 4.54	41.29 ± 5.85

***** The differences in least square (LS) means statistically significant ($p < 0.05$) from baseline (T0) within the treatment group.

† The differences in LS means statistically significant ($p < 0.05$) compared to Mor.

The differences in LS means statistically significant ($p < 0.05$) compared to Maro+Mor.

Table 5.5 Pharmacokinetic variables after IM administration of different combinations of a) morphine, b) dexmedetomidine, and c) maropitant.

Values are reported as mean ± SD. Treatment groups: 1) **Mor**: morphine 0.6 mg kg^{-1}; 2) **Dex+Mor**: morphine 0.3 mg kg^{-1}+ dexmedetomidine 10 µg kg^{-1}; 3) **Maro+Mor**: morphine 0.3 mg kg^{-1}+ maropitant 1 mg kg^{-1}; 4) **Dex+Maro+Mor**: morphine 0.2 mg kg^{-1}+ dexmedetomidine 7 µg kg^{-1}+ maropitant 0.7 mg kg^{-1}.

a)

Parameters	Units	Mor	Dex+Mor	Maro+Mor	Dex+Maro+Mor
$T_{1/2}$	minutes	39.68 ± 13.72	70.90 ± 41.14	58.35 ± 31.88	40.52 ± 16.09
Tmax	minutes	21.00 ± 7.75	16.87 ± 5.30	18 ± 6.32	18.75 ± 6.94
Cmax	ng mL^{-1}	18.99 ± 9.41	6.80 ± 4.56	9.30 ± 3.35	9.56 ± 8.29
$AUC_{0-\infty}$	ng minutes mL^{-1}	1221.43 ± 686.15	831.62 ± 555.52	838.68 ± 434.31	560.04 ± 315.64
MRT 0–∞	minutes	63.03 ± 19.60	286.52 ± 491.5	90.87 ± 46.75	64.97 ± 24.12
Vz	L kg^{-1}	36.87 ± 22.84	79.68 ± 82.57	32.12 ± 13.53	33.59 ± 27.39
CL	mL kg^{-1} $minutes^{-1}$	738.80 ± 606.13	629.51 ± 533.20	433.98 ± 199.75	631.23 ± 666.71

b)

Parameters	Units	Dex+Mor	Dex+Maro+Mor
$T_{1/2}$	minutes	54.24 ± 11.93	37.36 ± 11.93
Tmax	minutes	25.00 ± 7.50	20.00 ± 7.07
Cmax	ng mL^{-1}	2.44 ± 1.63	1.85 ± 2.01
$AUC_{0-\infty}$	ng minutes mL^{-1}	185.56 ± 111.16	116.55 ± 115.39
MRT 0–∞	minutes	134.33 ± 145.26	61.41 ± 14.55
Vz	L kg^{-1}	6.18 ± 4.66	7.32 ± 5.44
CL	mL kg^{-1} $minutes^{-1}$	79.53 ± 85.31	159.58 ± 137.06

c)

Parameters	Units	Maro+Mor	Dex+Maro+Mor
$T_{1/2}$	minutes	72.45 ± 17.78	161.82 ± 157.73
Tmax	minutes	21.67 ± 7.90	25.72 ± 7.32
Cmax	ng mL^{-1}	873.33 ± 385.26	716.62 ± 380.42
$AUC_{0-\infty}$	ng minutes mL^{-1}	100915.39 ± 43994.04	123720.94 ± 66346.60
MRT 0–∞	minutes	111.84 ± 24.15	243.38 ± 229.43
Vz	L kg^{-1}	987.44 ± 642.97	1175.43 ± 705.76
CL	mL kg^{-1} $minutes^{-1}$	11.85 ± 5.20	7.21 ± 3.84

Abbreviation: $T_{1/2}$, half-life of terminal elimination phase; C_{max}, maximum plasma concentration of the drug; T_{max}, time to reach C_{max}, $AUC_{0-\infty}$, area under the plasma concentration-time curve from time zero to infinity; $MRT_{0-\infty}$, mean resident time from time zero to infinity, V_z, volume of distribution based on the terminal slope; CL, Plasma clearance

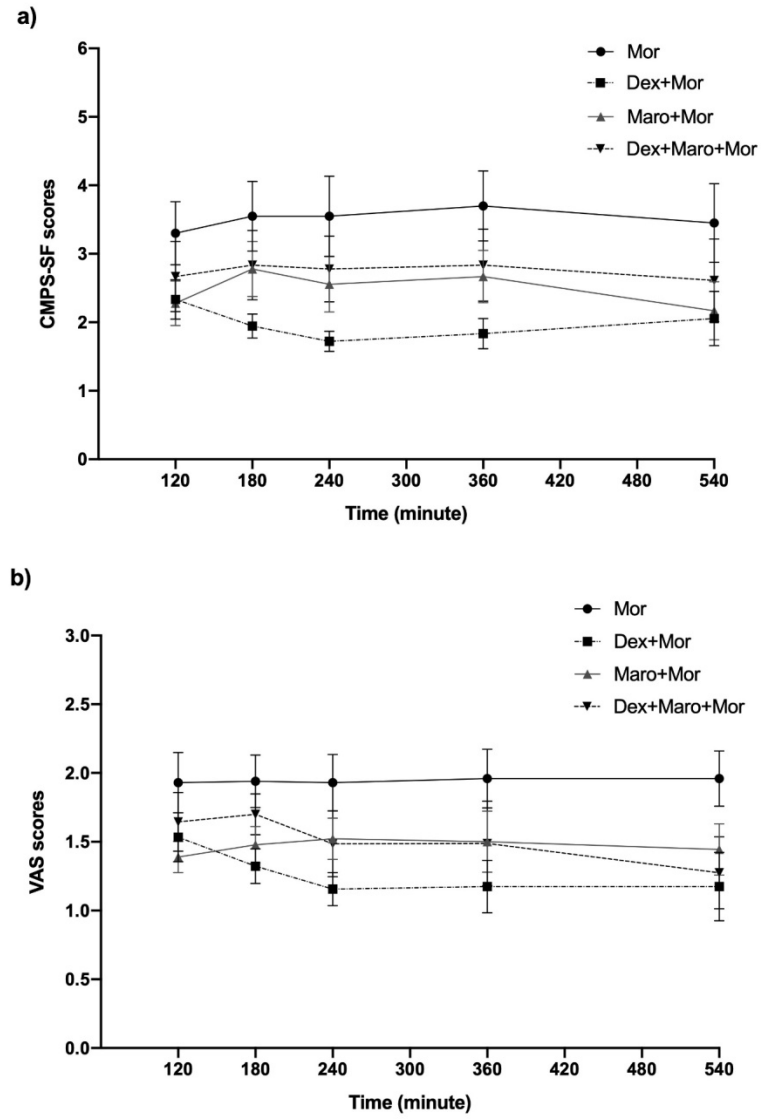

Figure 5.1 Glasgow composite measure pain scale (CMPS-SF) and b) Visual Analogue Scale (VAS) pain scores for the post-operative period in dogs after ovariohysterectomy (OHE).

Values are reported as mean ± SD. Treatment groups: 1) **Mor**: morphine 0.6 mg kg^{-1}; 2) **Dex+Mor**: morphine 0.3 mg kg^{-1}+ dexmedetomidine 10 µg kg^{-1}; 3) **Maro+Mor**: morphine 0.3 mg kg^{-1}+ maropitant 1 mg kg^{-1}; 4) **Dex+Maro+Mor**: morphine 0.2 mg kg^{-1}+ dexmedetomidine 7 µg kg^{-1}+ maropitant 0.7 mg kg^{-1}

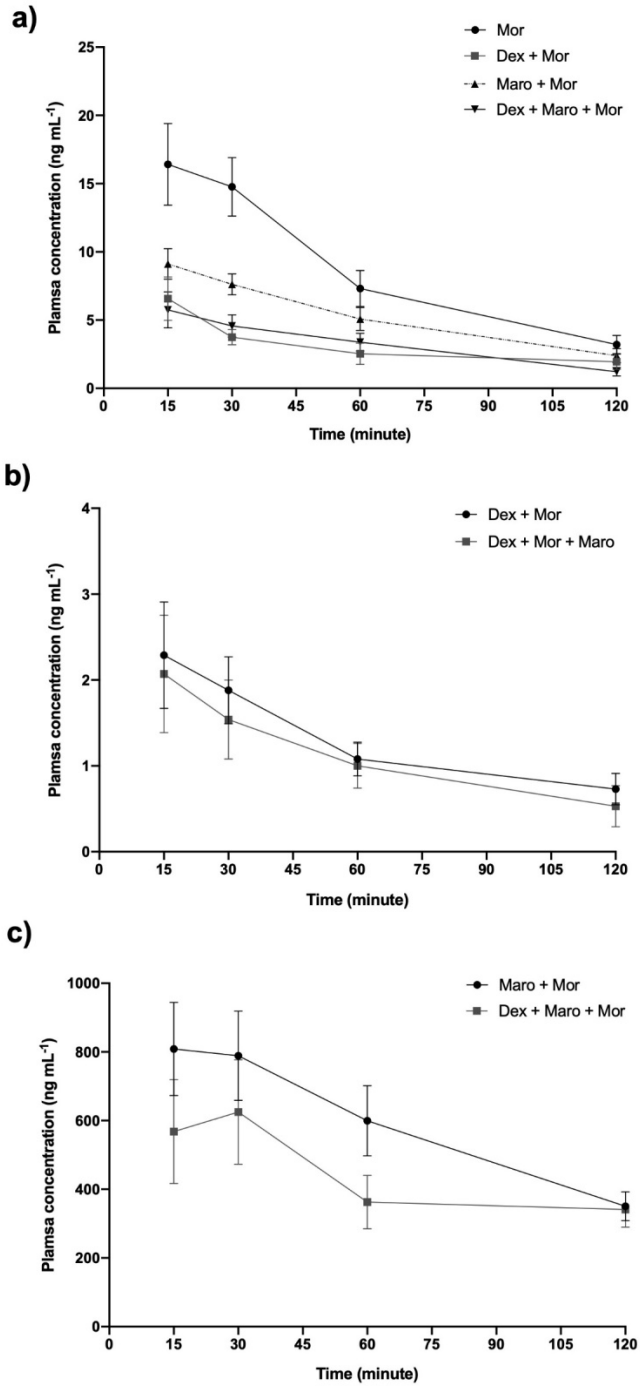

Figure 5.2 Concentration-time curve after IM administration of different combinations of a) morphine, b) dexmedetomidine, and c) maropitant in dogs.

Values are reported as mean ± SEM. Treatment groups: 1) **Mor**: morphine 0.6 mg kg^{-1}; 2) **Dex+Mor**: morphine 0.3 mg kg^{-1}+ dexmedetomidine 10 µg kg^{-1}; 3) **Maro+Mor**: morphine 0.3 mg kg^{-1}+ maropitant 1 mg kg^{-1}; 4) **Dex+Maro+Mor**: morphine 0.2 mg kg^{-1}+ dexmedetomidine 7 µg kg^{-1}+ maropitant 0.7 mg kg^{-1}

5.5 Discussion

This study shows that the lower dose of morphine (0.3 mg kg^{-1}) when combined with dexmedetomidine can provide superior analgesia compared to morphine alone at higher doses (0.6 mg kg^{-1}). Our results are in agreement with previous studies showing synergistic interaction between opioids and alpha-2-adrenoceptor agonists administered by different routes. Cardoso et al. (Cardoso *et al.*, 2014) showed that the antinociceptive effects of dexmedetomidine was enhanced when combined with morphine and methadone, all injected intramuscularly and shown by pedal withdrawal reflex test in dogs (Cardoso *et al.*, 2014). Adrenoceptor agonists (ST-9, clonidine and medetomidine) also potentiated the anti-nociceptive effect produced by opioids (morphine, fentanyl and meperidine) all injected intrathecally in primates (Yaksh and Reddy, 1981), cats (Murata *et al.*, 1989) and rats (Wang *et al.*, 1980; Ossipov *et al.*, 1989). The mechanism of synergistic interaction between opioids and alpha-2-adrenoceptor agonists is not completely understood but involvement of several spinal and supraspinal sites have been postulated. Both opioids and alpha-2-adrenoceptor agonists elicit membrane hyperpolarization by opening the same population of potassium channels through a G-protein coupled mechanisms resulting in inhibition of neurotransmitter release (Miyake *et al.*, 1989). Also, both opioid and alpha-2-adrenoceptors are present within the same superficial layer of the spinal cord inhibiting C fibre inputs (Sullivan *et al.*, 1987). Coexistence of these receptors in the same spinal region could also potentially lead to the modulation of opioid antinociceptive potency by alpha-2-adrenoceptor by allosteric mechanisms (Chabot-Doré *et al.*, 2015). In addition to the action on spinal cord, the synergy between the two classes of analgesics in producing antinociception by activating descending modulatory pathways from periaqueductal gray in the midbrain and via ventral medullary sites is also possible (Ossipov *et al.*, 1990).

A combination of morphine and maropitant also showed lower pain scores than morphine when used singly at higher doses but the analgesic effect of this combination was not as potent as the combination of morphine and dexmedetomidine. The three-drug combination also did not show superior analgesia. One of the mechanisms by which opioids and alpha-2-adrenoceptor agonists produce antinociception is by inhibiting the release of a widespread neurotransmitter, substance P, at primary afferents. Maropitant blocks the binding of substance P to NK-1 receptors. A synergy in analgesic effects between morphine, dexmedetomidine and maropitant is possible as all the three drugs can inhibit the effects of substance P at nociceptive primary afferents (Nicoll *et al.*, 1980; Sakurada *et al.*, 1995). Maropitant has been shown to be an effective analgesic in animal models (Sakurada *et al.*, 1995; Laird *et al.*, 2000; Ruggieri *et al.*, 2000) but the results from human clinical trials have been discouraging (Hill, 2000), and the reason for this remains unknown. A study in rats showed that the combination of morphine and maropitant was superior to morphine alone or in combination with dexmedetomidine in the tail flick (TF) test whereas no such effect was seen in the hot plate (HP) test (Karna *et al.*, 2019). A potential explanation for the difference in the effects of maropitant could be that a variation may exist in the relative density of NK-1 receptors and substance P in different somatic or pain pathways which can lead to the differences in antinociceptive effects of the NK-1 antagonists (Perry and Lawson, 1998). Multiple neurotransmitters may exist within the same neuron and many neurotransmitters are involved in the activation of a nociceptive pathway within the dorsal horn of spinal cord (Salt and Hill, 1983). The TF test in rats is the test of spinal analgesia whereas other complex pain pathways could be involved in the OHE model where substance P may not be the dominant neurotransmitter. Another possible reason

could be that the dose of the drugs in a three drug combination would have been too low to show clinically superior analgesic effects. Interspecies differences in distribution and involvement of NK-1 receptors and substance P in anti-nociception could also be the reason why combination of morphine and maropitant was superior to morphine in the HP and TF test in the rat study but not in this OHE study in dogs.

The results of this study show that the duration of surgery and the wait-time from the administration of drugs to the occurrence of surgical stimulus are important determinants of the pain scores. The study design used here was different from the common clinical practice used in routine OHE where analgesics are administered as premedication. The test drugs were not administered as a premedication but were administered 30 minutes before the expected time of the start of surgery. This was an attempt to synchronise *Tmax* with surgery start time as students teaching surgeries may have a long patient preparation and surgery time. A shorter duration of time between administration of test drugs and the start of surgery could be a reason for a lower number of dogs requiring rescue analgesia compared to other studies using the same dose of morphine in student teaching OHE (Kongara *et al.*, 2012). It is also important to note that the *Cmax* of dexmedetomidine, maropitant and morphine were observed at 15 and 30 minutes in this study. Thus, a protocol of IM administration of analgesics 15-30 minutes before the start of surgery could be a more effective especially in teaching surgeries where longer preparation and surgery time are expected. Administration of morphine after induction of anaesthesia as in this study will also reduce the incidence of preoperative vomiting and defecation, the common adverse effects seen after administration of morphine in conscious dogs (Valverde *et al.*, 2004).

Acepromazine was used as premedication to provide sedation to allow placement of intravenous catheters before surgery. Placement of catheters without sedation would not have been possible in many dogs. Acepromazine was chosen as premedication because it has no analgesic effect (Thurmon *et al.*, 1996) and thus would not interfere with comparing the analgesic effect of the test drugs. A concern over the use of acepromazine in this study could be its long lasting sedative effect which could still be present when pain scoring but the sedative effect of acepromazine is dose dependent and a very low dose of acepromazine (0.02 mg kg^{-1} IM) was used. Further, only the pain scores recorded after the sedation score of the dogs returned to zero (after 2 hours in all dogs) were used for the analysis.

The PK parameters of morphine in this study varied significantly compared to other reported studies in conscious and non-surgical dogs (Dohoo *et al.*, 1994; Barnhart *et al.*, 2000). In the present study, the treatment group which received 0.6 mg Kg^{-1} of morphine, had lower *Cmax* and $AUC_{0-\infty}$, CL, higher V*z*, and similar $T_{1/2}$ compared to the study conducted by Dohoo et al. (Dohoo *et al.*, 1994) after administering 0.5 mg Kg^{-1} of morphine. The pharmacokinetics of intramuscular dexmedetomidine and maropitant have not been previously studied; hence a comparison cannot be made. The average $T_{1/2}$ of morphine was longer when combined with dexmedetomidine which was also seen in a previous study (Karna *et al.*, 2019b). The disposition of a drug is affected by several factors such as perfusion at the injection site, anaesthesia, physiological state of animals, and co-administration of other drugs etc. (Zuidema *et al.*, 1988). Dexmedetomidine can decrease the cardiac output and the total flow of blood through the liver (Lawrence *et al.*, 1996; Dutta *et al.*, 2000) which potentially leads to a slower

metabolism and elimination of morphine leading to a longer residence time of the drug in plasma and longer duration of analgesia.

No correlation between plasma concentration and other pharmacokinetic variables of morphine with pain scores was observed in the group Mor. For the combination groups, the data were complicated because of the use of different dosage of two or three drugs. Previous human studies have also shown that no simple relationship exists between plasma concentration of morphine and pharmacodynamic variables (Berkowitz, 1976; Faura *et al.*, 1996). The plasma concentration of the drugs at the time of pain scoring was not available in this study; hence a pharmacokinetic-pharmacodynamics (PKPD) modelling could not be performed. Thus, the pharmacokinetics analysis here is limited to a descriptive study.

The dose rates of the drugs chosen in this study were based on clinical opinion of the authors as the drug combinations have not been studied before. The recommended dose range of morphine in cats and dogs is 0.3-1 mg/kg IM (Mathews *et al.*, 2014). We used lower doses of morphine in this study out of concern that higher dosage may might overshadow its interactions with other two drugs. In the absence of data, we initially assumed that 0.2 mg/kg morphine would give similar analgesia to 7 µg/kg dexmedetomidine or 1mg/kg maropitant and that the analgesia would be additive.

The drugs were given by the intramuscular route as this is preferred for administration of morphine (Hall and Clarke, 1983) because intravenous administration is associated with excitement reactions and release of histamines leading to cardiovascular changes (Maiante *et al.*, 2009). Dexmedetomidine and maropitant were administered IM so that

T_{max} of the drugs would be closer to each other to study any synergistic/supra-additive effect between them. It was not practical to administer dexmedetomidine by the subcutaneous route because of its vasoconstrictive effects, and thus IM route of administration was chosen for all drugs. In general practice, analgesics are likely to be given as a premedication and IM injection is often used. However, it is also important to consider that IM injection can lead to large inter-animal variation in the plasma drug concentration of the drugs which was observed in this study. The high inter-animal variability through IM route of administration can happen due to variation in the regional blood flow and unintentional administration into peri-muscular fat (Nordberg *et al.*, 1985; Autefage *et al.*, 1990). Some drugs may also bind to muscle and intersubjective variability in the binding could cause variation in their pharmacokinetics (Fichtl and Kurz, 1978). A wide variation in breed and age of the dogs could also have contributed to variation in pharmacokinetics of the drugs. Statistical difference in the PK parameters between treatment groups was also not identified because of this wide inter-animal variation. A low number of blood collection points, study over only two hours and unavailability of plasma concentration data at the time of pain scoring were the limitations of the pharmacokinetics study.

The physiologic parameters were within the expected range for anesthetized dogs. The cardiovascular effects of dexmedetomidine seen in this study agree with the biphasic cardiovascular response described for this drug (Gertler *et al.*, 2001; Murrell and Hellebrekers, 2005). Phase 1 shows an initial increase in blood pressure and a reflex decrease in heart rate due to increased vagal tone associated with activation of post-synaptic α_2-receptors in peripheral vascular smooth muscle. Increase in vagal tone can also lead to a period of sinus arrest, second-degree atrioventricular block and

arrhythmias. Phase 2 is characterized by a fall in blood pressure due to the decline of the vasoconstrictive effect and decrease in sympathetic tone leading to prolonged decreased but stabilized heart rate. Administration of a single dose of dexmedetomidine is also associated with a significant reduction in norepinephrine which could be another possible explanation of the cause of decrease in the heart rate after administration of dexmedetomidine (Aantaa *et al.*, 1990). Arrhythmias and second-degree atrioventricular block during anaesthesia may not be concerning if blood pressure is adequate (Hall *et al.*, 2014). These side effects of dexmedetomidine can also be prevented with the use of anticholinergics when given before administration of dexmedetomidine (Short, 1991). All dogs used in this study were screened for cardiovascular disease based on the clinical examination and history. Thus, the results of cardiovascular effects cannot be extrapolated for dogs with cardiovascular disease and caution should be used when using dexmedetomidine in those patients.

The present study has some limitations. Firstly, three different doses of morphine were used and there were no control groups with morphine alone at lower doses (0.3 and 0.2 mg kg^{-1}) or only dexmedetomidine and/or maropitant. Isobolographic studies have also shown that interaction between the opioids and alpha-2-adrenoceptor agonists may vary according to the dose ratio of the drugs (Ossipov *et al.*, 1990), thus the findings of the study apply only to the doses of the drugs used in this study. Future studies involving treatment groups at different drug dose ratios and control groups with morphine alone at lower doses (0.3 and 0.2 mg kg^{-1}) and dexmedetomidine and/or maropitant only are recommended. Another limitation of the present study is that both CMPS-SF and VAS used for performing pain assessment are based on subjective judgement of the assessor. Significant interobserver variability has been seen while using these pain scales (Holton

et al., 1998). A second pain assessor could have assessed the inter-observer variability but any variation between the assessors would have increased the complications in analysis. In addition, all surgeries were performed by different students in supervision of a veterinary surgeon. The assumption while using teaching surgeries as pain models was that all dogs will receive a supramaximal stimulus. However, it is possible that some degree of variability between the surgeons could have occurred in terms of tissue handling and the degree of noxious stimulus produced.

In summary, a combination of morphine with dexmedetomidine and/or maropitant provided post-operative analgesia significantly better than morphine alone. A bigger study with treatment groups at different dose combinations of the drugs and more control groups as stated above is recommended. Incidence of cardiovascular adverse effects of dexmedetomidine should be considered while incorporating the drug in the analgesic protocol.

5.6 References

Aantaa R, Kanto J, Scheinin M, Kallio, A, Acheinin, H. Dexmedetomidine, an alpha 2-adrenoceptor agonist, reduces anesthetic requirements for patients undergoing minor gynecologic surgery. *Anesthesiology* 73**,** 230-35, 1990

Autefage A, Fayolle P, Toutain P. Distribution of material injected intramuscularly in dogs. *American Journal of Veterinary Research* 51**,** 901-04, 1990

Barnhart MD, Hubbell JA, Muir WW, **Sams RA, Bednarski RM**. Pharmacokinetics, pharmacodynamics, and analgesic effects of morphine after rectal, intramuscular, and intravenous administration in dogs. *American Journal of Veterinary Research* 61**,** 24-28, 2000

Barr CA, Gianotti G, Graffeo CE, Drobatz KJ, Silverstein DC. Effect of blood collection by the push-pull technique from an indwelling catheter versus direct venipuncture on venous blood gas values before and after administration of alfaxalone or propofol in dogs. *Journal of the American Veterinary Medical Association* 251**,** 1166-74, 2017

Berkowitz BA. The relationship of pharmacokinetics to pharmacological activity: morphine, methadone and naloxone. *Clinical Pharmacokinetics* 1**,** 219-30, 1976

Bitti FS, Campagnol D, Rangel JP, Junior JSN, Loureiro B, Monteiro ER. Effects of three methadone doses combined with acepromazine on sedation and some cardiopulmonary variables in dogs. *Veterinary Anaesthesia and Analgesia* 44**,** 237-45, 2017

Boscan P, Monnet E, Mama K, Twedt DC, Congdon J, Steffey EP. Effect of maropitant, a neurokinin 1 receptor antagonist, on anaesthetic requirements during noxious visceral stimulation of the ovary in dogs. *American Journal of Veterinary Research* 72**,** 1576-79, 2011

Cardoso CG, Marques DR, da Silva THM, de Mattos-Junior E. Cardiorespiratory, sedative and antinociceptive effects of dexmedetomidine alone or in combination with methadone, morphine or tramadol in dogs. *Veterinary Anaesthesia and Analgesia* 41**,** 636-43, 2014

Chabot-Doré A-J, Millecamps M, Naso L, Devost D, Trieu P, Piltonen M, Diatchenko L, Fairbanks CA, Wilcox GL, Hébert TE. Dual allosteric modulation of opioid antinociceptive potency by α2A-adrenoceptors. *Neuropharmacology* 99**,** 285-00, 2015

Dohoo S, Tasker RAR, Donald A. Pharmacokinetics of parenteral and oral sustained-release morphine sulphate in dogs. *Journal of Veterinary Pharmacology and Therapeutics* 17**,** 426-33, 1994

Dutta S, Lal R, Karol MD, Cohen T, Ebert T. Influence of cardiac output on dexmedetomidine pharmacokinetics. *Journal of Pharmaceutical Sciences* 89**,** 519-27, 2000

Faura CC, Moore RA, Horga J, Hand CW, McQuay HJ. Morphine and morphine-6-glucuronide plasma concentrations and effect in cancer pain. *Journal of Pain and Symptom Management* 11**,** 95-02, 1996

Fichtl B, Kurz H. Binding of drugs to human muscle. *European Journal of Clinical Pharmacology* 14**,** 335-40, 1978

Fielder SE. Hematologic Reference Ranges. In: Seamamdg A (ed). *The Merck Veterinary Manual*. Merck & Co., Inc., Kenilworth, NJ, USA, 2016

Gaynor JS, Muir W. Acute pain management: a case-based approach. In: Gaynor JS, Muir W (eds). *Handbook of Veterinary Pain Management*. Elsevier, USA. Pp 444-46, 2014

Gertler R, Brown HC, Mitchell DH, Silvius, EN. Dexmedetomidine: a novel sedative-analgesic agent. *Proceedings of Baylor University Medical Center*. Pp 13-21, Taylor & Francis, USA, 2001

Guo T-Z, Jiang J-Y, Buttermann AE, Maze, M. Dexmedetomidine injection into the locus ceruleus produces antinociception. *The Journal of the American Society of Anesthesiologists* 84**,** 873-81, 1996

Hall L, Clarke K, Trim C. Special anaesthesia. In: Hall L, Clarke K, Trim C (eds). *Veterinary Anaesthesia*. Saunders Elsevier Limited, 2014

Hall LW, Clarke KW. Principles of sedation, analgesia and premedication. In: Hall LW, Clarke KW, Trim C (eds). *Veterinary Anaesthesia*. Pp 75-112. Bailliere Tindall, United Kingdom, 1983

Hansen BD. Assessment of pain in dogs: veterinary clinical studies. *Institute for Laboratory Animal Research Journal* 44**,** 197-05, 2003

Hill R. NK1 (substance P) receptor antagonists–why are they not analgesic in humans? *Trends in Pharmacological Sciences* 21**,** 244-46, 2000

Holton LL, Scott E, Nolan AM, Reid J, Welsh E, Flaherty D. Comparison of three methods used for assessment of pain in dogs. *Journal of the American Veterinary Medical Association* 212**,** 61-66, 1998

Kalso EA, Pöyhiä R, Rosenberg PH. Spinal Antinociception by Dexmedetomidine, a Highly Selective α2-Adrenergic Agonist. *Pharmacology & Toxicology* 68**,** 140-43, 1991

Karna S, Singh P, Chambers P, Kongara K. Pharmacokinetics of morphine in combination with dexmedetomidine and maropitant following intramuscular injection in dogs anaesthetized with halothane. *Journal of Veterinary Pharmacology and Therapeutics* 2019;00:1–9, 2019b

Karna SR, Kongara K, Singh PM, Chambers P, Lopez-Villalobos N. Evaluation of analgesic interaction between morphine, dexmedetomidine and maropitant using hot-plate and tail-flick tests in rats. *Veterinary Anaesthesia and Analgesia*, 2019a

Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biology* 8**,** e1000412, 2010

Kim YK, Lee SS, Suh EH, Lee L, Lee HC, Lee HJ, Yeon SC. Sprayed intraperitoneal bupivacaine reduces early postoperative pain behavior and biochemical stress response after laparoscopic ovariohysterectomy in dogs. *The Veterinary Journal* 191**,** 188-92, 2012

Kongara K, Chambers J, Johnson C. Effects of tramadol, morphine or their combination in dogs undergoing ovariohysterectomy on peri-operative electroencephalographic responses and post-operative pain. *New Zealand Veterinary Journal* 60**,** 129-35, 2012

Laird J, Olivar T, Roza C, De Felipe C, Hunt SP, Cervero F. Deficits in visceral pain and hyperalgesia of mice with a disruption of the tachykinin NK1 receptor gene. *Neuroscience* 98**,** 345-52, 2000

Lawrence C, Prinzen F, De Lange S. The effect of dexmedetomidine on nutrient organ blood flow. *Anesthesia & Analgesia* 83**,** 1160-65, 1996

Maiante A, Teixeira Neto FJ, Beier SL, Corrente, JE, Pedroso, CEBP. Comparison of the cardio-respiratory effects of methadone and morphine in conscious dogs. Journal of *Veterinary Pharmacology and Therapeutics* 32**,** 317-28; 2009

Marquez M, Boscan P, Weir H; Vogel P, Twedt DC. Comparison of NK-1 receptor antagonist (maropitant) to morphine as a pre-anaesthetic agent for canine ovariohysterectomy. *PloS One* 10**,** e0140734; 2015

Mathews K, Kronen PW, Lascelles D, Nolan A, Robertson S, Steagall PVM, Wright B, Yamashita K. Guidelines for recognition, assessment and treatment of pain: WSAVA Global Pain Council members and co-authors of this document. *Journal of Small Animal Practice* 55**,** E10-E68, 2014

Miyake M, Christie MJ, North RA. Single potassium channels opened by opioids in rat locus ceruleus neurons. *Proceedings of the National Academy of Sciences* 86**,** 3419-22, 1989

Murata K, Nakagawa I, Kumeta Y, Kitahata LM, Collins JG. Intrathecal clonidine suppresses noxiously evoked activity of spinal wide dynamic range neurons in cats. *Anaesthesia and Analgesia* 69**,** 185-91, 1989

Murrell JC, Hellebrekers LJ. Medetomidine and dexmedetomidine: a review of cardiovascular effects and antinociceptive properties in the dog. *Veterinary Anaesthesia and Analgesia* 32**,** 117-27, 2005

Nicoll R, Schenker C, Leeman S. Substance P as a transmitter candidate. *Annual Review of Neuroscience* 3**,** 227-68, 1980

Niyom S, Boscan P, Twedt DC, Monnet E, Eickhoff JC. Effect of maropitant, a neurokinin-1 receptor antagonist, on the minimum alveolar concentration of sevoflurane during stimulation of the ovarian ligament in cats. *Veterinary Anaesthesia and Analgesia* 40**,** 425-31, 2013

Nordberg G, Borg L, Hedner T, Mellstrand T. CSF and plasma pharmacokinetics of intramuscular morphine. *European Journal of Clinical Pharmacology* 27**,** 677-81, 1985

Ossipov MH, Harris S, Lloyd P, Messineo E, Lin BS, Bagley J. Antinociceptive interaction between opioids and medetomidine: systemic additivity and spinal synergy. *Anesthesiology* 73**,** 1227-35, 1990

Ossipov MH, Suarez LJ, Spaulding TC. Antinociceptive interactions between alpha-2-adrenergic and opiate agonists at the spinal level in rodents. *Anesthesia & Analgesia* 68**,** 194-00, 1989

Perry M, Lawson S. Differences in expression of oligosaccharides, neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. *Neuroscience* 85**,** 293-10, 1998

Reid J, Nolan A, Hughes JML, Lascelles D, Pawson P, Scott EM. Development of the short-form Glasgow Composite Measure Pain Scale (CMPS-SF) and derivation of an analgesic intervention score. *Animal Welfare-Potters Bar Then Wheathampstead* - 16**,** 97, 2007

Ruggieri MR, Filer-Maerten S, Hieble JP, Hay DWP. Role of neurokinin receptors in the behavioral effect of intravesical antigen infusion in guinea pig bladder. *The Journal of Urology* 164**,** 197-02, 2000

Sakurada T, Katsumata K, Yogo H, Tan-No K, Sakurada S, Ohba M, Kisara K. The neurokinin-1 receptor antagonist, sendide, exhibits antinociceptive activity in the formalin test. *Pain* 60**,** 175-80, 1995

Salt T, Hill R. Neurotransmitter candidates of somatosensory primary afferent fibres. *Neuroscience* 10**,** 1083-03, 1983

Short C. Effects of anticholinergic treatment on the cardiac and respiratory systems in dogs sedated with medetomidine. *The Veterinary Record* 129**,** 310-13, 1991

Slingsby LS, Murison PJ, Goossens L, Engelen M, Waterman-Pearson AE. A comparison between pre-operative carprofen and a long-acting sufentanil

formulation for analgesia after ovariohysterectomy in dogs. *Veterinary Anaesthesia and Analgesia* 33**,** 313-27, 2006

Sullivan AF, Dashwood MR, Dickenson AH. α2-Adrenoceptor modulation of nociception in rat spinal cord: location, effects and interactions with morphine. *European Journal of Pharmacology 138***,** 169-77, 1987

Thurmon JC, Tranquili W, Benson G. Preanesthetics and anesthetics adjuncts. In: Tranquili W, Benson G (eds). *Veterinary Anesthesia*, Pp 183-89, Williams & Wilkins, Baltimore, USA, 1996

Valverde A, Cantwell S, Hernández J, Brotherson C. Effects of acepromazine on the incidence of vomiting associated with opioid administration in dogs. *Veterinary Anaesthesia and Analgesia* 31**,** 40-45, 2004

Verbeke G, Molenberghs G. Linear mixed models in practice: a SAS-oriented approach. *Springer Science & Business Media*, Springer-Verlag, New York, USA, 1997

Wang J, Yasuoka S, Yaksh T. Studies on the analgetic effect of intrathecal ST-91 (2-[2, 6-diethyl-phenylamino]-2-imidazoline): antagonism, tolerance and interaction with morphine. *Pharmacologist* 22**,** 302, 1980

Yaksh TL, Reddy S. Studies in the primate on the analgetic effects associated with intrathecal actions of opiates, alpha-adrenergic agonists and baclofen. *Anesthesiology* 54**,** 451-67, 1981

Young A, Buvanendran A. Recent advances in multimodal analgesia. *Anesthesiology Clinics* 30**,** 91-00, 2012

Zhang Y, Huo M, Zhou J, Xie S. PKSolver: An add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Computer Methods and Programs in Biomedicine* 99**,** 306-14, 2010

Zuidema J, Pieters F, Duchateau G. Release and absorption rate aspects of intramuscularly injected pharmaceuticals. *International Journal of Pharmaceutics* 47**,** 1-12, 1988

5.7 Appendix

SHORT FORM OF THE GLASGOW COMPOSITE PAIN SCALE

Dog's name _____

Hospital Number _____ **Date** / / **Time**

Surgery Yes/No (delete as appropriate)

Procedure or Condition_____

_____

In the sections below please circle the appropriate score in each list and sum these to give the total score.

A. Look at dog in Kennel

Is the dog?

(i)		(ii)	
Quiet	0	Ignoring any wound or painful area	0
Crying or whimpering	1	Looking at wound or painful area	1
Groaning	2	Licking wound or painful area	2
Screaming	3	Rubbing wound or painful area	3
		Chewing wound or painful area	4

In the case of spinal, pelvic or multiple limb fractures, or where assistance is required to aid locomotion do not carry out section **B** and proceed to **C**
Please tick if this is the case ☐ then proceed to C.

B. Put lead on dog and lead out of the kennel.

When the dog rises/walks is it?

(iii)	
Normal	0
Lame	1
Slow or reluctant	2
Stiff	3
It refuses to move	4

C. If it has a wound or painful area including abdomen, apply gentle pressure 2 inches round the site.

Does it?

(iv)	
Do nothing	0
Look round	1
Flinch	2
Growl or guard area	3
Snap	4
Cry	5

D. Overall

Is the dog? *(v)*		*Is the dog?* *(vi)*	
Happy and content or happy and bouncy	0	Comfortable	0
Quiet	1	Unsettled	1
Indifferent or non-responsive to surroundings	2	Restless	2
Nervous or anxious or fearful	3	Hunched or tense	3
Depressed or non-responsive to stimulation	4	Rigid	4

Total Score (i+ii+iii+iv+v+vi) = _____

Figure 5.3 Glasgow composite measure pain scale-short form for dogs.

From: Reid et al. (2007)

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

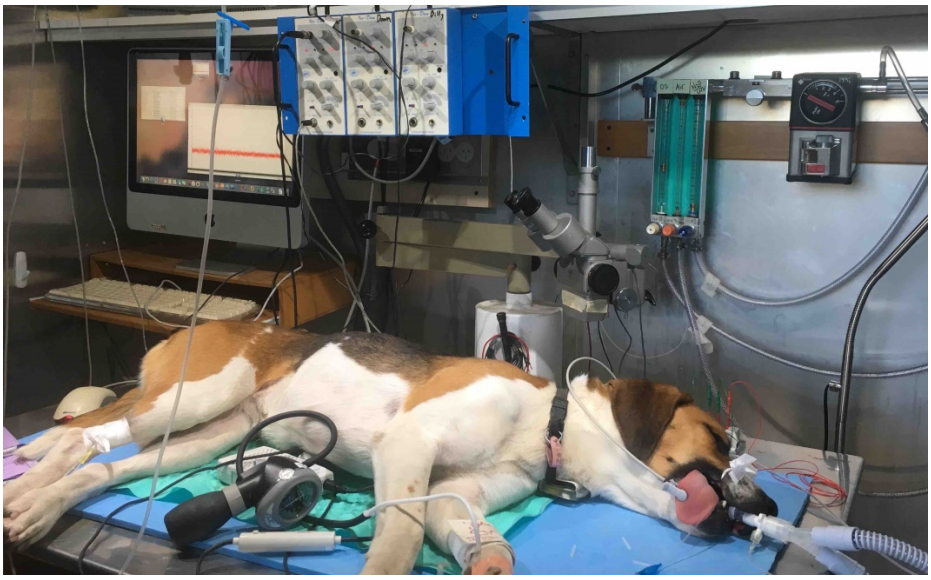
We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Sandeep Raj Karna
Name/title of Primary Supervisor:	Dr Kavitha Kongara
Name of Research Output and full reference:	
Evaluation of analgesic interaction between morphine, dexmedetomidine and maropitant dogs undergoing ovariohysterectomy.	
In which Chapter is the Manuscript /Published work:	Chapter 5
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	80%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Sandeep had a primary role in study design, conducting experiments, data collection, statistical analysis, interpretation and preparation of the manuscript, with guidance from supervisors.	
For manuscripts intended for publication please indicate target journal:	
To be submitted to the New Zealand Veterinary Journal	
Candidate's Signature:	
Date:	13/03/2020
Primary Supervisor's Signature:	Kavitha Kongara Digitally signed by Kavitha Kongara Date: 2020.03.15 20:42:19 +13'00'
Date:	

(This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis)

CHAPTER 6

Effect of combinations of morphine, dexmedetomidine and maropitant on the electroencephalogram in response to acute electrical stimulation in anaesthetised dogs



Author's note: Chapter 6 is presented in the style of *the Journal of Veterinary Pharmacology and Therapeutics* where it is intended to be submitted for publication as:

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6.1 Abstract

This study was conducted to compare the efficacy of combinations of morphine, dexmedetomidine and maropitant in preventing the changes in electroencephalographic (EEG) indices of nociception in anaesthetised dogs subjected to a noxious electrical stimulus. Eight healthy adult dogs weighing 25.76 ± 3.16 Kg and 3.87 ± 1.64 years of age were used in a crossover study. Dogs were randomly allocated to four groups: 1) morphine 0.6 mg/kg; 2) morphine 0.3 mg/kg + dexmedetomidine 5 µg/kg; 3) morphine 0.3 mg/kg + maropitant 1 mg/kg; 4) morphine 0.2 mg/kg + dexmedetomidine 3 µg/kg + maropitant 0.7 mg/kg. Anaesthesia was induced with intravenous propofol and maintained with halothane at a stable concentration between 0.85% and 0.95% which was followed by the EEG recording. Following intramuscular administration of test drugs after induction and maintenance of anaesthesia, a supramaximal electrical stimulus (50 V at 50 Hz for 2 sec) was applied using a Grass Stimulator and the EEG data were recorded. There were significant increases ($p < 0.05$) in the post-stimulus median frequency (F50) in groups Mor and Maro+Mor but not in groups Dex+Mor and Dex+Maro+Mor. There were no significant changes in the post-stimulation spectral edge frequency (F95) and total EEG power (Ptot) in any treatment groups. Dex+Mor group had a significantly lower change in F50 and F95 compared to all other treatment groups. There was no correlation of the changes in EEG frequencies with blood plasma concentration of the drugs during and after noxious stimulation. Combination of dexmedetomidine and morphine was most effective in abolishing the changes in EEG indices in response to a noxious stimulus which indicates a supra-additive interaction between these two drugs.

Keywords: Morphine, Dexmedetomidine, Maropitant, Anaesthesia, EEG

6.2 Introduction

Multimodal analgesia is a pharmacologic method of combining two or more classes of drugs or techniques to target different points in the pain pathway and is gaining popularity in the treatment of post-operative pain in veterinary patients. The main premise of using this technique is to decrease the dose of each individual drug and hence reduce their associated adverse effects.

Opioids such as morphine are the commonly used perioperative analgesics in dogs (Clarke, Trim, & Hall, 2014). A single dose of morphine administered preoperatively may not provide an adequate level of postoperative analgesia and frequent further dosing is required during the postoperative period (Hall, Clarke, & Trim, 2014). Morphine is also associated with adverse effects like prolonged sedation, respiratory depression, vomition, defecation and dysphoria (Barnhart, Hubbell, Muir, Sams, & Bednarski, 2000; Maiante, Teixeira Neto, Beier, Corrente, & Pedroso, 2009), which limits its use as a sole analgesic agent, especially at higher doses. Dexmedetomidine is often used in combination with morphine preoperatively to achieve better sedation and analgesia (Lemke, 2004). Dexmedetomidine is a highly selective alpha-2-adrenoceptor agonist (Vainio, 1989) and a commonly used sedative and analgesic in dogs and cats (Murrell & Hellebrekers, 2005). Maropitant is a neurokinin -1 (NK1) receptor antagonist used to control emesis of different aetiologies in dogs and cats (De la Puente-Redondo et al., 2007; Diemunsch & Grélot, 2000). Maropitant also has an anaesthetic dose sparing effect in dogs and cats undergoing ovariohysterectomy (OHE) (Boscan et al., 2011; Niyom, Boscan, Twedt, Monnet, & Eickhoff, 2013) and a supra-additive

analgesic effect in combination with morphine in a rat model (Karna, Kongara, Singh, Chambers, & Lopez-Villalobos, 2019).

A multimodal analgesia protocol combining morphine with dexmedetomidine and/or maropitant has been evaluated in dogs undergoing ovariohysterectomy (Chapter 5). Lower doses of morphine (0.2 and 0.3 mg/kg) combined with dexmedetomidine or maropitant provided analgesia similar to or better than a higher dose of morphine alone (0.6 mg/kg). The short-form of the Glasgow composite measure pain scale (CMPS-SF) and visual analogue scale (VAS) were used to assess pain in the study. These pain scales are based on the subjective judgement of the assessor and significant interobserver variability can be associated with using them (Holton et al., 1998).

Neurophysiological techniques such as electroencephalography are the objective tools that can be used to evaluate the antinociceptive properties of drugs. The electroencephalogram (EEG) is a record of the spontaneous electrical activity of the cerebral cortex and changes in variables derived from the EEG have been used as reliable indicators of nociception in nonverbal animals (Murrell & Johnson, 2006). In humans, EEG responses to phasic noxious stimuli correlates with perceived pain (Chen, Dworkin, Haug, & Gehrig, 1989). The change in EEG responses to a noxious stimulus in animals (Ong *et al.* 1997) has been found to be similar to those in humans (Chen et al., 1989). Johnson and co-workers (Johnson, Wilson, Woodbury, & Caulkett, 2005; Murrell et al., 2003) have developed a minimal anaesthesia model using which the changes in EEG power spectrum specifically, median frequency (F50), spectral edge frequency (F95) and total power (Ptot) in response to noxious stimulus under a light plane of anaesthesia have been shown to indicate nociception. This model has also been

used to evaluate antinociceptive efficacy of analgesics in different animal species (Kongara, Chambers, & Johnson, 2010; Murrell & Johnson, 2006; Singh et al., 2018).

The primary aim of this study was to evaluate and compare the antinociceptive effects of combinations of morphine with dexmedetomidine and maropitant on the electroencephalogram to noxious electrical stimulation. We hypothesised that if these drugs were additive, the combination of three drugs at one-third of the full dose or two drugs at half dose would be equally effective as morphine alone at the full dose in attenuating the change in the EEG power spectral indices of nociception (F50, F95 and Ptot) in response to a noxious electric stimulus. The secondary aim of the study was to investigate any association between the change in EEG frequencies with the blood plasma concentration of the drugs which is already published as a separate study (Karna, Singh, Chambers, & Kongara, 2019).

6.3 Materials and methods

6.3.1 Study design and animals

The study was approved by the Massey University Animal Ethics Committee (protocol no 17/57) and is reported in accordance with the ARRIVE guidelines for experiments involving animals (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010).

Eight healthy dogs (5 Harrier Hounds; 3 Shetland Sheepdogs) were used in a randomised crossover study where each dog received four treatments separated by a three week washout period. The sample size was estimated to achieve an experimental power of 90% with an alpha error of 0.05 to detect a difference of 10% between treatment means. These parameters were based on a previous study with similar study design (Kongara et al., 2010).

The dogs were judged to be in good health by physical examination and history. They were obtained from the Centre for Canine Nutrition (CCN) at Massey University, Palmerston North, New Zealand. They were housed in a larger outer pen during the day and indoor pens in pairs at night. Dogs were fed once a day and had unlimited access to water. The dogs were brought to the laboratory on the day of the experiment and were kept in the laboratory until the final blood collection time point. After that, they were housed at the MU Veterinary Teaching Hospital overnight and returned to the CCN facility, the next day.

The treatment groups were as follows: group 1 (Mor): morphine 0.6 mg/kg; group 2 (Maro+Mor): morphine 0.3 mg/kg + maropitant 1 mg/kg; group 3 (Dex+Mor): morphine 0.3 mg/kg + dexmedetomidine 5 μg/kg; group 4 (Dex+Maro+Mor): morphine 0.2 mg/kg + dexmedetomidine 3 μg/kg + maropitant 0.7 mg/kg, all injected intramuscularly.

The doses of these drugs have not been established for dogs and were based on their clinical and experimental use in animals.

6.3.2 Drug administration and anaesthetic monitoring

Dogs were fasted for 12 hr before each treatment and had unlimited access to water. They were pre-medicated with acepromazine 0.02 mg/kg IM 40 min prior to the anaesthetic induction. The right saphenous vein was catheterised percutaneously using a 22 gauge 1.00-inch catheter for anaesthesia induction, blood sampling and administration of Hartmann's solution. Anaesthesia was induced with Propofol (IV Propofol Injection; Mayne Pharma Pty Ltd, Australia) to effect.

After induction of anaesthesia, the trachea was intubated with a cuffed endotracheal tube. Anaesthesia was maintained by halothane (HalothaneVet; Merial NZ Limited, New Zealand) in oxygen delivered via a circle breathing system for 35 min. An established minimal anaesthesia model was followed (Kongara, Chambers, & Johnson, 2012; Murrell et al., 2003). The End-tidal halothane concentration (EtHal) was maintained between 0.85% and 0.95% and the dogs breathed spontaneously. The dogs were given Hartmann's solution (Baxter Healthcare Ltd, Australia) IV to maintain systolic arterial blood pressure above 100 mmHg throughout the anaesthetic period and their systolic arterial blood pressure was monitored using a Doppler ultrasound pressure transducer with cuff (Doppler flow detector, Parks Medical Electronics Inc., USA). Heart rate (HR), respiratory rate (RR), end-tidal halothane tension (EtHal) and end-tidal CO_2 tension ($EtCO_2$) of dogs were monitored using an anaesthetic agent monitor (Hewlett Packard M1025B; Hewlett Packard, Germany). Peripheral capillary oxygen saturation (SPO_2) was monitored using a pulse oximeter (Pulse Ox-Fisher & Paykel Healthcare Ltd, Auckland, NZ), and the rectal temperature was measured using a digital veterinary thermometer (Kamsay, USA). All of these parameters were recorded every 5 min.

Dogs were positioned into left lateral recumbency. Immediately after the target EtHal was achieved, test drugs were injected into lumbar epaxial muscles using a 2 ml syringe and 22- gauge, 1-inch needles. All drugs were injected separately within a minute of each other starting with morphine followed by maropitant and dexmedetomidine. The drugs used were morphine sulphate (DBL morphine sulphate injection BP; Hospira Australia Pty. Ltd., Australia) 5 mg/ml, dexmedetomidine hydrochloride (Dexdomitor;

Zoetis, New Zealand) 0.5 mg/ml and maropitant citrate (Cerenia; Zoetis, New Zealand) 10 mg/ml.

6.3.3 EEG recording and electrical stimulus:

The EEG recording was performed using the Chart 5.2.2 recording software connected to Powerlab 4/20 data recording system (Powerlab data acquisition system; AD Instruments Ltd, Australia). The active, reference and ground electrodes were employed using 27 standard wire gauge stainless steel needles (Medelec, New Zealand) placed subcutaneously over the zygomatic process of the left frontal bone, on the left mastoid process and caudal to the occipital process respectively. The sampling rate of the recording was 1 kHz and a low pass filter was applied to remove all the activity above the limit frequency. The signals were amplified with a gain of 1000x.

The EEG recording was started as soon as the dogs were stabilised under halothane anaesthesia. A 10-second baseline was recorded immediately preceding the electrical stimulus. A supramaximal electrical stimulus (50 volts at 50 H_z for 2 sec, (Kongara et al., 2010; Valverde, Dyson, & McDonell, 1989) was applied using a Grass Stimulator (S48K square pulse stimulator, Astro-Med Inc.,USA). The stimulus was applied to the lateral aspect of the distal metatarsus of the right hind limb using two stainless steel needle electrodes placed 2 cm apart. EEG data were collected for 5 min following the application of the stimulus. Halothane was discontinued and the endotracheal tube was removed when the dogs were able to maintain their airway.

Dogs were monitored for any adverse effects throughout the study period.

Blood samples (2 ml) were collected before injection of the treatment drugs and 0.25, 0.5, 1, 2, 3 4, 6 and 8 hr afterwards. The samples were taken to measure plasma concentrations of the drugs and the data were used to study the pharmacokinetics of the drugs.

6.3.4 Data analysis:

Analysis of the raw EEG data was carried out after the completion of experiments. The F50, F95 and Ptot were calculated for consecutive 1-second epochs using purpose-written software (Spectral Analyser, CB Johnson, Massey University, Palmerston North, New Zealand).

The pre-stimulation mean heart rate was obtained from the recordings 1 min before stimulation. The post-stimulation mean heart rates were obtained from the recordings immediately after the stimulation and every 15 min until the dog recovered from anaesthesia.

6.3.5 Statistical analysis:

All statistical analyses were performed using SAS for Windows v 9.4 (SAS Institute Inc Cary NC, USA). The distribution of data was tested for normality by using the Kolmogorov-Smirnov test and logarithmic (log) transformed values were used for the analysis of the variables which did not follow a normal distribution.

The EEG data averaged from the last 10 sec block before the electric stimulus was used as the baseline. Post stimulus data comprised of the 10-sec blocks after the electrical stimulus (after excluding two-sec blocks immediately after the stimulus to avoid the contamination due to movement of the dog).

The repeated measures of the EEG spectral variables on the same dog were analysed using the MIXED procedure with a mixed linear model that included the fixed effect of time, treatment and interaction between time and treatment and the random effect of the week and animal to account for the crossover study and repeated measures in the same animal. Least square (LS) means and standard errors for treatment time and times and combinations between treatments and times were obtained and used for multiple mean comparisons using the Fisher's least significant difference as implemented in the MIXED procedure. There was a significant difference between the baseline of the treatment groups and thus the baselines were used as a covariate in the model.

The means of post-stimulation data from the three EEG spectral variables of interest were also expressed as percent of baseline values calculated directly.

The pharmacokinetic data from this study have already been published separately (Karna, Singh, Chambers, & Kongara, 2019). The relationship between percentage changes in EEG frequencies and plasma concentration of the drugs at the time of application of electrical stimulus was assessed using the Pearson correlation coefficients.

All results were considered significant if $p < 0.05$ for all analyses.

6.4 Results

The mean age and body weight of dogs were 3.87 ± 1.64 years and 25.76 ± 3.16 kg respectively. There was no association of body weight, age, breed or the day of testing with the change in EEG parameters.

The baselines of F50 and F95 were significantly lower ($p < 0.0001$) in Dex+Mor and Dex+Maro+Mor groups compared to all other treatment groups. Similarly, Dex+Mor and Dex+Maro+Mor had significantly higher baselines of Ptot compared to Mor ($p < 0.0001$). There were no significant differences between the baselines of Dex+Mor and Dex+Maro+Mor for any EEG frequencies.

The differences in LS means of the EEG frequencies before and after noxious electric stimulus for each treatment groups are reported in the Table 6.1 and the differences between the treatment groups for the change in the EEG frequencies are reported in the Table 6.2.

6.4.1 Median frequency (F50)

There were statistically significant increases ($p < 0.05$) in the post-stimulation F50 in groups Mor and Maro+Mor, and groups Dex+Maro+Mor and Dex+Mor showed no statistically significant change. The rank order of the treatment group for the percentage change in F50 after stimulus compared to their baseline was Dex+Mor (0.54 ± 6.51%) < Dex+Maro+Mor (14.32 ± 6.55%) < Maro+Mor (18.55 ± 6.50 %) < Mor (21.85 ± 6.88%). The group Dex+Mor had significantly lower change ($p < 0.0001$) in F50 compared to all other treatment groups.

6.4.2 Spectral edge frequency (F95)

There were no significant changes in the F95 after an electrical stimulus in any treatment group. The rank order of the treatment group for the percentage change in F95 after stimulus compared to the baseline was Dex+Mor (0.18 ± 1.12%) < Maro+Mor (0.41 ± 1.13 %) < Mor (1.17 ± 1.18%) < Dex+Maro+Mor (1.92 ± 1.13%). The group

Dex+Mor had a significantly lower change in F95 ($p < 0.0001$) compared to all other treatment groups.

6.4.3 Total power (Ptot)

There were no significant changes in the Ptot after an electrical stimulus in any treatment groups. The rank order of the treatment group for the percentage change in Ptot after stimulus compared to the baseline was Dex+Maro+Mor (-1.39 ± 5.20%) < Maro+Mor (-2.12 ± 5.19%) < Dex+Mor (-2.36 ± 5.18%) < Mor (-8.12 ± 5.55%). The group Mor had a significantly larger decrease ($p < 0.0001$) in Ptot compared to all other treatment groups.

The percentage change in the LS means of the post-stimulation F50, F95 and Ptot is illustrated in Figure 6.1.

6.4.4 Physiological parameters

All dogs in treatment groups Dex+Mor and Dex+Maro+Mor showed a significant decrease in the heart rate, sinus arrhythmia and second-degree heart block 1-2 min after administration of the drugs. The change in the heart rate from before the administration of the drugs to 5 min post-stimulation is illustrated in Figure 6.2. The groups Dex+Mor and Dex+Maro+Mor showed a significant decreases ($p < 0.0001$) in heart rate from their baselines and compared to other treatment groups 1-2 min after administration of dexmedetomidine and remained stable at the decreased level until recovery from anaesthesia. All other physiological parameters were within expected values for anaesthetised dogs.

6.4.5 Correlation of EEG frequencies with plasma concentration of drugs:

There were no correlations of the changes in EEG frequencies and plasma concentrations of the drugs overall in any of the treatment groups.

Table 6.1 Differences of Least Squares Means and standard error of the EEG frequencies (F50, F95 and Ptot) before and after electric stimulation in different treatment groups.

Treatment groups: 1) **Mor**: morphine 0.6 mg/kg; 2) **Dex+Mor**: morphine 0.3 mg/kg + dexmedetomidine 5 μg/kg; 3) **Maro+Mor**: morphine 0.3 mg/kg + maropitant 1 mg/kg; 4) **Dex+Maro+Mor**: morphine 0.2 mg/kg + dexmedetomidine 3 μg/kg + maropitant 0.7 mg/kg

Treatment group	Difference	Standard error	*p*-value
F50			
Dex+Maro	-0.25	1.08	0.82
Dex+Maro+Mor	-1.63	1.08	0.14
Maro+Mor	-2.44	1.08	0.03
Mor	-2.81	1.15	0.02
F95			
Dex+Maro	-0.03	0.35	0.94
Dex+Maro+Mor	-0.46	0.35	0.20
Maro+Mor	-0.11	0.35	0.75
Mor	-0.41	0.37	0.28
Ptot			
Dex+Maro	0.04	0.16	0.81
Dex+Maro+Mor	0.02	0.16	0.86
Maro+Mor	0.03	0.16	0.86

Mor	0.12	0.18	0.51

Table 6.2 Differences of Least Squares Means and standard errors of the differences of EEG frequencies (F50, F95 and Ptot) between different treatment groups after electric stimulation.

Treatment groups: 1) **Mor**: morphine 0.6 mg/kg; 2) **Dex+Mor**: morphine 0.3 mg/kg + dexmedetomidine 5 μg/kg; 3) **Maro+Mor**: morphine 0.3 mg/kg + maropitant 1 mg/kg; 4) **Dex+Maro+Mor**: morphine 0.2 mg/kg + dexmedetomidine 3 μg/kg + maropitant 0.7 mg/kg

Treatment group	Difference	Standard error	*p*-value
<u>F50</u>			
Dex+Mor *vs* Maro+Mor	-0.06	0.01	<.0001
Dex+Mor *vs* Dex+Maro+Mor	-0.06	0.01	<.0001
Dex+Mor *vs* Mor	-0.07	0.01	<.0001
Dex+Maro+Mor *vs* Mor	-0.01	0.01	0.26
Maro+Mor *vs*			
Dex+Maro+Mor	0.01	0.01	0.62
Maro+Mor *vs* Mor	-0.01	0.01	0.47
<u>F95</u>			
Dex+Mor *vs* Maro+Mor	-0.01	0.00	0.02
Dex+Mor *vs* Dex+Maro+Mor	-0.01	0.00	0.01
Dex+Mor *vs* Mor	-0.01	0.00	<.001
Dex+Maro+Mor *vs* Mor	0.00	0.00	0.36
Maro+Mor *vs*			
Dex+Maro+Mor	0.00	0.00	0.64
Maro+Mor *vs* Mor	0.00	0.00	0.16
<u>Ptot</u>			
Dex+Mor *vs* Maro+Mor	0.01	0.01	0.52
Dex+Mor *vs* Dex+Maro+Mor	0.00	0.01	0.71
Dex+Mor *vs* Mor	0.06	0.01	<.0001
Dex+Maro+Mor *vs* Mor	0.06	0.01	<.0001

Maro+Mor *vs*			
Dex+Maro+Mor	0.00	0.01	0.79
Maro+Mor *vs* Mor	0.05	0.01	<.0001

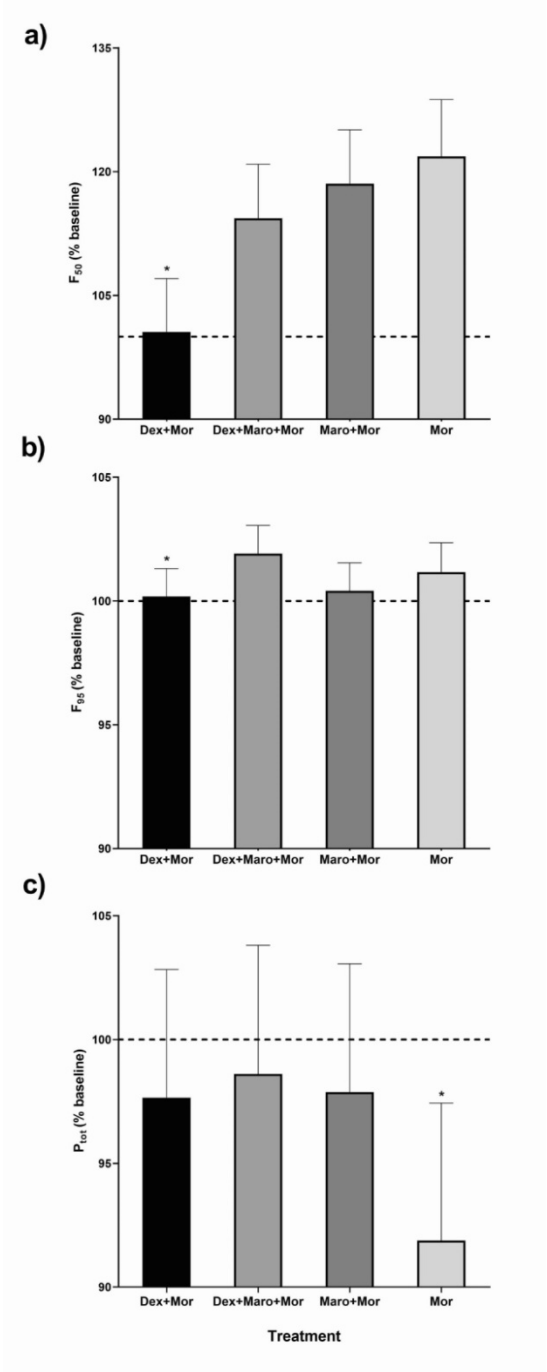

Figure 6.1 Least Squares means ± standard errors of the means for post-stimulation a) F50, b) F95 and c) Ptot expressed as percent of baseline values.

*Significantly different from all other treatment groups

Treatment groups: 1) **Mor**: morphine 0.6 mg/kg; 2) **Dex+Mor**: morphine 0.3 mg/kg + dexmedetomidine 5 µg/kg; 3) **Maro+Mor**: morphine 0.3 mg/kg + maropitant 1 mg/kg; 4) **Dex+Maro+Mor**: morphine 0.2 mg/kg + dexmedetomidine 3 µg/kg + maropitant 0.7 mg/kg

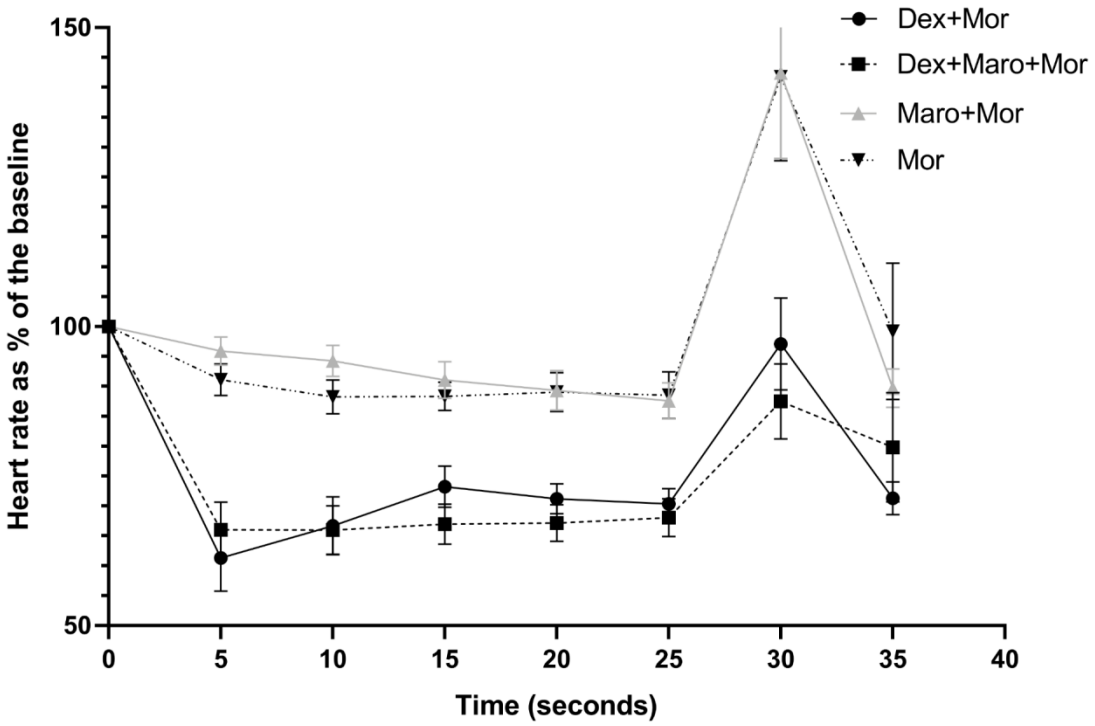

Figure 6.2 Least Squares means ± standard errors of the means for the heart rate (beats per min) expressed as percent of baseline values. Baseline was obtained 5 min before administration of drugs (T0).

Electrical stimulus applied 30 min after adminstration of drugs (T30).

Treatment groups: 1) **Mor**: morphine 0.6 mg/kg; 2) **Dex+Mor**: morphine 0.3 mg/kg + dexmedetomidine 5 µg/kg; 3) **Maro+Mor**: morphine 0.3 mg/kg + maropitant 1 mg/kg; 4) **Dex+Maro+Mor**: morphine 0.2 mg/kg + dexmedetomidine 3 µg/kg + maropitant 0.7 mg/kg.

6.5 Discussion

The objective of this study was to evaluate and compare the changes in EEG indices of nociception in response to noxious electrical stimulation in dogs anaesthetised with halothane and treated with different combinations of morphine with dexmedetomidine and maropitant. Changes in the EEG frequency variables in response to acute noxious stimulation have been previously used to evaluate and compare analgesic regimens and techniques in animals (Johnson, Wilson, Woodbury, & Caulkett, 2005; Kells, Beausoleil, Sutherland, Morrison, & Johnson, 2017; Kongara et al., 2010). A response to nociception is characterized by a significant increase in F50 as shown in a variety of animals (Gibson, Johnson, Stafford, Mitchinson, & Mellor, 2007; Johnson, Wilson, Woodbury, & Caulkett, 2005; Murrell et al., 2003; Sylvester, Johnson, Stafford, Ward, & Mellor, 2002) including dogs (Kaka et al., 2015; Kongara, McIlhone, Kells, & Johnson, 2014). Administration of effective analgesia prior to a noxious stimulus can attenuate or abolish such response.

In the present study, groups Dex+Mor and Dex+Maro+Mor abolished the rise in F50 after noxious stimulation which suggests that these combinations were effective in preventing the afferent flow of noxious stimulus to the brain after an acute noxious stimulus. After the noxious stimulation, the group Dex+Mor demonstrated a significantly lower change in F50 compared to all other treatment groups. Similarly, the group Dex+Maro+Mor also showed a significantly lower change in F50 compared to all other treatment groups except Dex+Mor and Mor. The differences between the pre and post stimulation LS means of F50 were lower in Dex+Mor compared to Dex+Maro+Mor which indicates that the antinociceptive efficacy of the combination increases with the increase in the dose of dexmedetomidine.

There was no statistically significant change in the magnitude of F95 and Ptot after noxious stimulation unlike that of F50 in any of the treatment groups. Previous studies have shown that increase in F50 is accompanied by an increase in F95 and a decrease in Ptot following a noxious stimulation (Murrell et al. 2003). However, F95 and ptot are not as sensitive indicator as F50 to detect nociception and the activity of antinociceptive drugs (Murrell et al., 2003; Murrell, Mitchinson, Waters, & Johnson, 2007). Kongara *et al.* (Kongara et al., 2010) who compared the antinociceptive effects of morphine, tramadol or parecoxib in dogs under similar study design also showed that the differences in F50 between the treatment groups did not correlate with changes in F95 and Ptot. The changes in F95 are more associated with the CNS depression (Johnson, Bloomfield, & Taylor, 2004). Similarly, the changes in Ptot may represent a different component of nociception than F50; changes in Ptot may reflect a decrease in the adequacy of anaesthesia whereas F50 reflect the noxious component of the stimulus (Murrell et al., 2003; Traast & Kalkman, 1995).

The results of the current study are in agreement with our previous clinical study (Chapter 5) evaluating similar treatment groups in ovariohysterectomised dogs using Glasgow and VAS pain scoring systems. It was shown that a combination of morphine (0.3 mg/kg) and dexmedetomidine had significantly lower pain scores compared to morphine alone at higher doses (0.6 mg/kg). Based on these reports, it is likely that there exists a supra-additive interaction in antinociceptive effect between co-administered morphine and dexmedetomidine. The mechanism of synergistic interaction between opioids and alpha-2-adrenoceptor agonists is not completely understood but the involvement of several spinal and supraspinal sites have been postulated. Both opioids and alpha-2-adrenoceptor agonists elicit membrane hyperpolarization by opening the

same population of potassium channels through G-protein coupled mechanisms resulting in inhibition of neurotransmitter release (Miyake, Christie, & North, 1989). Also, both opioid and alpha-2-adrenoceptors are present within the same superficial layer of the spinal cord inhibiting C fibre input (Sullivan, Dashwood, & Dickenson, 1987). In addition to the action on the spinal cord, the synergy between the two classes of analgesics in producing antinociception is also possible via activation of descending modulatory pathways from periaqueductal gray in the midbrain and ventral medullary sites (Ossipov et al., 1990).

The cardiovascular effects of dexmedetomidine seen in this study agree with the cardiovascular response described for this drug (Gertler, Brown, Mitchell, & Silvius, 2001; Murrell & Hellebrekers, 2005). Initially, there is an increase in the blood pressure and a reflex decrease in heart rate due to increased vagal tone associated with activation of post-synaptic alpha-2-receptors in peripheral vascular smooth muscle. The increase in vagal tone can also lead to a period of sinus arrest, second-degree atrioventricular block and arrhythmias. This is followed by a fall in blood pressure due to the decline of the vasoconstrictive effect and decrease in sympathetic tone leading to prolonged decreased but stabilized heart rate. Arrhythmias and second-degree atrioventricular block during anaesthesia may not be concerning if blood pressure is adequate (Hall et al., 2014). The dogs used in this study were screened for cardiovascular disease based on the clinical examination and history and the results of cardiovascular effects cannot be extrapolated for dogs with cardiovascular diseases.

This study was performed in conjunction with a pharmacokinetics study of the drugs which has been published separately (Karna, Singh, Chambers, & Kongara, 2019). No correlation of plasma concentration of any of the three drugs with the percentage change

in F50, F95 or Ptot was observed. Previous human studies have also shown little to no correlation between plasma concentration of morphine and its pharmacodynamic variables (Berkowitz, 1976; Faura, Moore, Horga, Hand, & McQuay, 1996). Further, the data in this study were complicated because three different drugs were used at different doses. An ideal way to establish the relationship between plasma concentration and its effects is the pharmacokinetic-pharmacodynamic (PK-PD) modelling which can also be used to determine the minimum effective dose of the drugs to produce optimum antinociceptive effects (Derendorf & Meibohm, 1999). PK-PD modelling requires multiple simultaneous PK and PD data points. Since PD data (change in EEG variables in response to a noxious stimulus) was available at only one time point, this analysis couldn't be performed.

The dose rates of the drugs were based on their clinical use in dogs and also based on experience from our previous clinical study. No other studies have been conducted to test the combination of the drugs used. We tend to use the lower doses of the drugs so that higher doses of any drug do not interfere with the interaction. Acepromazine was chosen as premedication because it has no analgesic effect (Thurmon, Tranquili, & Benson, 1996) and thus would not interfere with comparing the analgesic efficacy of the test drugs. Halothane is used as the inhalant anaesthetic of choice in the minimum anaesthesia model because it causes significantly less cortical activity depression compared to newer agents such as isoflurane, sevoflurane and desflurane at equivalent multiples of MAC (Murrell, Waters, & Johnson, 2008). Propofol, which was used as anaesthetic induction agent in this study is associated with EEG burst suppression and unresponsiveness to noxious stimulation (Lichtner, 2018). However, it has a short duration of action (Fulton & Sorkin, 1995) and the noxious stimulus in this study was

applied at 30 minutes after induction of anaesthesia. Therefore, propofol would have had minimal effects on the EEG activity.

It is important to note that the baseline (pre-stimulus) values of F50 and F95 were lower in Dex+Mor and Dex+Maro+ Mor compared to other treatment groups. The end-tidal halothane tension was tightly controlled in the pre and post-treatment period. The lower baselines in the two groups could be because the combination of dexmedetomidine and morphine have caused more general central nervous system depression (Johnson, Bloomfield, & Taylor, 2004). The baselines of the F50 and F95 were included as covariates in the model to account for their differences between the treatment groups.

In conclusion, this study showed that a combination of morphine with dexmedetomidine was more potent in controlling the changes in EEG indices of nociception following noxious electrical stimulation in dogs under minimal halothane anaesthesia. This indicates that a supra-additive interaction can occur between these two drugs which could be utilised in clinical settings to reduce the doses and enhance the analgesic effects of the individual drugs.

6.6 References

Barnhart, M. D., Hubbell, J. A., Muir, W. W., Sams, R. A., & Bednarski, R. M. (2000). Pharmacokinetics, pharmacodynamics, and analgesic effects of morphine after rectal, intramuscular, and intravenous administration in dogs. American Journal of Veterinary Research, 61(1), 24-28. https://doi.org/10.2460/ajvr.2000.61.24

Berkowitz, B. A. (1976). The relationship of pharmacokinetics to pharmacological activity: morphine, methadone and naloxone. Clinical Pharmacokinetics, 1(3), 219-188. https://doi.org/10.2165/00003088-197601030-00004

Boscan, P., Monnet, E., Mama, K., Twedt, D. C., Congdon, J., & Steffey, E. P. (2011). Effect of maropitant, a neurokinin 1 receptor antagonist, on anesthetic

requirements during noxious visceral stimulation of the ovary in dogs. American Journal of Veterinary Research, 72(12), 1576-1579. https://doi.org/10.2460/ajvr.72.12.1576

Chen, A. C., Dworkin, S. F., Haug, J., & Gehrig, J. (1989). Topographic brain measures of human pain and pain responsivity. Pain, 37(2), 129-141. https://doi.org/10.1016/0304-3959(89)90125-5

Clarke, K., Trim, C., & Hall, L. (2014). Anaesthesia of the dog. In Veterinary Anaesthesia (11th ed., pp. 405-498): Oxford: WB Saunders.

De la Puente-Redondo, V., Siedek, E., Benchaoui, H., Tilt, N., Rowan, T., & Clemence, R. (2007). The anti-emetic efficacy of maropitant (Cerenia™) in the treatment of ongoing emesis caused by a wide range of underlying clinical aetiologies in canine patients in Europe. Journal of Small Animal Practice, 48(2), 93-98. https://doi.org/10.1111/j.1748-5827.2006.00321.x

Derendorf, H., & Meibohm, B. (1999). Modeling of pharmacokinetic/pharmacodynamic (PK/PD) relationships: concepts and perspectives. Pharmaceutical Research, 16(2), 176-185. https://doi.org/10.1023/A:1011907920641

Diemunsch, P., & Grélot, L. (2000). Potential of substance P antagonists as antiemetics. Drugs, 60(3), 533-546. https://doi.org/10.2165/00003495-200060030-00002

Ebner, L. S., Lerche, P., Bednarski, R. M., & Hubbell, J. A. (2013). Effect of dexmedetomidine, morphine-lidocaine-ketamine, and dexmedetomidine-morphine-lidocaine-ketamine constant rate infusions on the minimum alveolar concentration of isoflurane and bispectral index in dogs. American Journal of Veterinary Research, 74(7), 963-970. https://doi.org/10.2460/ajvr.74.7.963

Faura, C. C., Moore, R. A., Horga, J., Hand, C. W., & McQuay, H. J. (1996). Morphine and morphine-6-glucuronide plasma concentrations and effect in cancer pain. Journal of Pain and Symptom Management, 11(2), 95-102. https://doi.org/10.1016/0885-3924(95)00148-4

Fulton, B., & Sorkin, E. M. (1995). Propofol. Drugs, 50(4), 636-457. https://doi.org /10.2165/00003495-199550040-00006

Gertler, R., Brown, H. C., Mitchell, D. H., & Silvius, E. N. (2001). Dexmedetomidine: a novel sedative-analgesic agent. Paper presented at the Baylor university Medical Center Proceedings

Gibson, T., Johnson, C., Stafford, K., Mitchinson, S., & Mellor, D. (2007). Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning. New Zealand Veterinary Journal, 55(4), 152-157. https://doi.org/10.1080/00480169.2007.36760

Hall, L., Clarke, K., & Trim, C. (2014). Analgesia. In Veterinary Anaesthesia (pp. 112): Saunders Elsevier Limited.

Holton, L. L., Scott, E., Nolan, A. M., Reid, J., Welsh, E., & Flaherty, D. (1998). Comparison of three methods used for assessment of pain in dogs. Journal of the American Veterinary Medical Association, 212(1), 61-66.

Johnson, C. B., Bloomfield, M., & Taylor, P. M. (2004). Effects of ketamine on the equine electroencephalogram during anesthesia with halothane in oxygen. Veterinary Surgery, 28(5), 380-385. https://doi.org/10.1111/j.1532-950X.1999.00380.x

Johnson, C. B., Wilson, P. R., Woodbury, M. R., & Caulkett, N. A. (2005). Comparison of analgesic techniques for antler removal in halothane-anaesthetized red deer (Cervus elaphus): electroencephalographic responses. Veterinary Anaesthesia and Analgesia, 32(2), 61-71. https://doi.org/10.1111/j.1467-2995.2005.00228.x

Kaka, U., Hui Cheng, C., Meng, G. Y., Fakurazi, S., Kaka, A., Behan, A. A., & Ebrahimi, M. (2015). Electroencephalographic changes associated with antinociceptive actions of lidocaine, ketamine, meloxicam, and morphine administration in minimally anaesthetized dogs. BioMed Research International, 2015. https://doi.org/10.1155/2015/305367

Karna, S. R., Kongara, K., Singh, P., Chambers, P., & Lopez-Villalobos, N. (2019). Evaluation of analgesic interaction between morphine, dexmedetomidine and maropitant using hot-plate and tail-flick tests in rats. Veterinary Anaesthesia and Analgesia. https://doi.org/10.1016/j.vaa.2018.12.009

Karna, S. R., Singh, P., Chambers, P., & Kongara, K. (2019). Pharmacokinetics of morphine in combination with dexmedetomidine and maropitant following intramuscular injection in dogs anaesthetized with halothane. Journal of Veterinary Pharmacology and Therapeutics, 2019;00:1–9. https://doi.org/10.1111/jvp.12831

Kells, N. J., Beausoleil, N. J., Sutherland, M. A., Morrison, R. M., & Johnson, C. B. (2017). Electroencephalographic assessment of oral meloxicam, topical anaesthetic cream and cautery iron for mitigating acute pain in pigs (Sus scrofa) undergoing tail docking. Veterinary Anaesthesia and Analgesia, 44(5), 1166-1174. https://doi.org/10.1016/j.vaa.2017.02.004

Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biology, 8(6), e1000412. https://doi.org/10.1371/journal.pbio.1000412

Kongara, K., Chambers, J. P., & Johnson, C. B. (2010). Electroencephalographic responses of tramadol, parecoxib and morphine to acute noxious electrical stimulation in anaesthetised dogs. Research in Veterinary Science, 88(1), 127-133. https://doi.org/10.1016/j.rvsc.2009.05.012

Kongara, K., Chambers, J. P., & Johnson, C. B. (2012). Effects of tramadol, morphine or their combination in dogs undergoing ovariohysterectomy on peri-operative electroencephalographic responses and post-operative pain. New Zealand Veterinary Journal, 60(2), 129-135. https://doi.org/10.1080/00480169.2011.641156

Kongara, K., McIlhone, A. E., Kells, N. J., & Johnson, C. B. (2014). Electroencephalographic evaluation of decapitation of the anaesthetized rat. Laboratory Animals, 48(1), 15-19. https://doi.org/10.1177/0023677213502016

Lemke, K. A. (2004). Perioperative use of selective alpha-2 agonists and antagonists in small animals. The Canadian Veterinary Journal, 45(6), 475.

Lichtner, G., Auksztulewicz, R., Kirilina, E., Velten, H., Mavrodis, D., Scheel, M., Blankenburg, F. and von Dincklage, F., (2018). Effects of propofol anesthesia on the processing of noxious stimuli in the spinal cord and the brain. Neuroimage, 172, 642-653. https://doi.org/10.1016/j.neuroimage.2018.02.003

Maiante, A., Teixeira Neto, F. J., Beier, S., Corrente, J., & Pedroso, C. (2009). Comparison of the cardio-respiratory effects of methadone and morphine in conscious dogs. Journal of Veterinary Pharmacology and Therapeutics, 32(4), 317-328. https://doi.org/10.1111/j.1365-2885.2008.01042.x

Miyake, M., Christie, M. J., & North, R. A. (1989). Single potassium channels opened by opioids in rat locus ceruleus neurons. Proceedings of the National Academy of Sciences, 86(9), 3419-3422.

Murrell, J. C., & Hellebrekers, L. J. (2005). Medetomidine and dexmedetomidine: a review of cardiovascular effects and antinociceptive properties in the dog. Veterinary Anaesthesia and Analgesia, 32(3), 117-127. https://doi.org/10.1111/j.1467-2995.2005.00233.x

Murrell, J. C., & Johnson, C. B. (2006). Neurophysiological techniques to assess pain in animals. Journal of Veterinary Pharmacology and Therapeutics, 29(5), 325-335. https://doi.org/10.1111/j.1365-2885.2006.00758.x

Murrell, J. C., Johnson, C. B., White, K. L., Taylor, P. M., Haberham, Z. L., & Waterman–Pearson, A. E. (2003). Changes in the EEG during castration in horses and ponies anaesthetized with halothane. Veterinary Anaesthesia and Analgesia, 30(3), 138-146. https://doi.org/10.1046/j.1467-2995.2003.00138.x

Murrell, J. C., Mitchinson, S. L., Waters, D., & Johnson, C. B. (2007). Comparative effect of thermal, mechanical, and electrical noxious stimuli on the electroencephalogram of the rat. British Journal of Anaesthesia, 98(3), 366-371. https://doi.org/10.1093/bja/ael377

Niyom, S., Boscan, P., Twedt, D. C., Monnet, E., & Eickhoff, J. C. (2013). Effect of maropitant, a neurokinin-1 receptor antagonist, on the minimum alveolar concentration of sevoflurane during stimulation of the ovarian ligament in cats. Veterinary Anaesthesia and Analgesia, 40(4), 425-431. https://doi.org/10.1111/vaa.12017

Ossipov, M. H., Harris, S., Lloyd, P., Messineo, E., Lin, B., & Bagley, J. (1990). Antinociceptive interaction between opioids and medetomidine: systemic additivity and spinal synergy. Anesthesiology, 73(6), 1227-1235. https://doi.org/10.1097/00000542-199012000-00022

Singh, P., Kongara, K., Harding, D., Ward, N., Dukkipati, V. S. R., Johnson, C., & Chambers, P. (2018). Comparison of electroencephalographic changes in response to acute electrical and thermal stimuli with the tail flick and hot plate test in rats administered with opiorphin. BMC Neurology, 18(1), 43. https://doi.org/10.1186/s12883-018-1047-y

Sullivan, A. F., Dashwood, M. R., & Dickenson, A. H. (1987). α2-Adrenoceptor modulation of nociception in rat spinal cord: location, effects and interactions with morphine. European Journal of Pharmacology, 138(2), 169-177. https://doi.org/10.1016/0014-2999(87)90430-4

Sylvester, S. P., Johnson, C. B., Stafford, K. J., Ward, R. N., & Mellor, D. J. (2002). Effects of age on pain sensation following castration in lambs. Paper presented at The Physiological Society of New Zealand.

Thurmon, J. C., Tranquili, W., & Benson, G. (1996). Preanestheticsand anesthetics adjuncts. In W. Tranquili & G. Benson (Eds.), Veterinary Anesthesia (3rd ed., pp. 183-189). Baltimore, USA: Williams & Wilkins.

Traast, H. S., & Kalkman, C. J. (1995). Electroencephalographic characteristics of emergence from propofol/sufentanil total intravenous anesthesia. Anesthesia & Analgesia, 81(2), 366-371. https://doi.org/10.1097/00000539-199508000-00027

Vainio, O. (1989). Introduction to the clinical pharmacology of medetomidine. Acta Veterinaria Scandinavica. Supplementum, 85, 85-88.

Valverde, A., Dyson, D. H., & McDonell, W. N. (1989). Epidural morphine reduces halothane MAC in the dog. Canadian Journal of Anaesthesia, 36(6), 629-632.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
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We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

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Primary Supervisor's Signature:	Kavitha Kongara
Date:	16/03/2020

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CHAPTER 7 GENERAL DISCUSSION

7.1 Major findings, limitations and future directions

This project evaluated and compared the efficacy of combinations of morphine, dexmedetomidine and maropitant against a variety of nociceptive stimuli with the goal of using them in dogs undergoing routine elective surgery or other surgical procedures.

This dissertation presents four experiments:

a) a pilot study to evaluate analgesic interaction between morphine, dexmedetomidine and maropitant in rats,

b) studies comparing the pharmacokinetics (PK) of the test drug combinations in dogs under anaesthesia,

c) a clinical study to compare the efficacy of the test drug combination in dogs undergoing ovariohysterectomy (OHE), and

d) a study to compare efficacy of the test drug combinations in preventing the changes in electroencephalographic (EEG) indices of nociception subjected to a noxious electrical stimulus in anaesthetised dogs.

7.1.1 Pilot study in rats

There are no previous studies evaluating the analgesic interaction between all the three drugs tested in this study. A pilot study (**Chapter 3**) was performed in rats to compare the analgesic efficacy of different dose combinations of the test drugs using hot-plate (HP) and tail-flick (TF) tests in rats. Eight treatment groups were used which consisted of the treatment groups with each drug used singly at the full dose, combinations of two

drugs at the half of the full dose and a combination of three drugs at one-third of the full dose.

The advantage of HP and TF tests is that they are simple to execute and are sensitive experimental models of nociception based on response thresholds to acute thermal stimuli (D'Amour and Smith 1941; Eddy and Leimbach 1953). The results showed that the combination of morphine and maropitant provided potent antinociceptive effect superior to the effect produced by the drugs when used singly or in other combinations. This supra-additive effect for analgesia between morphine and maropitant was demonstrated by TF but not the HP test. TF is based on spinally mediated response whereas HP test involves supraspinal pathways, which could be the potential explanation for the differences in results from the two tests.

A limitation of this study was that the experiment was not designed to prove the synergy between any drug combinations. Rather the study aimed to achieve some indicative results to test if any analgesic interactions between the drugs exist. An isobolographic analysis comprising different dose ratios of the drugs to determine the best drug ratios for the optimal synergistic interaction is recommended. Another limitation of this study was that the thermal pain model used in this study cannot be equated with the clinical pain and thus the isobolographic analysis in models mimicking the clinical pain is recommended.

In conclusion, this study provided the evidence that there is a supra-additive interaction between morphine and maropitant which encouraged me to test the combinations of the drugs in clinical and experimental EEG studies in dogs.

Results of the pilot study in rats have also led me to narrow the eight treatment groups down to four treatment groups for the subsequent dog studies.

7.1.2 Pharmacokinetic studies

Two pharmacokinetic studies were performed, one in conjunction with the EEG study (lab PK study, **Chapter 4**) and another in the dogs undergoing OHE (clinical PK study, **Chapter 5**). A common method to detect plasma drug concentrations was developed and validated, and used for analysing drug concentrations in test samples. The aim of conducting the PK studies was to describe the pharmacokinetic profile of the drugs at different doses and combinations. With the combinations of drugs, variation in analgesic efficacy could be attributed to synergy or antagonism, or PK interactions. The PK study would also help to determine if there was any alteration in the pharmacokinetics of co-administered drugs, which could influence their analgesic efficacy.

7.1.2.1 PK method development

A simple and inexpensive method to determine the concentration of morphine, dexmedetomidine and maropitant in blood plasma of dogs using the liquid chromatography-mass spectrometry (LCMS) technique was developed in this study. The precision and accuracy observed in our method were similar to previously published studies for morphine (Dohoo *et al.* 1994), dexmedetomidine (Cui *et al.* 2018) and maropitant (Berryhill *et al.* 2019). Internal standards were not used in this study. Internal standards have the advantage of correcting the precision issues arising due to

variation in the injection volumes. However, the precision was within the acceptable limits and thus internal standards would not have provided an extra advantage.

7.1.2.2 Two pharmacokinetic studies

There were some differences in the design and results between the laboratory PK (**chapter 4**) and the clinical PK (**chapter 5**) studies. First, there were eight blood collection time-points in the lab PK study but the clinical PK study involved only four blood collection points. Also, significant differences in the pharmacokinetic parameters were observed between the two studies. In the clinical PK study, the dogs were anaesthetised with isoflurane, were in the deeper plane of anaesthesia for a longer duration of time and underwent surgical manipulation. However, the dogs in the lab PK study were minimally anaesthetised with halothane for a shorter duration of anaesthesia and received a brief electrical stimulus (for 2 sec). Also, the clinical PK study involved diverse groups in terms of subjects, breed and age compared to the lab PK study which involved only two breeds of dogs in a crossover study design. Disposition of drugs after IM injection depends on multiple factors such as perfusion at the site of injection and physiological state of the animal (Zuidema *et al.* 1988) which could be influenced by the depth and duration of anaesthesia (Nimmo and Peacock 1988; Sinclair and Dyson 2012). Thus, the differences in experimental conditions and physiological state of animals in these two studies could have led to the differences in the pharmacological parameters.

The most remarkable finding in the lab PK study (chapter 4) was that the elimination half-life of morphine in combination with dexmedetomidine was longer and the clearance rate was lower compared to its combination with maropitant, or morphine

when administered singly at higher doses. This could be a contributing factor for lower pain scores in the dogs receiving the combination of dexmedetomidine and morphine in the clinical study. This may also have a clinical advantage of prolonging the dosing interval of morphine.

There are some limitations in the PK studies, that arose primarily due to logistic reasons and financial constraints. The maximum plasma concentration (C_{max}) of the drugs was observed at 15 min in majority of animals which was the first blood collection time. It is possible that the actual C_{max} was achieved before 15 min. Thus, future studies should include the blood collection point before 15 min. Further, the blood sampling could not be performed for longer than two hours in the clinical PK study and eight hours for the lab PK study. The PK data were not available at the time of pain scoring in the clinical study which would have allowed us to investigate any correlation between plasma concentration of the drugs and the pain scores. Therefore, future studies with earlier sampling points, longer sampling duration, and sampling during pain scoring in clinical studies are also recommended.

This study for the first time investigated the pharmacokinetics of the combination of morphine, dexmedetomidine and maropitant under anaesthesia in dogs. It was also shown that the drug combinations are safe for use in dogs. The pharmacokinetics data obtained from this study will help in determining dose rates and regimen of the drugs combinations for their use in the clinical practice. Further studies investigating the pharmacokinetics of the drugs at other dose ratios and involving control groups with only dexmedetomidine and/or maropitant are recommended.

7.1.3 Clinical Study

Chapter 5 compared the analgesic efficacy of combinations of morphine, dexmedetomidine and maropitant in dogs undergoing ovariohysterectomy (OHE). OHE was chosen because it is a common and clinically available model of pain in dogs. In addition to the somatic pain, OHE also involves the visceral pain arising from the manipulation of abdominal visceral organs and stretching of the suspensory ligaments (Guerrero *et al.* 2016). Previous studies have shown high expression of the neurokinin (NK)-1 receptor in the neural pathways involved in visceral pain (Perry and Lawson 1998; Laird *et al.* 2000). One of the mechanisms by which the synergy between the three test drugs was expected was via their effect in antagonising the NK-1 receptor in pathways carrying visceral nociceptive signals.

In contrast to the pilot study (in rats) which showed a supra-additive interaction between morphine and maropitant in the tail flick test, this study showed that the lower dose of morphine (0.3 mg/kg) when combined with dexmedetomidine can provide superior post-operative analgesia for up to 9 hours compared to morphine alone at higher doses (0.6 mg/kg). The potential mechanism for the explanation of this result and the disagreement with the results of our rat studies have been discussed in Chapter 5. The supra-additive interaction between morphine and dexmedetomidine observed in this study has a clinical advantage as enhanced analgesic effects can be achieved by using lower dose of morphine in combination with dexmedetomidine leading to lower potential morphine associated adverse effects.

OHE is a complex pain model involving acute somatic and visceral pain (Fitzpatrick *et al.* 2010; Muir *et al*. 2002). The results of analgesic interaction of drugs from this OHE

study may not be directly applicable to other clinical situations involving chronic, inflammatory and neuropathic pain. Further, the analgesic effects of the test drugs only up to 9 hours from the extubation were analysed in this study. Therefore, further investigations using different animal models of clinical pain and studies assessing the analgesic effects of the drug combination for longer duration are recommended.

This study had several limitations. The study did not include control groups using morphine alone at lower doses (0.3 and 0.2 mg/kg) and dexmedetomidine or maropitant used singly. There was no negative control group (without any analgesic treatment) in the study due to the ethical concerns. Further, the doses of the drugs were chosen based on their usage in clinical and experimental studies in animals. Given that the pain response in any animal is a compromise between noxious stimulus intensity and the dose of analgesic, the findings of the study only apply to the doses of the drugs used in this study. Because the outcome of the interaction between the drugs may vary according to the dose ratio of the drugs (Ossipov *et al.* 1990; Woode *et al.* 2015), studies involving the drug combinations at different doses and ratios are recommended. A wide variation in the breed and age of the dogs could also have resulted in a larger variation in the pain scores. Therefore, it is recommended to test the combination of these drugs in a larger study to account for the variation between the subjects.

All dogs in that received dexmedetomidine showed a significant decrease in the heart rate and second-degree atrioventricular block (AV block). Arrhythmias and second-degree atrioventricular block during anaesthesia may not be concerning if blood pressure is adequate (Hall *et al.* 2014). It is also important to note that all dogs used in this study were fit and healthy (American Society of Anaesthesiologists 1 category) and

at low risk of cardiovascular diseases. Thus, the results of cardiovascular effects cannot be extrapolated for dogs with cardiovascular diseases and caution should be used when using dexmedetomidine in such patients.

7.1.4 EEG study

The study in Chapter 6 was conducted to test the findings of the OHE study by using an alternative and objective technique. The efficacy of the drug combinations which were tested in the OHE study was evaluated based on reducing the changes in EEG indices of nociception (F50, F95 and Ptot) in the dogs subjected to a noxious electrical stimulus using a minimal anaesthesia model.

The results showed that the combination of dexmedetomidine and morphine showed no significant increase in F50 after noxious stimulation. Also, this group had significantly lower change in F50 in response to the noxious stimulus compared to all other treatment groups. These results were consistent with the previous studies which showed that changes in F50 is a sensitive indicator of nociception in a variety of animal species (Sylvester *et al.* 2002; Murrell *et al.* 2003; Johnson *et al.* 2005; Gibson *et al.* 2007; Kongara *et al.* 2010; Kaka *et al.* 2015). Also, the finding of the EEG study was in agreement with the OHE study and supports that that the combination of dexmedetomidine and morphine is the most potent analgesic combination among all the combinations of the drugs tested.

The EEG study also involved four treatment groups with the same dose of morphine and maropitant as in the OHE but a lower dose of dexmedetomidine (5 and 3 μg/kg in EEG study contrary to 10 and 7 μg/kg in OHE study). This was done because of a concern that the dose of the dexmedetomidine would be too high and might cause serious bradycardia in the EEG study contrary to the clinical study, which involved more sympathetic stimulation during patient preparation and surgery, leading to increase in the heart rate (Bantel and Trapp 2011). Therefore, the dose rates of dexmedetomidine in clinical and EEG studies were different.

A limitation of EEG study is that the brief noxious stimuli used in this study cannot be equated to clinical pain. Therefore, making assessments about the analgesic drugs based solely on the EEG model using phasic noxious stimuli may not be appropriate. Further, the antinociceptive effect of the test drug combinations were assessed only at 30 minutes after the administration. Thus, studies investigating the antinociceptive effects of the drug combination for longer duration after their administration is recommended.

This study was conducted together with the pharmacokinetic study (chapter 4). It would be very beneficial to perform a PK/PD modelling study which would require simultaneous EEG (PD) and PK data for multiple time points. Such study would link the change in the concentration of drugs over time to the intensity of the observed antinociceptive response. However, the change in EEG in response to the stimulus was only available for one-time point that did not allow us to perform a PK/PD modelling. Therefore, a PK/PD modelling study is highly recommended.

7.2 Conclusion:

The following conclusions can be drawn from the results of the research presented in this thesis:

- There is a supra-additive interaction in spinal analgesia between morphine and maropitant as shown by the tail flick test in rats.
- The combination of morphine and dexmedetomidine provided the most potent analgesic effect among the tested combinations of morphine, dexmedetomidine and maropitant in dogs undergoing OHE.
- Administration of dexmedetomidine at the dose as low as 3 μg/kg is associated with second-degree heart block and a significant decrease in the heart rate without clinically significant change in the blood pressure. The cardiovascular changes produced by dexmedetomidine may be clinically insignificant in fit and healthy dogs.
- Dexmedetomidine can increase the elimination half-life and decrease the clearance rate of co-administered morphine, which may have a clinical advantage of the increase in the dosing interval of morphine and a consequent decrease in the occurrence of adverse effects.
- Median frequency of the EEG is a sensitive indicator of nociception. The combination of morphine and dexmedetomidine was more potent compared to other treatment groups in controlling the changes in EEG indices of nociception following noxious electrical stimulation in dogs under minimal halothane anaesthesia.

7.3 References:

Bantel C, Trapp S. The role of the autonomic nervous system in acute surgical pain processing–what do we know? *Anaesthesia* 66, 541-4, 2011

Berryhill EH, Knych H, Edman JM, Magdesian KG. Pharmacokinetics of single doses of maropitant citrate in adult horses. *Journal of Veterinary Pharmacology and Therapeutics*, 2019

Cui W, Liu Q, Xiong S, Qiao L. LC-MS/MS method for simultaneous quantification of dexmedetomidine, dezocine, and midazolam in rat plasma and its application to their pharmacokinetic study. *Journal of Analytical Methods in Chemistry* 2018, 2018

D'Amour FL, Smith DL. A method for determining loss of pain sensation. *Journal of Pharmacology and Experimental Therapeutics* 72, 74-9, 1941

Dohoo S, Tasker RAR, Donald A. Pharmacokinetics of parenteral and oral sustained-release morphine sulphate in dogs. *Journal of Veterinary Pharmacology and Therapeutics* 17, 426-33, 1994

Eddy NB, Leimbach D. Synthetic analgesics. II. Dithienylbutenyl-and dithienylbutylamines. *Journal of Pharmacology and Experimental Therapeutics* 107, 385-93, 1953

Gibson T, Johnson C, Stafford K, Mitchinson S, Mellor D. Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning. *New Zealand Veterinary Journal* 55, 152-7, 2007

Guerrero KSK, Campagna I, Bruhl-Day R, Hegamin-Younger C, Guerrero TG. Intraperitoneal bupivacaine with or without incisional bupivacaine for postoperative analgesia in dogs undergoing ovariohysterectomy. *Veterinary Anaesthesia and Analgesia* 43, 571-8, 2016

Hall L, Clarke K, Trim C. Analgesia. In: Dethioux F (ed). *Veterinary Anaesthesia*. Pp 112. Elsevier Saunders, Oxford, United Kingdom, 2014

Holton LL, Scott E, Nolan AM, Reid J, Welsh E, Flaherty D. Comparison of three methods used for assessment of pain in dogs. *Journal of the American Veterinary Medical Association* 212, 61-6, 1998

Johnson CB, Wilson PR, Woodbury MR, Caulkett NA. Comparison of analgesic techniques for antler removal in halothane-anaesthetized red deer (Cervus elaphus): electroencephalographic responses. *Veterinary Anaesthesia and Analgesia* 32, 61-71, 2005

Kaka U, Hui Cheng C, Meng GY, Fakurazi S, Kaka A, Behan AA, Ebrahimi M. Electroencephalographic changes associated with antinociceptive actions of lidocaine, ketamine, meloxicam, and morphine administration in minimally anaesthetized dogs. *BioMed Research International* 2015, 2015

Kongara K, Chambers JP, Johnson CB. Electroencephalographic responses of tramadol, parecoxib and morphine to acute noxious electrical stimulation in anaesthetised dogs. *Research in Veterinary Science* 88, 127-33, 2010

Laird J, Olivar T, Roza C, De Felipe C, Hunt S, Cervero F. Deficits in visceral pain and hyperalgesia of mice with a disruption of the tachykinin NK1 receptor gene. *Neuroscience* 98, 345-52, 2000

McKune CM, Pascoe PJ, Lascelles BDX, Kass PH. The challenge of evaluating pain and a pre-incisional local anesthetic block. *PeerJ* 2, e341, 2014

Muir WW, Gaynor JS. Handbook of Veterinary Pain Management. Mosby: St. Louis, Missouri, 2002

Murrell JC, Johnson CB, White KL, Taylor PM, Haberham ZL, Waterman–Pearson AE. Changes in the EEG during castration in horses and ponies anaesthetized with halothane. *Veterinary Anaesthesia and Analgesia* 30, 138-46, 2003

Nimmo WS, Peacock JE. Effect of anaesthesia and surgery on pharmacokinetics and pharmacodvamics. *British Medical Bulletin* 44, 286-301, 1988

Ossipov MH, Harris S, Lloyd P, Messineo E, Lin B, Bagley J. Antinociceptive interaction between opioids and medetomidine: systemic additivity and spinal synergy. *Anesthesiology* 73, 1227-35, 1990

Perry M, Lawson S. Differences in expression of oligosaccharides, neuropeptides, carbonic anhydrase and neurofilament in rat primary afferent neurons retrogradely labelled via skin, muscle or visceral nerves. *Neuroscience* 85, 293-310, 1998

Sinclair MD, Dyson DH. The impact of acepromazine on the efficacy of crystalloid, dextran or ephedrine treatment in hypotensive dogs under isoflurane anesthesia. *Veterinary Anaesthesia and Analgesia* 39, 563-73, 2012

Sylvester S, Johnson C, Stafford K, Ward R, Mellor D. Effects of age on pain sensation following castration in lambs. *Proceedings of the New Zealand Physiolical Society*. Pp 72, 2002

Woode E, Ameyaw EO, Abotsi WKM, Boakye-Gyasi E. An isobolographic analysis of the antinociceptive effect of xylopic acid in combination with morphine or diclofenac. *Journal of Basic and Clinical Pharmacy* 6, 103, 2015

Zuidema J, Pieters F, Duchateau G. Release and absorption rate aspects of intramuscularly injected pharmaceuticals. *International Journal of Pharmaceutics* 47, 1-12, 1988