EFFICACY OF ARTICAINE HYDROCHLORIDE FOR DISBUDDING IN GOAT KIDS AND VELVET ANTLER REMOVAL IN RED DEER, AND NOVEL DISBUDDING METHODS FOR GOAT KIDS

A thesis presented in partial fulfilment of the requirements
for the degree of

Doctor of Philosophy
in
Veterinary Science

at Massey University, Palmerston North,
New Zealand

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2019

This thesis is dedicated to my late father

Venkatachalam Suprayan

ABSTRACT

Painful husbandry procedures are routinely performed in farm animals all over the world. Most of these procedures can be humanely performed under local anaesthesia. Lignocaine is the most commonly used local anaesthetic in veterinary medicine. Even though lignocaine is a cheap and effective local anaesthetic, its use in goat kids and deer has been a concern. In goat kids, lignocaine has been reported to produce toxicity following cornual nerve block. In deer, the presence of lignocaine residue in the harvested velvet antlers following ring block has been a concern as one of its metabolites, 2,6-dimethylaniline (DMA) has been classified as a possible carcinogen in humans.

Articaine hydrochloride is an amide-type local anaesthetic with unique pharmacological properties such as rapid hydrolysis in plasma to an inactive metabolite and high lipid solubility. It is widely used in humans for local and regional nerve blocks in dentistry. Several studies in humans suggested that articaine hydrochloride was effective and safer than lignocaine. Given concerns on the use of lignocaine in goat kids and deer, a series of studies were conducted to evaluate the safety and efficacy of articaine hydrochloride as an alternative to lignocaine hydrochloride for disbudding in goat kids and velvet antler removal in deer. As there is a paucity of data on the toxicity of lignocaine in goat kids, the thesis has also investigated the toxicity of lignocaine hydrochloride in goat kids. In addition, novel analgesic and disbudding techniques for goat kids were evaluated.

The dose-ranging studies in goat kids suggested that doses up to 8 mg kg^{-1} and 7 mg kg^{-1} of articaine hydrochloride and lignocaine hydrochloride, respectively, can be safely used for perineural injections. Pharmacokinetic studies demonstrated that articaine hydrochloride was rapidly hydrolysed and eliminated in goat kids. The elimination half-life of articaine (1.26 ± 0.34 hours) was determined to be shorter than the elimination half-lives of lignocaine (1.71 ± 0.51 hours) and lignocaine's metabolite, monoethylglycinexylidide (3.19 ± 1.21 hours) in goat kids. The total dose of articaine (16.24 ± 1.79 mg kg^{-1}) required to produce convulsions in goat kids was higher than that of lignocaine (12.31 ± 1.42 mg kg^{-1}). The mean convulsive plasma concentrations of articaine and lignocaine were 9.90 ± 2.38 µg mL^{-1} and 13.59 ± 2.34 µg mL^{-1}, respectively. Both pharmacokinetic and toxicity data indicate that articaine has a greater margin of safety than lignocaine in goat kids. Cornual nerve block (0.5 mL/site) using articaine hydrochloride (1.5%) and lignocaine hydrochloride (1%) alleviated the acute pain during disbudding in goat kids. However, both the drugs provided analgesia only for a short

time which necessitates the use of non-steroidal anti-inflammatory drugs (NSAIDs) for postoperative analgesia. In addition, the injection of these drugs at four sites to anaesthetise both the horn buds caused stress and pain in goat kids. Therefore, it is recommended to use sedatives and NSAIDs along with local anaesthetics for disbudding goat kids. However, future studies should evaluate the safety and efficacy of this protocol for disbudding in goat kids.

Similar to goat kids, articaine was rapidly hydrolysed to the inactive metabolite, articainic acid, and rapidly eliminated in red deer. A ring block around the base of the antlers using 4% articaine hydrochloride (1 mL/cm pedicle circumference) provided effective analgesia for velvet antler removal in red deer. The results of the studies in red deer suggested that articaine could be a safe and effective local anaesthetic for velvet antler removal. Residue analysis of harvested antlers using liquid chromatography–mass spectrometry (LC–MS) method revealed that the concentrations of articaine and lignocaine in the harvested velvet antlers were similar. Further studies to evaluate the safety of articaine and its metabolites are warranted in target species before recommending articaine hydrochloride as an alternative to lignocaine hydrochloride for velvet antler removal.

The analgesic efficacy of methoxyflurane and a novel topical local anaesthetic formulation for disbudding in goat kids were evaluated. Both methoxyflurane and the novel topical formulation provided cutaneous analgesia but did not provide sufficient analgesia for disbudding in goat kids. Further research is required to evaluate the efficacy of these novel analgesic techniques.

The efficacy of mepacrine and eugenol for disbudding in goat kids were investigated following subcutaneous injection (0.2 mL) under the horn buds. Both eugenol and mepacrine produced necrosis of horn buds in goat kids but failed to stop horn bud growth. Injection of these compounds using a needle (26 G) and syringe was painful but no pain-related behaviours were seen after the injection. Future studies should evaluate different injection volumes and different non-invasive or minimally invasive administration techniques to increase the efficacy of this novel technique. Refinement of this novel technique might provide a simple, fast, safe and effective way to stop horn bud growth in goat kids.

Acknowledgements

I am grateful to my supervisors; Associate Professor Paul Chambers, Dr Kavitha Kongara and Dr Preet Singh for their tremendous support throughout my PhD research. I have been fortunate to have them as my supervisors. Thank you, to Professor David Harding, for allowing me to work in his lab and helping me to prepare novel formulations.

Many thanks to Anne Tunnicliffe and Collin for assisting me with animal housing and care. Dr Mathew Perrot is acknowledged for the gross pathological examination of study animals. Thank you, to Geoff Purchas, for deer husbandry, handling and sample collection.

I am thankful to Antony Jacob and Neil Ward for the technical assistance. I am also thankful to the members of the Animal Welfare Science and Bioethics Centre; Dr Craig Johnson, Dr Ngaio Beausoleil, Juliet Cayzer, Dr Nikki Kells, Erin Wilson, Lauren Stewart and Heidi Lehmann for their assistance and encouragement. I wish to thank David Lun and Trevor Loo for helping me with LC-MS analysis.

I also owe my gratitude to Debbie Hill for her support in academic administrative matters. I am grateful to the Massey University Scholarship committee for providing me a doctoral scholarship. This PhD research was funded by the Dairy Goat Co-operative and School of Veterinary Science, Massey University.

To my dear friends and colleagues; David, Eden, Alex, Sandeep, Farzin, Renata, Jay, Jabin, Baby, Mande, Sashi and Sagara who helped me throughout this journey.

Finally, to my family, who without, this PhD research would not have been possible. To my Mum, sister, brother and brother-in-law for the care, support and advice.

"There is little that separates humans from other sentient beings – we all feel pain, we all feel joy, we all deeply crave to be alive and live freely, and we all share this planet together"

(Mahatma Gandhi – 1869-1948)

LIST OF PUBLICATIONS

Venkatachalam D, Chambers JP, Kongara K, Singh P (2019). Analgesic efficacy of articaine hydrochloride for velvet antler removal in red deer (*Cervus elaphus*) and analysis of drug residues in the harvested velvet antlers. *New Zealand Veterinary Journal*, 67(5): 228-233

Venkatachalam D, Chambers JP, Kongara K, Singh P (2018). Toxicity and pharmacokinetic studies of lidocaine and its active metabolite, monoethylglycinexylidide, in goat kids. *Animals*, 8(8): 142.

Venkatachalam D, Chambers JP, Kongara K, Singh P (2018). Pharmacokinetics of articaine hydrochloride and its metabolite articainic acid after subcutaneous administration in red deer (*Cervus elaphus*). *New Zealand Veterinary Journal*, 66(1):16-20.

Venkatachalam D, Chambers JP, Kongara K, Singh P (2017). A dose-ranging study and a pilot intravenous pharmacokinetic study of articaine hydrochloride in goat kids. Abstract presented at Science Week - *Australia and New Zealand College of Veterinary Scientists*, Gold Coast, Australia.

Studies completed in parallel with this thesis research

***Novel disbudding methods for dairy calves*.**
Sponsor: Bayer Animal Health, New Zealand

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List of Abbreviations

%CV	Percent coefficient of variation
%RE	Percent relative error
5-HT	5-hydroxytryptamine
AUC	Area under the curve
AUMC	Area under the first moment curve
BW	Body weight
Ca^{++}	Calcium
CL	Total body clearance
C_{max}	Maximum plasma concentration
CNS	Central nervous system
CPEs	Chemical penetration enhancers
DMAP	4-amino-3,5-dimethylphenol
DMHA	N-(2,6-dimethylphenyl)hydroxylamine
DNA	Deoxyribonucleic acid
GABA	Gamma-Aminobutyric acid
GX	Glycinexylidide
HCD	Higher energy collisional dissociation
HIV	Human immunodeficiency viruses
HPLC	High-performance liquid chromatography
IL-1β	Interleukin 1 beta
INN	International non-proprietary name
J_{ss}	Steady-state flux
K^{+}	Potassium
K_p	Apparent permeability coefficients
LC-MS	Liquid chromatography-mass spectrometry
LD_{50}	Median lethal dose
LLOQ	Lower limit of quantification
m/z	Mass-to-charge ratio
MAC	Minimum alveolar concentration
MEGX	Monoethylglycinexylidide

MPI	Ministry for Primary Industries
MRL	Maximum residue level
MRT	Mean residence time
Na^+	Sodium
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl-D-aspartate
NSAIDs	Non-steroidal anti-inflammatory drugs
NZ	New Zealand
PAG	Periaqueductal gray region
PGE_2	Prostaglandin E2
pK_a	Dissociation constant
PRM	Parallel reaction monitoring
RNA	Ribonucleic acid
SD	Standard deviation
$t_{½\lambda z}$	Elimination half-life
T_{max}	Time to achieve maximum plasma concentration
TNF_α	Tumor necrosis factor alpha
TRP	Transient receptor potential channels
UK	The United Kingdom
USA	The United States of America
USAN	United States Adopted Name
USP	The United States Pharmacopeia
V_d	Apparent volume of distribution
λ_z	Rate constant of the terminal phase

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Thesis Structure

This thesis is composed of six chapters including the present chapter (General introduction). Following Chapter 1, the thesis presents the results of several studies in Chapters 2-5. All the studies are in the form of manuscripts as per the format of peer-reviewed journals. Two manuscripts have been published, one has been accepted for publication and the rest are either submitted or yet to be submitted for publication. Additional information that could not be included in the manuscripts is presented as an appendix at the end of each chapter.

Chapter 2 presents the results of the studies conducted to determine the pharmacokinetics, safety, and efficacy of articaine hydrochloride for disbudding in goat kids. In Chapter 3, the findings of the studies conducted to investigate the pharmacokinetics, toxicity, and efficacy of lignocaine hydrochloride for disbudding in goat kids are presented. Chapter 4 presents the findings of the novel disbudding and analgesic techniques in goat kids. In Chapter 5, the results of the studies conducted to evaluate the efficacy of articaine hydrochloride for velvet antler removal in red deer are presented. The final chapter (General discussion) summarises the results of the studies conducted in the thesis, discusses the limitations of the studies, and includes suggestions for future research.

1.2 Pain

Pain has been defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (International Association for the Study of Pain 2018). Animal-specific pain has been defined as “an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal’s physiology and behaviour to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery; unnecessary pain occurs when the intensity or duration of the experience is inappropriate for the damage sustained or when the physiological and behavioural responses to it are unsuccessful at alleviating it” (Molony and Kent 1997). Pain is a complex, multi-dimensional experience involving both psychological and sensory components (Fernandez and Turk 1992). It is a subjective experience, the perception of which varies from individuals to individuals (McKune *et al.* 2015). Several types of pain have been described; the common types of pain are discussed below.

1) *Nociceptive pain*

   Nociceptive pain is the pain that develops due to the increased firing and sensitization of nociceptors (high threshold peripheral sensory neurons) in response to mechanical, chemical or thermal noxious stimuli (Kehlet *et al.* 2006). It is also called as physiological pain as it alerts the body to protect from the painful stimuli by signaling the presence, location, intensity, and duration of the stimuli (Kehlet *et al.* 2006). Nociceptive pain usually disappears once the stimulus is removed, provided there has been no tissue damage (Kehlet *et al.* 2006). Initial application of a hot-iron to destroy the horn buds in ruminants is one example of nociceptive pain.

2) *Inflammatory pain*

Inflammatory pain occurs in response to tissue damage and the resulting inflammation (Kehlet *et al.* 2006; Costigan *et al.* 2009). The release of inflammatory mediators such as prostanoids (prostaglandins (particularly PGE_2), leukotrienes, hydroxy-acids), cytokines (TNF_α, IL-1β, IL-6), histamine, and substance P from the site of tissue damage activate and sensitise the nociceptors that innervate the inflamed tissue which in turn propagate pain signals to the central nervous system (CNS) (Costigan *et al.* 2009). Inflammatory pain usually has a rapid onset and lasts for several hours or days depending on the severity and duration of tissue damage (McKune *et al.* 2015). Inflammation is responsible for the acute postoperative pain following a tissue injury. The postoperative pain that may persist following dehorning in animals is an example of inflammatory pain.

3) *Neuropathic pain*

Neuropathic pain occurs following injury to the somatosensory system of peripheral nerves or the CNS (Colloca *et al.* 2017). Damaged or regenerating neurones fire abnormally resulting in hypersensitivity or sensory abnormalities in the affected area (Baron *et al.* 2010; Tian *et al.* 2013). Some of the manifestations of neuropathic pain include hypoalgesia, paraesthesia, hyperalgesia and allodynia (Tian *et al.* 2013). Neuropathic pain is commonly manifested in humans suffering from diabetic neuropathy, post-herpetic neuralgia, trigeminal neuralgia, HIV infection, and injuries where nerves are disrupted by stretching, such as some road traffic accidents. It has not been commonly described in animals as no specific diagnostic method is available to show the presence of neuropathic pain in animals.

1.2.1 Pain pathways

Nociception involves the transduction and transmission of pain signals to the brain via a pain pathway. Basic knowledge of pain pathways will help in the selection of appropriate analgesics/analgesic protocol for the effective treatment of pain. The pathways involved in the perception and modulation of pain from peripheral nociceptors to the brain are shown in Figure 1.1.

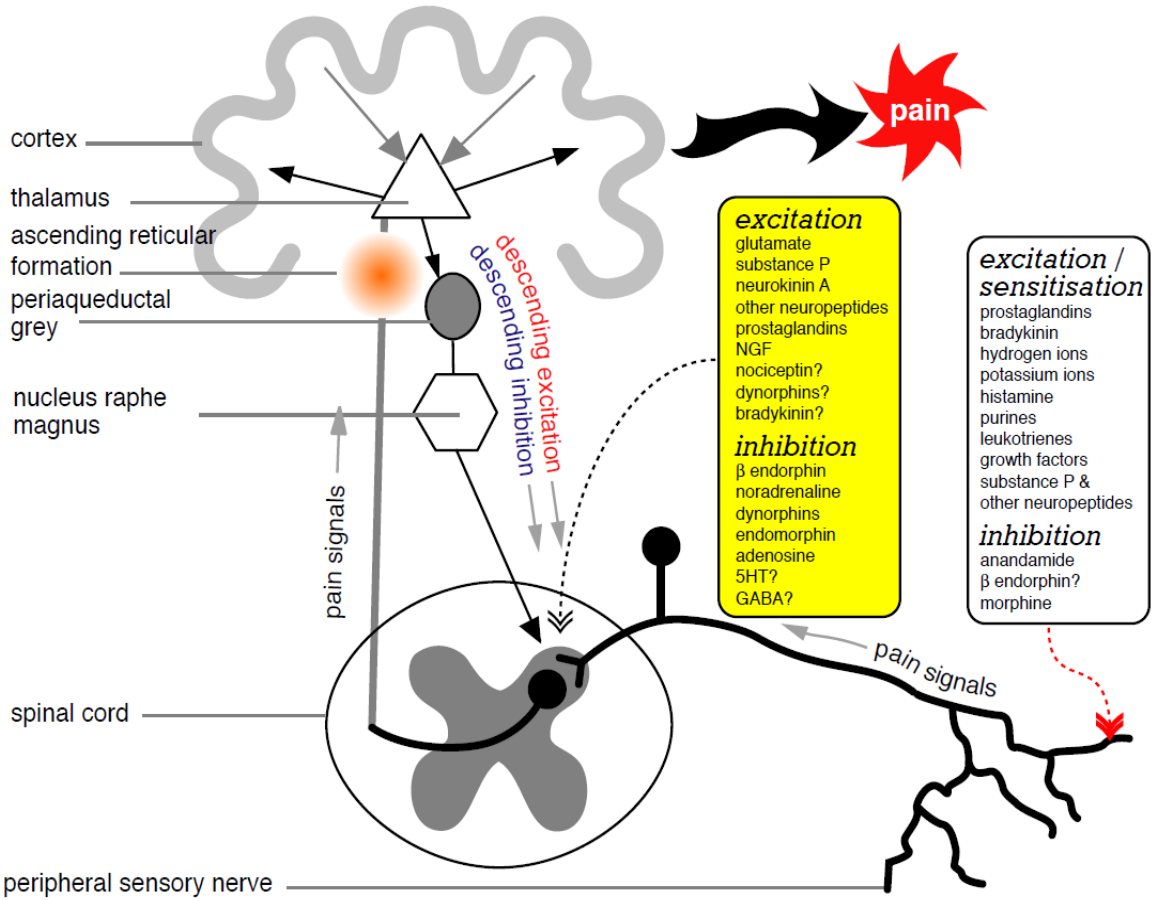

Figure 1.1: Pain pathways (Picture courtesy of Dr. Paul Chambers, Massey University). *Noxious stimuli activate the nociceptors (peripheral sensory nerve) which in turn transduce the information into action potentials and transmit the information to the dorsal horn of the spinal cord. Nociceptors can be excited/stimulated or inhibited by various stimuli, inflammatory mediators and substances (listed in the figure). Several ion channels including sodium (Na^+), calcium (Ca^{++}), potassium (K^+) and transient receptor potential (TRP) ion channels are involved in the transduction of the noxious stimuli. The transmission of the signals from the nociceptors to the central nervous system (CNS) is primarily carried out by Na^+ ion channels. From the dorsal horn of the spinal cord, the signals may be propagated directly to the brain without change or may be stimulated or inhibited (modulation) by interneurons or descending pathways. Several neurochemicals including glutamate, substance P and neurokinin A are released in the ganglion of the spinal cord which*

stimulates the second-order or projection neurons located at the dorsal horn of spinal cord to propagate the signals to the integration sites in the brain via the ascending tracts such as spinothalamic and spinoreticular tracts. The ascending signals from the spinal cord reach brain particularly at the thalamus, hypothalamus, and structures of the midbrain. From these sites, the signals are relayed to various cortical and subcortical regions including somatosensory cortex, periaqueductal gray region (PAG), reticular formation and the limbic system. In these sites, a variety of coordinated reactions occur including pain perception, learning, and memory, protective somatic and autonomic reflexes and endocrine actions. In addition to these processes, these sites also initiate the descending excitation and descending inhibition (endogenous analgesic system) of the noxious signal which will accentuate or reduce pain, respectively. This descending inhibition is mainly controlled by PAG. The PAG receives inputs from the higher centres such as thalamus, cortex, and hypothalamus, and excite the cells in the nucleus raphe magnus, from which nerve fibres descend to synapse at the dorsal horn of the spinal cord and release various neurotransmitters, including endorphins, serotonin (5-HT), GABA and noradrenaline. These neurotransmitters bind to presynaptic and postsynaptic sites of the dorsal horn of the spinal cord to alleviate the transmission of the pain signals by decreasing the release of excitatory neurotransmitters and by decreasing the propagation of the pain signals on the projection neurons. The central modulation system can also attenuate pain by descending excitation.

1.2.2 Pain in farm animals

Farm animals are routinely subjected to painful procedures such as castration, disbudding/dehorning, velvet antler removal, ear tagging/notching, branding, mulesing and tail docking for production and management purposes. Pain significantly affects the welfare of the animals and can have negative effects on production which in turn can affect farm economics (Mclennan 2018). Several studies have been carried out in the past two decades to avoid these painful procedures (e.g. breeding polled livestock and sexing sperm to produce female piglets) and to alleviate or minimize the pain caused by these painful procedures. All the painful procedures can be humanely performed by appropriate use of anaesthetics and analgesics.

Anaesthetics are drugs or chemicals that depress the activity of nerves locally, regionally, or in the CNS. Anaesthesia (derived from the Greek word *anaisthaesia* - insensibility) is defined as “the loss of sensation to the entire or any part of the body” (McKune *et al.* 2015). Analgesics (*an* – without, and *alges(is)* - pain) are drugs or chemicals that reduce the pain sensation to painful stimuli (McKune *et al.* 2015). Many anaesthetic and analgesic drugs have

been used in veterinary medicine. Since pain usually results when a noxious stimulus is transmitted from nerve endings to the spinal cord and then brain, a drug or combination of drugs which reduces transmission at one or more of these sites can cause analgesia. Analgesia can be produced by blocking sensitisation of nerve endings (NSAIDs and sometimes local anaesthetics), blockade of nerve conduction (local anaesthetics) and by altering signal processing in the brain or spinal cord (opioids, alpha$_2$ agonists, general anaesthetics). Local anaesthetics are the most commonly used analgesic drugs in farm animals as they are relatively safe, cost-effective, and require less skill to administer successfully than general anaesthesia.

1.3 Local anaesthetics

Local anaesthetics are a class of drugs that cause a reversible loss of sensation of an area of the body by transiently inhibiting the conduction of nerve impulses. Cocaine was the first drug to be used as a local anaesthetic for corneal anaesthesia by Karl Köller and Sigmund Freud in 1884 (Garcia 2015). Within a few years, cocaine was introduced as an anaesthetic in general surgery and dentistry. Its use in veterinary medicine was made popular by a veterinarian, Sir Frederick Hobday (Garcia 2015). Later, cocaine was found to be toxic and addictive, so new agents with better safety and pharmacological properties were discovered. Today, local anaesthetics are widely used in veterinary medicine for various local and regional nerve blocks especially in large animals.

Local anaesthetic techniques are usually simple to perform and cause desensitization to a distinct region of the body. They are inexpensive and relatively safe methods that allow surgical procedures to be performed on conscious animals. Local anaesthetics are the preferred drugs for appropriate analgesia in commercial farming conditions. The use of general anaesthetics in farm animals (cattle and small ruminants) is limited in most

circumstances because generalised depression of the brain and spinal cord produces side effects such as respiratory and cardiovascular depression, which are potentiated by recumbency, and equipment for the delivery of oxygen and assisted ventilation are required (Garcia 2015). Local anaesthetic techniques allow surgeries to be performed standing thereby minimizing the risks associated with recumbency in adult ruminants such as bloat, regurgitation, hypoxaemia, and myopathy. Some of the most commonly performed local anaesthetic techniques in large animals include lumbosacral epidural analgesia, cornual nerve block, intratesticular anaesthesia, and intravenous regional anaesthesia of the foot (Garcia 2015). In addition to the perineural injections, the systemic administration of local anaesthetics particularly lignocaine has been used for the management of ventricular arrhythmias, multimodal analgesia, and for reducing the requirement of general anaesthetics.

Most of the local anaesthetics are weak bases consisting of a lipophilic aromatic ring (mostly benzene ring) and a hydrophilic amine group (tertiary or quaternary amine), linked by an ester or an amide bond. Depending on the type of linkage, local anaesthetics are classified into amino-amide type or amino-esters type. Based on the type of linkage, local anaesthetics are metabolized by hepatic microsomes or plasma esterases. The amino-amide local anaesthetics are biotransformed in the liver whereas amino-esters are rapidly hydrolysed by esterases in plasma and tissues (Becker and Reed 2006). The metabolic profiles of local anaesthetics can vary between species. For example, the metabolites of lignocaine, monoethylglycinexylidide and glycylxylidide found in most species including dogs, cats, goats, sheep and horse were not detected in bovines (Cox *et al.* 2011).

Local anaesthetics are generally safe but toxicity can occur when high plasma concentrations are achieved. Various factors that determine the plasma concentrations of local anaesthetics include dose, site and route of injection, vasculature and rate of absorption from the site of injection (Garcia 2015). Chemical structures of different local anaesthetics are shown in

Figure 1.2. Properties of various local anaesthetics are listed in Table 1.1. Most local anaesthetics are weak bases that are poorly soluble in water. Local anaesthetics are commercially formulated as hydrochloride salts (acidic pH) to increase their aqueous solubility and improve stability. Buffering the local anaesthetic solutions with sodium bicarbonate has been shown to reduce the pain associated with infiltration, speed up the onset of action and increase the duration of action (Pascuet *et al.* 2009). Addition of sodium bicarbonate increases the pH of the local anesthetic solution and thus increasing the proportion of unionized-lipid soluble forms. This results in increased rate of penetration of the anaesthetic into the nerve cells.

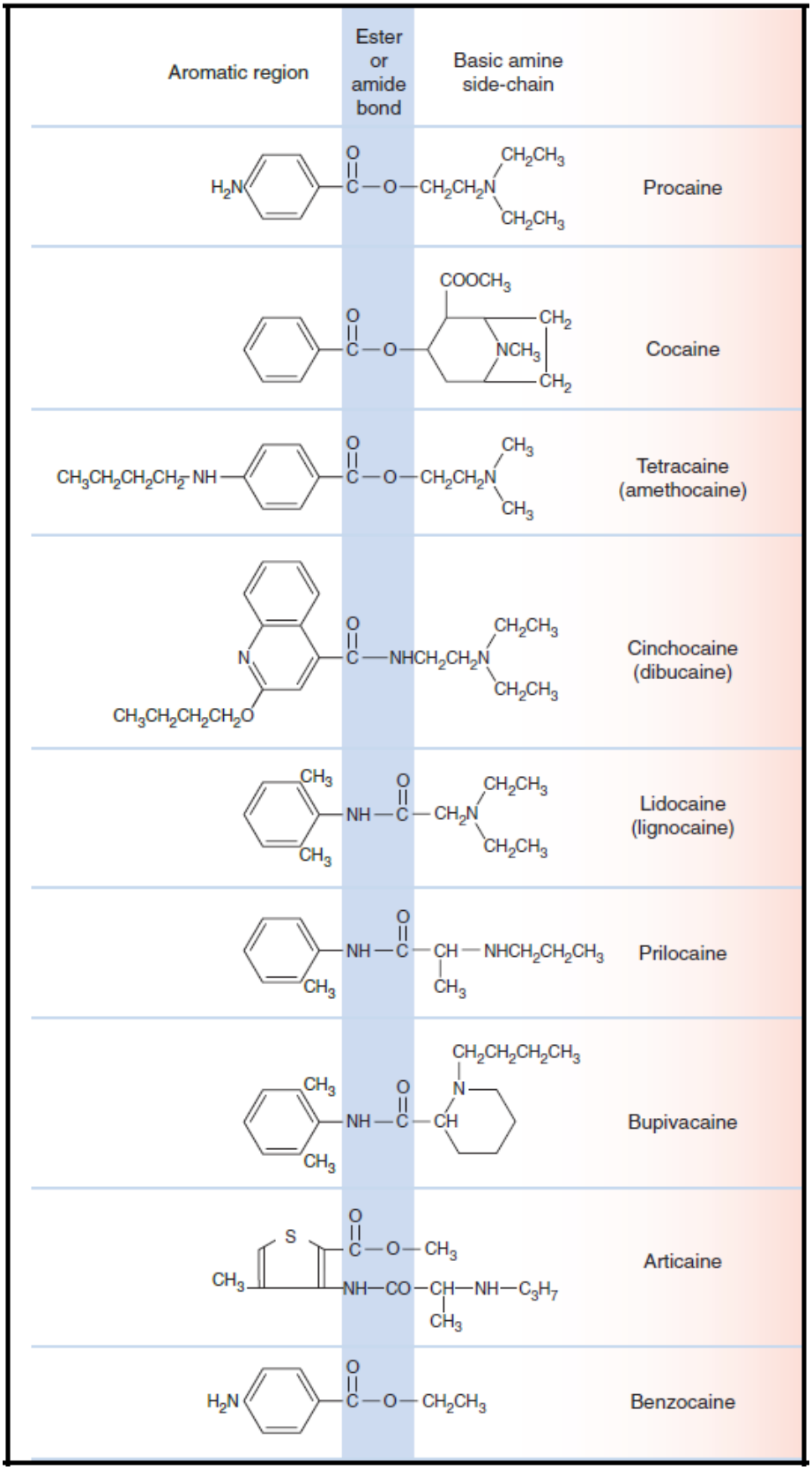

Figure 1.2: Chemical structure of various local anaesthetics: *Aromatic group (left), ester or amide group (shaded) and amine group (right) (from Ritter et al. 2016)*

Table 1.1. Physicochemical and pharmacological properties of local anaesthetic agents (*Modified from Garcia 2015*)

Local Anaesthetics	*pKa*	Lipid solubility[a]	% Protein binding	Relative anaesthetic potency	Onset of action	Duration of action	Relative potency for CNS toxicity[b]	CV:CNS ratio[c]
Ester-linked								
Procaine	8.89	100	6	1	Medium	Short	0.3	3.7
Chloroprocaine	9.06	810	7	1	Medium	Short	0.3	3.7
Tetracaine	8.38	5822	94	8	Very slow	Long	2	ND
Amide-linked								
Lidocaine	7.9	366	64	2	Rapid	Medium	1	7.1
Articaine	7.8	ND	94	2	Rapid	Medium	1	ND
Mepivacaine	7.72	130	77	2	Rapid	Medium	1.4	7.1
Prilocaine	8.02	129	55	2	Medium	Medium	1.2	3.1
Ropivacaine	8.16	775	94	6	Slow	Long	2.9	2
Bupivacaine	8.1	3420	95	8	Slow	Long	4	2
Levobupivacaine	8.1	3420	>97	8	Slow	Long	2.9	2
Etidocaine	7.87	7317	94	6	Rapid	Long	2	4.4

a Partition coefficients expressed as relative concentrations (mol/L) in octanol and buffer.
b Potency relative to procaine.
c Potency relative to lidocaine.
ND – no data.

1.3.1 Mechanism of action

Local anaesthetics prevent the initiation and conduction of action potentials primarily by blocking the voltage-gated sodium (Na^+) channels, and with low affinity to K^+ and Ca^{2+} channels (Scholz 2002). The Na^+ channel is a multimolecular complex consisting of a large alpha subunit. The alpha subunit is a single polypeptide protein composed of around 2000 amino acids that traverse the cell membrane several times to form the channel's pore and gating machinery (Lipkind and Fozzard 2005). The alpha subunit is made of four homologues domains (named the DI to DIV), each with six helices (S1-S6) arranged in a clockwise manner around the central pore (Lipkind and Fozzard 2005). Local anaesthetics reversibly bind to the S6 transmembrane helical domain (DIV) and physically block the transmembrane pore of the Na^+ channels, thereby affecting the influx of Na^+ ions and impulse generation from the site of action (Lipkind and Fozzard 2005). The S6 transmembrane helical domain (DIV) is also the target of antiarrhythmic and anticonvulsant drugs.

Depending on the membrane potential and time, the voltage-gated Na^+ channels exist in three different states: closed (resting), open, and inactivated (Scholz 2002). At membrane potentials below -70 mV, the channels exist in the closed state during which the Na^+ ions cannot enter. During depolarization of the membrane potential (above -40 mV), the channels open (open state) to allow diffusion of Na^+ ions down their concentration gradient through the pore and after a few milliseconds, the third state (inactivated) follows to allow membrane repolarization. Following repolarization of the membrane, the channels return to a closed state (Scholz 2002). The blockage of sodium channels by most local anaesthetics (except benzocaine) is use-dependent (Hille 1977). That is the blockage of Na^+ channels is greater when the channels are open and in inactivated states, as these states have a higher affinity to local anaesthetics than that of resting state (Hille 1977). Another hypothesis (guarded-receptor hypothesis) is that the local anaesthetics bind to the receptor located inside the

channel with constant affinity, and the access to the receptor is regulated by the channel gates (m and h gates) (Starmer *et al*. 1984). The channels with both gates in open state leave the receptor unguarded and so are accessible to the local anaesthetics, whereas the channels with gates in closed state restrict the accessibility of the local anaesthetics to the receptors and possibly trap the drug in the channel.

Local anaesthetics exhibit differential block or selective nerve block i.e., local anaesthetics demonstrate different patterns of sensory and motor blockade depending on various factors including type of nerve fibre (size and myelination), frequency of stimulation, length of nerve, and concentration of local anaesthetic e.g. local anaesthetic concentration required to block C‐fibres is usually higher than Aδ‐fibres (Scholz 2002).

1.3.2 Lignocaine hydrochloride

Among the several available local anaesthetics, 2% lignocaine (lidocaine) hydrochloride has been the most commonly used local anaesthetic in veterinary medicine owing to its efficacy, limited toxicity, and low cost. Lignocaine hydrochloride has been shown to be effective in providing anaesthesia for various painful procedures in farm animals including disbudding, castration, tail docking, and velvet antler removal. Although lignocaine is an effective and commonly used local anaesthetic, its use in goat kids and deer for disbudding and velvet antler removal, respectively, has been a concern. In goat kids, lignocaine has been reported to produce toxicity and mortality, and in deer, the presence of its residue in the harvested velvet antlers has been a concern (Taylor 1991; Bagonluri *et al.* 2005).

The details are discussed in the following sections.

1.4 Disbudding in goat kids

Cranial appendages such as horns and antlers have evolved in animals to protect against predators and to establish social ranking within a herd (Smith and Sherman 2009). Their presence may be advantageous in the wild but not desirable in a commercial farming condition. Goats with horns are dangerous to herd mates and handlers. They often engage in fighting to establish dominance during which severe injuries may occur. In addition, horned goats are difficult to handle and transport, cause damage to farm infrastructures such as fences and pen partitions, require more feeding and trough space, and make it difficult to use head bails or automatic feeders (Loretz *et al.* 2004; Smith and Sherman 2009). To avoid all these problems disbudding is commonly performed in goats, particularly in dairy goats.

Disbudding involves the removal or destruction of the horn buds before they attach to the underlying skull (Winder *et al.* 2016). The removal of horn after it has attached to the skull is called dehorning. Dehorning is much more stressful and painful than disbudding, and has complications such as epistaxis, sinusitis, and delayed wound healing (Hartnack et al. 2018). Therefore, disbudding is more humane and preferred over dehorning. Horns of goats grow rapidly compared to calves and so it is recommended to disbud between 2 and 4 weeks of age (Hartnack *et al.* 2018). Several disbudding methods have been reported in goat kids including thermal cauterization (most commonly used), application of caustic paste or liquid nitrogen, and injection of clove oil (Molaei *et al.* 2015; Hempstead *et al.* 2018). Irrespective of the method or age of the animal, disbudding causes behavioural, physiological and neuroendocrine changes which indicate that the procedure is stressful and painful (Hempstead *et al.* 2017; Alvarez *et al.* 2015). Therefore, appropriate anaesthesia and analgesia are required for humane prevention of horn growth. Disbudding can be avoided by selectively breeding for polledness, while this works in cattle, unfortunately, this technique can lead to serious reproductive disorders in some dairy goats of European extraction such as

Saanen, Alpine, and Toggenburg (Smith and Sherman 2009). The presence of horns in these breeds is determined by a recessive gene for infertility, hence the homozygous polled female will develop into a sterile intersex and the homozygous polled male will have increased risk of developing sperm granulomas (Smith and Sherman 2009).

Several anaesthetics and analgesics have been studied for disbudding in goat kids. Local anaesthetic nerve block has been described as one of the effective ways to alleviate the pain during disbudding in goat kids (Smith and Sherman 2009). Unfortunately, the most commonly used local anaesthetic, lignocaine, has been reported to produce toxicity in goat kids (Taylor 1991). This may be because goat kids can be easily overdosed because of their smaller body weight. For example, goat breeds such as pygmy and Nigerian Dwarf breeds can weigh less than 3 kg, injecting 2 mL of 2% lignocaine (20 mg/mL), equivalent to 12 mg/kg for nerve block can easily result in convulsive plasma concentrations and toxicity. In addition to lignocaine, its active metabolites (monoethylglycinexylidide and glycinexylidide) can also contribute to the toxicity when high doses of lignocaine are administered (Blumer *et al.* 1973; Rygnestad and Samdal 2000). Monoethylglycinexylide has been reported to produce convulsions in animals with convulsive doses similar to lignocaine (Smith and Duce 1971; Blumer *et al.* 1973). The convulsive effects of monoethylglycinexylide and lignocaine are additive. Glycinexylidide does not produce convulsions even at lethal doses but can potentiate the convulsive effects of lignocaine and monoethylglycinexylidide (Blumer *et al.* 1973). Other factors that increase the risk of toxicity in goat kids are the need for blocking four nerves for both the horn buds (only one nerve each side in calves) and the high vascularity at the site of nerve block (Figure 1.3) (Harwood 2012).

Both injectable and gaseous general anaesthetics have shown to be effective in alleviating the pain during disbudding, however, they may not be useful in a commercial farming situation as their use requires a veterinarian and may also increase the cost of disbudding (Hemstead *et*

al. 2018; Wagmann *et al.* 2018). Nevertheless, in the United Kingdom and other European countries, disbudding in goats must be performed only by a veterinarian using appropriate anaesthetic and analgesic (Wagmann *et al.* 2018). Nonsteroidal anti-inflammatory drugs have shown to reduce the post-disbudding pain but failed to prevent the acute pain produced during disbudding (Ingvast-Larsson *et al.* 2011). Sedatives such as xylazine reduce the stress during restraining and provide some analgesic effect but they fail to provide complete analgesia for disbudding (Stilwell *et al.* 2010; Caray *et al.* 2015). Therefore, cornual nerve block using local anaesthetics appears to be the most cost-effective on-farm method for disbudding in goat kids. Unfortunately, there is a risk of toxicity associated with the commonly used lignocaine. A local anaesthetic with a wider margin of safety is preferred over lignocaine for cornual nerve block in goat kids.

Figure 1.3: Innervation of the horn and injection sites for disbudding/dehorning in goats. *The cornual branch of the lacrimal nerve (zygomaticotemporal) is blocked behind the caudal ridge of the supraorbital process (**1**) and the cornual branch of the infratrochlear nerve is blocked at the dorsomedial rim of the orbit (**2**) (from Garcia 2015)*

1.5 Velvet antler removal in deer

Deer have been domesticated in many countries for the commercial production of venison, velvet antler and by-products. New Zealand has the greatest number of farmed deer in the world and is the largest producer of velvet antlers (Wilson 2002). Velvet antlers are cartilaginous blood-filled tissues covered with a soft, hairy, velvet-like skin (hence the term "velvet antler"). They develop from the pedicle of the frontal bone and possess the unique feature to cast and regrow annually (Bagonluri *et al.* 2005). The growth of velvet antlers is fast with average growth rates of 2 to 2.5 cm/day (Bagonluri *et al.* 2005). They are found mostly in male cervids except for reindeer where females also develop antlers (Donaldson and Doutt 1965). Velvet antlers are composed of proteins, amino acids, collagen, lipids, polyamines, growth factors like insulin-like growth factors, glycosaminoglycans, vitamins (Vitamin A and E), minerals including magnesium, potassium, sodium, sulphur, calcium and phosphorus, and trace minerals like cobalt, copper, iron, magnesium and selenium (Anonymous 2009).

Velvet antlers have been used in traditional Chinese medicine for several centuries for the treatment of various diseases including mammary hyperplasia, mastitis, uterine fibroids and mumps (Wu *et al.* 2013). In the western world, the harvested velvet antlers are processed and sold as nutraceuticals (Moreau *et al.* 2004). It was claimed that velvet antlers can improve immune system functioning, athletic performance and strength, muscle recovery and sexual functions (Gilbey and Perezgonzalez 2012). Several studies have been conducted to study the pharmacological properties of velvet antlers. Oral administration of elk velvet antler powder has been reported to be effective in the treatment of osteoarthrosis in dogs (Moreau *et al.* 2004). However, clinical trials in humans do not support the claimed benefits of velvet antler supplements (Allen *et al.* 2008). Nevertheless, velvet antlers are an economically viable commercial product from farmed deer.

Velvet antler removal (velvetting) is a surgical procedure that involves the removal of growing velvet antlers. Velvet antlers are highly innervated structures that require effective analgesia for humane removal (Webster and Matthews 2006). The regulation on the removal of velvet antlers varies from countries to countries. In the United Kingdom, the removal of velvet antler is permitted only for emergency situations whereas in New Zealand, Australia, Canada and the United States of America it can be carried out for commercial purposes. In New Zealand, velvet antler removal is a controlled surgical procedure that must be performed only by a veterinarian or farmer certified by the National Velvetting Standards Body under veterinary authorization using the approved methods of analgesia. A high dose complete ring block around the base of antlers using the local anaesthetic lignocaine hydrochloride is the most reliable and commonly used technique for velvetting (Woodbury *et al.* 2002; Johnson *et al.* 2005). Although lignocaine produces effective anaesthesia for humane removal of velvet antlers, concerns were raised on the presence of its residues in the harvested velvet antlers as one of its metabolites, 2,6-dimethylaniline (DMA; 2,6-xylidine) is reported to be carcinogenic and toxic (Anonymous 1990).

DMA has been classified as a possible carcinogen (Group B) by The International Agency for Research on Cancer (Anonymous 1993) based on the results of the toxicology studies in rats. In that study, chronic oral administration of DMA (3000 mg/kg) produced several cancers including nasal papilloma and carcinoma, rhabdomyosarcoma, subcutaneous fibromas and fibrosarcomas in rats (Anonymous 1990). In addition, the mortality rates were higher in animals that received 1000 and 3000 mg/kg of DMA than the control group. Based on these results, it was concluded that DMA was a carcinogen in rats. In dogs, chronic oral administration of DMA caused a reduction in body weight, hyperbilirubinemia, hypoproteinemia, and marked fatty degeneration. In vitro studies confirmed the genotoxic and mutagenic potential of DMA (Tydén *et al*. 2004). Several studies in humans suggested

that DMA was associated with an increased risk of bladder cancer (Duan *et al.* 2008; Tao *et al*. 2013). All these reports suggest that DMA may be a carcinogen hence the presence of its precursor lignocaine in the velvet antlers is undesirable.

The metabolic pathway and production of DMA are shown in Figure 1.4. Lignocaine and other amide-type local anaesthetics undergo hepatic biotransformation by cytochrome P450 isoforms (Tydén *et al*. 2004). Lignocaine is metabolized to many metabolites including monoethylglycinexylidide (MEGX) and glycinexylidide (GX) *via* oxidative N-dealkylation in the liver (Bill *et al*. 2004). The metabolite MEGX is further biotransformed into DMA (Figure 1.4). DMA undergoes oxidization in the liver to N-(2,6-dimethylphenyl) hydroxylamine (DMHA) and 4-amino-3,5-dimethylphenol (DMAP). DMHA undergoes phase II biotransformation (acetylation) to reactive esters that are converted to reactive nitrenium ion by phase 2 metabolism (Tydén *et al*. 2004). DMHA and nitrenium ion can covalently bind to DNA and cause tumours. DMHA also has the potential to react with haemoglobins which can be demonstrated *in vivo* by the presence of haemoglobin adducts that were formed by covalent binding of these reactive amines to the cysteine residues of the haemoglobin (Tydén *et al*. 2004). The other metabolite, DMAP undergoes oxidation to an iminoquinone, a strong electrophile with genotoxic potential (Gan *et al.* 2001). DMAP can also be formed from nitrenium ion or DMHA.

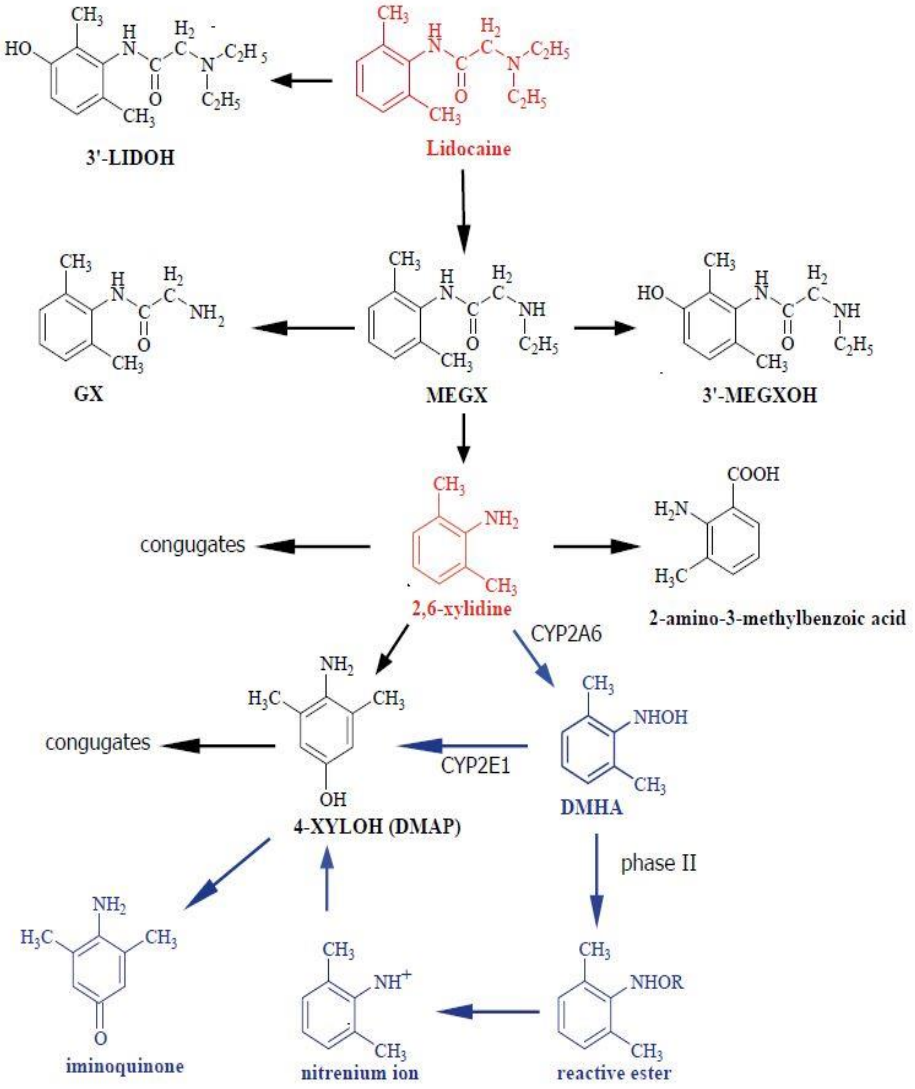

Figure 1.4: Metabolic pathway of lignocaine and proposed activation pathways of 2,6-xylidine. *Lignocaine is metabolized to monoethylglycinexylidide (MEGX) and then to 2,6-xylidine (DMA). DMA undergoes oxidation to form N-(2,6-dimethylphenyl)hydroxylamine (DMHA) and 4-amino-3,5-dimethylphenol (DMAP). DMHA may undergo acetylation to form reactive esters that are converted to a reactive nitrenium ion by spontaneous decomposition. DMHA has the potential to react with DNA and hemoglobin, and nitrenium ion can react with DNA and other macromolecules. The nitrenium ion can be converted to DMAP. DMAP can undergo oxidation to form iminoquinone. Iminoquinone is a strong electrophile with genotoxic potential. DMHA can also be formed to DMAP via CYP2E1-catalyzed reactions (from Flint 2012).*

In New Zealand, velvet antlers are the only food product for which a maximum residue level (MRL) for lignocaine has been specified by the Ministry for Primary Industries (MPI) (Anonymous 2018). The MRL of lignocaine and its metabolite DMA in the harvested deer

velvet antlers was set at 0.1 mg/kg, which was the default MRL for compounds for which residue levels were not otherwise specified. The MRL of lignocaine (0.1 mg/kg) was usually exceeded in the harvested antlers even after application of tourniquets (Flint 2012). In a limited survey of harvested velvet antlers, the concentration of lignocaine was found to be higher than the MRL (0.1 mg/kg) in 50% samples, with levels ranging from 0.12 to 18 mg/kg (Clear and Morris 2005). Flint (2012) reported that two third of the analysed samples exceed the MRL of 0.1 mg/kg with maximum levels up to 23 mg/kg. The MPI has very recently (1st December 2018) changed the MRL of lignocaine in the harvested velvet antlers from 0.1 mg/kg to 5 mg/kg (Anonymous 2018). The MPI mentioned that rationale to increase the MRL to 5 mg/kg was based on the review that confirmed the previous MRL 0.1 mg/kg was not fit for the management of lignocaine residues in the harvested velvet antlers when velvet antler removal was used according to the established good agricultural practice in New Zealand (Anonymous 2017). The document mentioned that the residues levels of lignocaine in 98% of the velvet samples were less than 4.5 mg/kg, and the current MRL level of lignocaine (5 mg/kg) is equivalent to less than 35% of the acceptable daily intake of 0.021 mg/kg (Anonymous 2017). Even though the levels of lignocaine in harvested velvet antlers may remain below the current MRL of 5 mg/kg, the presence of lignocaine in the food products will be a concern because of the reported carcinogenicity of DMA. The MRL of lignocaine and DMA was 0.1 mg/kg when the studies in this thesis were conducted.

Non-chemical alternatives such as electro-analgesia and compression analgesia were evaluated for velvet antler removal (Woodbury *et al.* 2002; Johnson *et al.* 2005). However, both the methods were not effective in providing sufficient analgesia for velvet antler removal. Moreover, the application of electro-analgesia and compression itself were noxious (Woodbury *et al.* 2002; Johnson *et al.* 2005). In New Zealand, a compression band (NaturO ring) has been approved for velvet antler removal in yearlings but not for animals older than a

year. Flint (2012) reported that NaturO ring provided analgesia during antler removal in yearlings but may cause discomfort on application. Long waiting times (60 minutes) are required for achieving effective analgesia using NaturO ring whereas analgesia is produced within a few minutes following lignocaine ring block. For all these reasons, local anaesthetic nerve block using lignocaine hydrochloride is the most effective, reliable, and commonly used technique for velvet antler removal although the presence of its residue has been a concern. A local anaesthetic that is equally effective as lignocaine and does not leave toxic residues in the velvet antlers may be preferred over lignocaine for velvet antler removal.

1.6 Hypothesis

To address the issues associated with the commonly used local anaesthetic lignocaine for disbudding goat kids and velvet antler removal in deer, a literature search was carried out to find a safer alternative to lignocaine. This led us to examine the unique features of a more modern local anaesthetic drug, articaine hydrochloride. Several studies in humans reported that articaine hydrochloride was safer and possessed greater analgesic efficacy than lignocaine (Oertel *et al.* 1997; Scully 2014). Even though articaine is an amide-type local anaesthetic like lignocaine, the metabolic pathway of articaine is different from lignocaine and has not been reported to produce DMA in humans or dogs. Neither articaine nor its metabolite has been reported to be carcinogenic. Hence, we hypothesize that articaine hydrochloride can be a good choice of local anaesthetic for velvet antler removal in deer and disbudding in goat kids. Articaine was chosen over other local anaesthetics because it is an amide type local anaesthetic with properties similar to the most commonly used lidocaine. Also, it is the newest local anesthetic that has been proposed as an alternative to lidocaine in humans (Wells and Beckett 2008). The pharmacological and toxicological properties of articaine are discussed in the next section.

1.7 Articaine hydrochloride

1.7.1 Introduction

Articaine, formerly known as carticaine, is the most recent local anaesthetic drug in current use. It was synthesized by Muschaneau in 1969 (Yapp *et al.* 2011). It entered clinical practice first in Germany in 1976, and then in Canada in 1984 (Malamed *et al.* 2001; Yapp *et al.* 2011). Later, it was introduced in the United Kingdom (1998), the United States (2000) and Australia (2005). Articaine is an intermediate-acting local anaesthetic with fast onset of action and intermediate potency (Snoeck 2012; Scully 2014). It is commonly used in humans for local infiltration and peripheral nerve blocks in dentistry (Yapp *et al.* 2011). Articaine has been shown to be effective for spinal, epidural, ocular, and intravenous regional anaesthesia (Oertel *et al.* 1997). It is considered a safe local anaesthetic with a wide safety margin which is related to its pharmacological properties.

1.7.2 Pharmacology of articaine

Articaine hydrochloride (3-n-propylamino-α-propionylamino-2-carbomethoxy-4-methyl-thiophen hydrochloride) is a unique amino-amide type local anaesthetic that differs from other members of the group by having a thiophene ring (instead of a benzene ring), and an ester group (Figure 1.5). The thiophene ring enhances the lipid solubility of articaine thereby facilitating faster and better penetration across nerve fibres (Yapp *et al.* 2011). Its unique molecular structure allows it to penetrate bone and soft tissues better than other local anaesthetics (Skjevik *et al.* 2011; Oertel *et al.* 1997). The presence of ester group makes it a special amino-amide type local anaesthetic to undergo rapid hydrolysis by esterases in tissues and plasma (Yapp *et al.* 2011; Oertel *et al.* 1997).

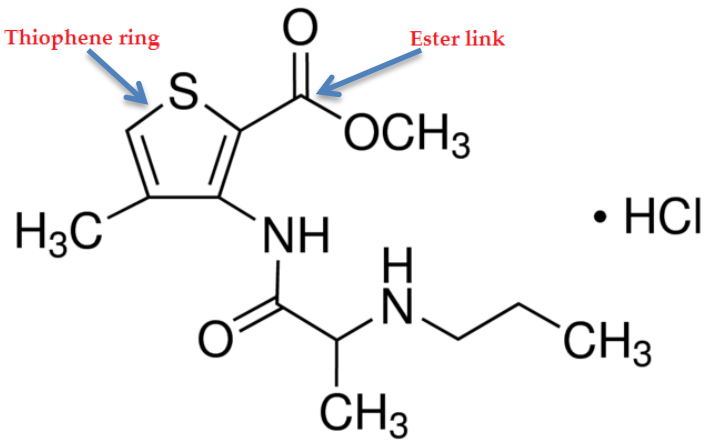

Figure 1.5: Structure of articaine hydrochloride (molecular weight 320.84).

1.7.3 Mechanism of action

Articaine acts similar to other local anaesthetics by reversibly binding to the S6 transmembrane helical domain (DIV) of the voltage-gated Na_+ channels and preventing the initiation and conduction of action potentials (Snoeck 2012). Similar to other local anaesthetics, articaine blocks the Na_+ channels in a state dependent manner: the affinity is greater when the channels are in the open and inactivated states than when they are at resting state (Snoeck 2012).

1.7.4 Pharmacokinetics

Pharmacokinetics describes "what the body does to a drug" after administration (Westhouse and Car 2013). It provides information on the time-course of the drugs in the body. Unlike most drugs, local anaesthetics are usually administered at the site of action to achieve their clinical effect: traditional pharmacokinetics has more of a role in understanding the adverse effects, specifically CNS and cardiac toxicity (Arthur and Covino 1991). Systemic absorption, distribution, metabolism, and elimination serve mainly to terminate the action of local anaesthetics. Knowledge of pharmacokinetic parameters can help to select safe doses and the right local anaesthetic for a particular procedure.

1.7.4.1 Absorption and distribution

Absorption of local anaesthetics is one of the important processes determining the blood concentration and thus the risk of systemic toxicity. The rate of absorption of local anaesthetics from the site of injection is determined by various factors including the injection site, dose administered, co-administration of vasoconstrictors and the pharmacological properties of the drug (Arthur and Covino 1991). Absorption of articaine has been reported to be rapid following various perineural injections in humans (Oertel *et al.* 1997). Following submucosal administration in humans, articaine was rapidly absorbed with C_{max} occurring at 10 to 15 minutes (Oertel *et al.* 1997). Articaine, like most local anaesthetics, possesses vasodilatory property which contributes to its rapid absorption into systemic circulation from the site of injection (Snoeck 2012). To reduce the absorption and to prolong the duration of action of articaine, adrenaline (1:60,000, 1:100,000, and 1:200,000) has been added in the commercial dental cartridges (eg, Ubistesin, 3M, NZ). The distribution of local anaesthetics depends on the degree of protein binding to tissues and plasma (Snoeck 2012). The volume of distribution of articaine has been reported to be similar to lignocaine (Oertel *et al.* 1997).

1.7.4.2 Metabolism and excretion

Unlike other amino-amide type local anaesthetics, articaine is rapidly hydrolysed by plasma and tissue esterases (figure 1.6) (Snoeck 2012). The ester linkage in the molecular structure of articaine undergoes rapid hydrolysis by esterases, and the amide linkage undergoes slow biotransformation in the liver (Yapp *et al.* 2011). Almost 90% of articaine is rapidly hydrolysed in plasma into articainic acid, most of which is excreted unchanged in urine and part of articainic acid undergoes further metabolism in the kidney into articainic acid glucuronide and excreted in urine (Yapp *et al.* 2011; Oertel *et al.* 1997). Both these metabolites lack local anaesthetic activity. In contrast, other amino amide type local anaesthetics including lignocaine depend mostly on hepatic metabolism and some of their

metabolites are known to possess local anaesthetic properties. Several pharmacokinetic studies in humans have reported that articaine was rapidly metabolized and eliminated with elimination half-lives less than 20 minutes (Oertel *et al.* 1997; Yapp *et al.* 2011). Rate of metabolism of local anaesthetics is one of the important factors that determine the systemic toxicity. Rapid metabolism of articaine in plasma decreases the risk of toxicity and increases the margin of safety. Although articaine is rapidly absorbed into systemic circulation its rapid metabolism to an inactive metabolite in the plasma reduces the risk of toxicity.

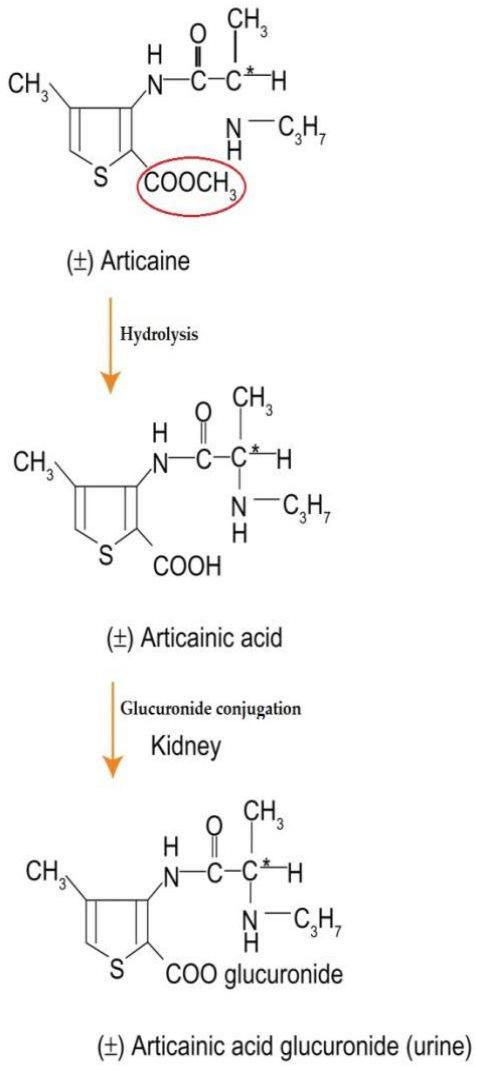

***Figure 1.6**: **Metabolism of articaine.** Articaine is hydrolysed by plasma esterases to articainic acid, which is then metabolized to articainic acid glucuronide in the kidney and excreted (from Snoeck 2012).*

1.7.5 Clinical Pharmacology

1.7.5.1 Onset of action

Most local anaesthetics are weak bases which exist in equilibrium between unionised (lipophilic) and ionised (hydrophilic) forms. The time for onset of action of most local anaesthetics depends on the proportion of lipophilic, unionised forms at the site of application as only the unionised forms can penetrate the epineurium and neuronal membrane to reach the axoplasm. However, after entering the axoplasm, the unionized forms gain a hydrogen ion to become ionized forms and it is the ionized forms that bind to the sodium channels (Becker and Reed 2006). Aqueous solutions of local anesthetics have hydrochloride added to reduce the pH (4-7) to increase the stability of the drug in solution. The ratio of unionised to ionised forms at a given pH is determined by the pKa of the drug. Local anesthetics with pKa values closer to the tissue pH (normally 7.4) act faster than the local anesthetics with higher pKa values. The weak basic nature of articaine (pKa = 7.8) along with greater lipid solubility enables the drug to penetrate the nerve fibres quickly and induce anaesthesia in a short time. Rapid onset of anaesthesia has been reported to occur following various articaine nerve blocks in humans. Following maxillary infiltration of 4% articaine hydrochloride, pulpal anaesthesia was achieved within 1.4 minutes in humans (Nizharadze *et al*. 2011). The onset of action of articaine was slightly faster than lignocaine (pKa = 7.9) in humans and rabbits (Miyoshi *et al.* 2000; Costa *et al.* 2005).

1.7.5.2 Potency

The potency of a local anaesthetic depends on its lipid solubility. The presence of a thiophene ring enhances the lipid solubility and hence the potency of articaine as a higher proportion of drug penetrates the neurons. The lipid solubility of articaine was slightly higher than lignocaine. *In vivo* studies in rabbits showed that the anaesthetic potency of articaine was 1.5

times and 1.9 times higher than that of lignocaine and procaine, respectively (Miyoshi *et al.* 2000).

1.7.5.3 Duration of action

Duration of action of local anaesthetics is predicted by the degree of protein binding. Higher protein binding increases the affinity and duration of binding to the receptors in the Na^+ channels (Becker and Reed 2006; Garcia 2015). The plasma protein binding of articaine was reported to be similar to lignocaine ranging from 57 to 73% (Oertel and Richter 1998). Articaine is an intermediate-acting local anaesthetic. A mean duration of action of 56.7 minutes has been reported following maxillary infiltration in humans (Costa *et al* 2005). Like other local anaesthetics, the addition of vasoconstrictors increases the duration of anaesthesia and decreases the rate of absorption of articaine (Oertel *et al.* 1997). Several studies have shown that the duration of action of 4% articaine was greater than 2% lignocaine (Coasta *et al.* 2005; Tortamano *et al.* 2013). Articaine is commercially available as 4% solution either with adrenaline or without adrenaline. Studies have shown that even 2% articaine produced effective nerve blockade however, the duration of analgesia was significantly lower than 4% solution (Hintze and Paessler 2006).

1.7.5.4 Toxicity and safety

Articaine is generally considered as a safe local anaesthetic with a wide safety of margin (Snoeck 2012). Preclinical toxicity of articaine has been evaluated both *in vitro* and *in vivo*. Repeated intramuscular injection of 25 mg/kg/day for 30 days was tolerated without major adverse effects in rats and dogs (FDA 2000). No pathomorphological changes were produced following repeated subcutaneous administration of articaine for 4 weeks in rats and dogs (FDA 2000). The no-observed-adverse-effect-level (NOAEL) value of articaine following subcutaneous administration in rats and dogs was estimated to be 25 mg/kg and 40 mg/kg, respectively (Leuschner and Leblanc 1999). The average LD_{50} values of articaine following

intravenous administration were 23.2 mg/kg, 19.6 mg/kg and 56 mg/kg for rats, rabbits and dogs, respectively (FDA 2000). The intramuscular LD_{50} values for rats, rabbits and dogs were 278 mg/kg, 20.6 mg/kg and 160 mg/kg, respectively (FDA 2000). The signs of toxicity reported during the acute toxicity study were tremor, tonic and clonic convulsions (FDA 2000). Reproductive toxicity studies in rats and rabbits reported that doses 10 times the maximum recommended dose of humans did not show any pathological changes in foetus or dams (Leuschner and Leblanc 1999). Both *in vitro* and *in vivo* mutagenic studies showed that articaine lacks mutagenic potential (Leuschner and Leblanc 1999). No carcinogenicity studies were conducted for articaine or its metabolites. Studies on local tolerance were carried out in rabbits and dogs following different routes of administration. The results suggested that articaine had good local tolerance without producing any lesions at the injection site (Leuschner and Leblanc 1999). The maximum recommended dose in humans was 7 mg/kg which is almost 3 times (based on body surface area) lower than the NOAEL observed in dogs (FDA 2000). The preclinical data indicate that articaine does not possess any relevant side-effects such as muscle tremor, sedation, convulsion or gross toxicity at the maximum recommended dose and can be considered a safe local anaesthetic.

There are no reports on the toxicity of articaine in humans except for a rare condition called paraesthesia. Paraesthesia is defined as a neuropathy with persistent of anaesthesia or altered sensations (Piccinni *et al.* 2018). The pathogenesis of paraesthesia is still not known, however, it is related to the concentration of the local anaesthetics. Paraesthesia has been reported to occur more commonly after the use of 4% local anaesthetic formulations such as articaine and prilocaine than following the use of 2% local anaesthetic formulations (Piccinni *et al.* 2014).

Even though articaine is considered as a safe local anaesthetic, systemic toxicity (CNS and cardiovascular toxicity) can occur like other local anaesthetics when toxic plasma

concentrations are achieved particularly during unintentional intravenous administration or during an overdose. However, the risk of systemic toxicity during overdosage is low compared to lignocaine and other amide-type local anaesthetics as articaine undergoes rapid hydrolysis following systemic absorption (Yapp *et al.* 2011).

1.7.6 Rationale for investigating articaine hydrochloride and lignocaine hydrochloride

Based on the reported human studies and the pharmacological properties of articaine including efficacy, rapid hydrolysis and elimination, articaine may be considered as a safe and effective local anaesthetic in animals. The ability of articaine to undergo rapid hydrolysis to the inactive metabolite, articainic acid, suggests that articaine may be safer than lignocaine. Because of the better therapeutic index in regard to systemic toxicity, articaine is used as a 4% solution, whereas lignocaine is used as 2% solution (Oertel *et al.* 1997). Moreover, there are no reports on the carcinogenicity of articaine or its metabolites unlike the metabolite of lignocaine, which would be a major advantage in food animals. Considering all these, it was hypothesized that articaine is a safe alternative to lignocaine for disbudding in goat kids and velvet antler removal in deer. Hence, one of the objectives of this thesis was to investigate the efficacy of articaine hydrochloride for disbudding in goat kids and velvet antler removal in deer.

Although lignocaine was reported to be toxic in goats, there are no data on the doses or the plasma concentrations of lignocaine that caused toxicity in goat kids. Therefore, another objective was to determine the toxic dose of lignocaine and its plasma concentrations in goat kids.

1.8 Novel analgesic and disbudding methods for goat kids

Local anaesthetic nerve block may be an effective way to alleviate or minimize the pain during disbudding, but the injection of local anaesthetics at four sites for both the horns was found to be stressful and probably painful. In addition, injection of local anaesthetics requires skill and expertise which may not be available in a commercial farming setting. Disbudding methods that are simple, cost-effective, and cause minimal pain to animals would be preferred in a commercial forming setting. Hence, the other objective of this thesis was to evaluate several novel disbudding and analgesic techniques.

1.8.1 Novel Analgesia Methods

1.8.1.1 Methoxyflurane

Methoxyflurane is an old inhalation anaesthetic which was widely used in people and animals from 1960 to 1990 (Steffey *et al.* 2015; Dayan 2016). It was one of the preferred volatile anaesthetics for orthopaedic surgery and ophthalmic surgery as it provided effective post-operative analgesia and extra ocular muscle relaxation, respectively (Steffey *et al.* 2015; Massey 1971). It is a non-irritant, non-inflammable, non-explosive fluorinated hydrocarbon anaesthetic (Massey 1971). It was discontinued as an anaesthetic because of many disadvantages (Steffey *et al.* 2015). The important disadvantages are discussed below:

1) *Nephrotoxicity*

   Methoxyflurane was reported to produce irreversible, dose-dependent renal toxicity (Grindlay and Babl 2009). The pathogenesis of renal toxicity was related to its metabolism. It undergoes extensive hepatic and renal biotransformation leading to the release of free inorganic fluoride ions which in turn causes damage to renal tubular structures and renal failure (Kharasch *et al.* 2006; Steffey *et al.* 2015). Nephrotoxicity following the administration of methoxyflurane has been reported in humans, dogs, and rats (Kharasch *et al.* 2006; Steffey *et al.* 2015). Interestingly, the renal toxicity of

methoxyflurane is characterized by polyuria and not oliguria. Species and strain differences have been reported to occur in the rate of metabolism of methoxyflurane and its nephrotoxicity. Injection of same doses of methoxyflurane produced greater renal toxicity in Fischer 344 rats than in Buffalo rats (Mazze *et al.* 1973). Renal toxicity of methoxyflurane was not commonly reported in animals, except in dogs where nephrotoxicity has been reported to occur when used in combination with tetracycline and flunixin (Mathews *et al.* 1990; Steffey *et al.* 2015)

2) *Slow anaesthetic induction and recovery*

   The high boiling point (104.65˚C) and low vapour pressure (25 mmHg; 3.1 kPa) of methoxyflurane reduce the evaporation rate and the maximal concentration which can be produced by a conventional vaporiser (Massey 1971). The blood:gas partition of methoxyflurane is higher than other anaesthetics, hence the anaesthetic induction and recovery are slower than other anaesthetics (Pawson and Forsyth 2008). In addition, it is highly soluble in rubber that leads to the loss of anaesthetic to the anaesthetic equipment and rubber hoses which delays the development of an inspired anaesthetic concentration (Steffey *et al.* 2015).

3) *Chemically unstable*

   As methoxyflurane is unstable, butylated hydroxytoluene was added as a preservative. Butylated hydroxytoluene is not highly volatile and so collects in the vaporization chambers affecting vaporiser efficiency (Steffey *et al.* 2015).

Even though methoxyflurane had many issues as an anaesthetic it was a very good analgesic even at sub-anaesthetic doses and the analgesic effects lasted for many hours after inhalation (Dayan 2016; Gaskell *et al.* 2016). At low doses, methoxyflurane produces rapid and effective analgesia without causing deep sedation or respiratory depression (Jephcott *et al.* 2018). The low doses that produced effective analgesia were reported to be well tolerated and

do not produce nephrotoxicity (Grindlay and Babl 2009; Dyan 2016). Self-administration of low concentration of methoxyflurane in labour produced rapid, effective and safe analgesia without any adverse effects (Bodley 1966). It has been widely used as a self-administered inhalation analgesic in Australasia for acute trauma associated pain and minor surgical procedures such as burn dressings and drainage of abscesses (Gaskel *et al.* 2016). For many years, it has been used as a battlefield analgesic by the New Zealand and Australian army (McLennan 2007). It is starting to be used in European countries as an analgesic for moderate to severe pain associated with trauma (Porter *et al.*2018). Methoxyflurane is commercially available as a small hand-held inhaler (Penthrox) that can be easily self-administered by people. Several studies suggested that methoxyflurane administered via Penthrox inhaler was safe and produced immediate pain relief in humans (Dyan 2016; Gaskel *et al.* 2016).

1.8.1.2 Hypothesis

Based on the reported analgesic efficacy of methoxyflurane and the safety of subanaesthetic doses, it was hypothesised that methoxyflurane can be an effective analgesic for disbudding in goat kids. Using the commercially available Penthrox inhaler, the analgesic efficacy of methoxyflurane was evaluated for disbudding in goat kids (Chapter 4).

1.8.1.3 Topical local anaesthetic formulation

Topical local anaesthetic formulations have been used in humans to anaesthetise skin for needle insertion, intravenous catheterisation, and minor surgical procedures such as split skin grafting. Other uses include topical anaesthesia of eyes, anaesthesia of mucous membranes for endoscopy and urethral catheterization, management of neuropathic pain, and analgesia of wounds. The most commonly used topical local anaesthetic cream, EMLA, has been reported to produce cutaneous analgesia to a depth of 6 mm (Tadicherla and Berman 2006). Recently, anaesthetic efficacy of EMLA was evaluated for the anaesthesia of horn buds in calves (Fierheller *et al.* 2012). In that study, EMLA cream was reported to be ineffective in providing anaesthesia of horn buds. EMLA is a eutectic mixture of 2.5% lignocaine and 2.5% prilocaine without any penetration enhancers. The low concentration of local anaesthetics in the cream and insufficient application time may be the reason for anaesthetic failure in calves (Fierheller *et al.* 2012).

Skin is a protective barrier that prevents the entry of pathogens and foreign substances into the body. It is made up of epidermis, dermis, and hypodermis (Figure 1.7). The outermost layer of the epidermis, stratum corneum, plays a significant role in preventing the entry of pathogens and foreign bodies. Stratum corneum consists of a thick layer of keratinocytes and lipid bilayers with hydrophilic regions in between (Meinardi 2000). These lipid layers limit the permeation of water-soluble substances but allow the permeation of lipophilic compounds with molecular weight less than 500 Da (Bos and Meinardi 2000). Most local anaesthetics are lipophilic with molecular weights less than 500 Da, however, they exist as crystals at normal body temperature which limits their permeation through the stratum corneum. Several methods have been applied to disrupt the stratum corneum safely and reversibly in order to allow permeation of drugs through the skin. The methods can be broadly classified as physical methods and chemical methods (Karande and Mitragotri 2009). Some of the

physical methods used to deliver drugs through skin include the use of iontophoresis, application of ultrasound, needleless jet injectors, lasers and microneedle arrays (Bos and Meinardi 2000; Karande and Mitragotri 2009). Physical methods need special equipment or devices to deliver the drugs and hence are expensive.

The chemical methods use chemicals (chemical permeation enhancers or sorption promoters) to disrupt the stratum corneum to allow drug permeation. Chemical penetration enhancers (CPEs) are commonly used for topical and transdermal formulations as they are inexpensive and easy to formulate (Karande and Mitragotri 2009; Lopes 2015). There are more than 300 chemical permeation enhancers with different mechanisms of action. The details of the CPEs are not discussed in this thesis, an extensive review on different types of chemical permeation enhancers can be found in several research articles including Karande and Mitragotri (2009), Smith and Maibach (2005) and Lopes (2015).

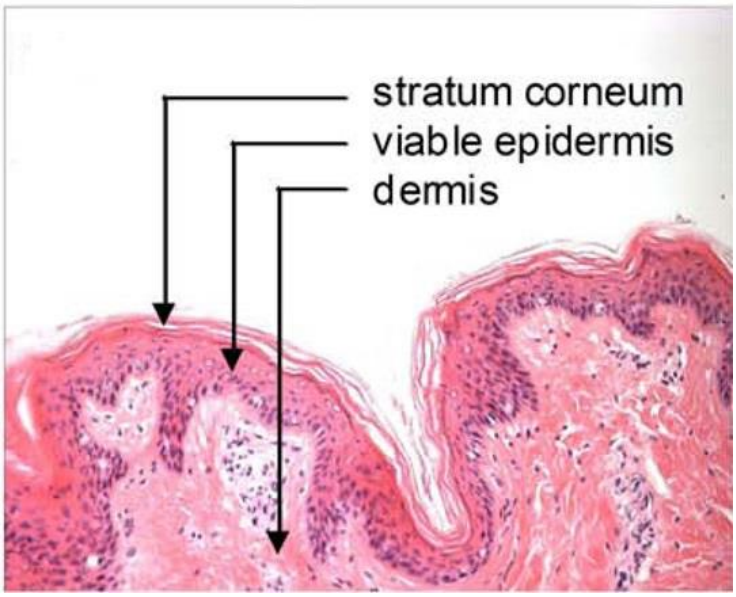

Figure 1.7: Skin histology. *Stratum corneum is the outermost layer of skin, composed of keratinocytes that act as the main barrier for the entry of pathogens and foreign bodies. The next layer, viable epidermis is made up of epithelial layer that renews the stratum corneum continuously. The third layer, dermis consists of fibrous layer, blood vessels, sweat glands, and hair follicles. The function of dermis is to provide mechanical support to the skin (from Prausnitz and Langer 2008).*

1.8.1.4 Hypothesis

A topical formulation with high concentration of local anaesthetics along with potent chemical permeation enhancers may effectively penetrate goat skin and anaesthetize horn buds for disbudding. If the application of topical local anaesthetic formulation provides effective analgesia, it can be easily applied by farmers without the need for any special skill. Various topical local anaesthetic formulations were formulated and evaluated *in vitro* using Franz cells. The formulation that showed higher diffusion in the *in vitro* study was tested on goat kids for anaesthesia of horn buds. The details of the experiments are discussed in chapter 4.

1.8.2 Novel Disbudding methods

1.8.2.1 Mepacrine

Mepacrine (INN), also known as quinacrine (USAN) (4-N-(6-chloro-2-methoxyacridin-9-yl)-1-N,1-N-diethylpentane-1,4-diamine) is an acridine derivative, was first synthesized in 1928 in Germany (Lippes 2015; Ehsanian *et al.* 2011). It was extensively used as an antimalarial agent for many years until it was substituted by better tolerated compounds like chloroquine (Ehsanian *et al.* 2011). Mepacrine was taken daily by millions of military personnel as a prophylaxis for malaria during the Second World War (Ehsanian *et al.* 2011). It has also been used for the treatment of giardiasis, tapeworm infection, lupus erythematosus, rheumatoid arthritis, and as an adjuvant chemotherapeutic agent for cancer (Lippes 2015; Ugarte *et al.* 2018). In addition, owing to its painless sclerotic potential it was widely used for the treatment of pleural effusion, and for non-surgical sterilisation of women (Lippes 2015). Mepacrine has been clinically evaluated for prostate cancer, Creutzfeldt-Jakob disease, and other prion diseases (Collinge *et al.* 2009). It is one of the thoroughly studied drugs and has been used for more than 70 years (Ehsanian *et al.* 2011; Lippes 2015).

Mepacrine was reported to possess sclerotic, anti-inflammatory, anti-cancer, analgesic, and local anaesthetic-like properties (Volpi *et al.* 1981; Abdel-Latif *et al.* 1983; Lippes 2015). Intratumour injection of mepacrine produced a unique type of inflammatory reaction which leads to necrosis and subsequent fibrosis (Sotelo *et al.* 2004). The mechanism of this action of mepacrine is not well understood (Kulkarny *et al.* 2014). The proposed mechanism of action includes inhibition of phospholipase A2, DNA intercalation interference with RNA transcription and translation, inhibition of NF- κB and cholinesterase (FDA 2016; Kulkarny *et al.* 2014). Mepacrine induces apoptosis and cell cycle arrest in cancer cells and has been reported to modify the expression of microRNAs involved in tumorigenesis (Chumanevich *et al.* 2016).

Mepacrine is generally considered as a safe compound but toxicity can occur following chronic administration (Lippes 2015; Sarin and Sarin 2003). Adverse effects reported in humans following chronic ingestion include gastrointestinal upset, yellowing of conjunctiva, cornea, sclera and skin, and aplastic anaemia (Lippes 2015). Intrauterine administration of high dose mepacrine produced cancers in the reproductive tract of rats and *in vitro* studies reported that it is mutagenic (Cancel *et al.* 2010). However, the epidemiological studies in humans suggest that no risk of cancers of genital tract was associated with mepacrine (Lippes 2015). Nevertheless, mepacrine has still been evaluated for various clinical conditions such as Creutzfeldt-Jakob disease and prostate cancer (Lippes 2015).

1.8.2.2 Hypothesis

Based on the reported pharmacological properties particularly asymptomatic sclerosis, apoptosis, and local anaesthetic-like properties, we hypothesise that injection of mepacrine into the horn buds will destroy horn producing cells with minimum pain and arrest horn bud growth.

1.8.2.3 Eugenol

Clove oil injection has been recently reported to be effective in destroying the horn buds of goats and calves (Molaei *et al.* 2014; Molaei *et al.* 2015). The active ingredient of clove oil is eugenol (4-allyl-2-methoxyphenol), a polyphenolic compound present in cloves and other plants (Esmail *et al.* 2015). Eugenol possesses several pharmacological properties including local anaesthetic, analgesic, anti-inflammatory, antitumor, antioxidant, antibacterial and insecticidal properties (Park *et al.* 2011; Chung and Oh 2013). It has been widely used in human dentistry as filling material, endodontic sealers, and dental cement for its local anaesthetic effect. It is also used as an anaesthetic/sedative for fish and shrimps (Esmail *et al.* 2015). Aqui-S (New Zealand) is a sedative/anaesthetic containing eugenol used during harvest and transport of fish and shrimps to avoid stress and unnecessary suffering (Bosworth 2007).

Eugenol acts by binding to the voltage-gated Na^{+}, K^{+}, and Ca^{2+} ion channels and preventing the transmission of nerve impulses (Park *et al.* 2011, Chung and Oh 2013). It is also shown to inhibit GABAa and NMDA receptors (Chung and Oh 2013). Eugenol produces cytotoxicity and necrosis of tissues at higher concentrations (Webb and Bussell 1981; Kamatou *et al.* 2012).

Molaei *et al* (2015) reported that clove oil injection was effective in preventing the growth of horn buds but did not provide much detail on the degree of distress or adverse effects observed during the procedure. Eugenol has been reported to produce necrosis and inflammation following subcutaneous injection in rats, and hypersensitivity reactions following mucosal application of dental materials containing eugenol in humans (Webb and Bussel 1981; Sarrami *et al.* 2002). Therefore, one of the objectives of this study was to evaluate the efficacy and the pain associated with the injection of eugenol (the active ingredient of clove oil) for disbudding in goat kids.

1.9 Thesis Objectives

The objective of this thesis was to evaluate various novel, and potentially safer, methods of producing analgesia for surgical disbudding in goat kids and velvet removal in deer, and to assess if medical treatment can stop horn bud growth without the pain of surgery.

To achieve these objectives the following studies were conducted:

1) Dose-ranging study, pharmacokinetics, toxicity, and efficacy of articaine hydrochloride in goat kids.

   Hypothesis: Based on the reported pharmacological properties of articaine including its efficacy, rapid hydrolysis to inactive metabolites, and rapid elimination, it was hypothesized that articaine would be a safe and effective local anaesthetic for cornual nerve block in goat kids.

2) Toxicity, pharmacokinetics, and efficacy of lignocaine and its active metabolite, monoethylglycinexylidide, in goat kids.

   Rationale: Even though lignocaine was reported to be toxic in goat kids, there are no data on the doses or the plasma concentrations of lignocaine that produced toxicity in goat kids. Therefore, this study was planned to determine the toxic dose of lignocaine and its plasma concentrations in goat kids.

3) Investigation of the efficacy of mepacrine, eugenol and methoxyflurane for disbudding in goat kids.

   Hypothesis: Considering the pharmacological properties of mepacrine and eugenol particularly necrosis, and local anaesthetic properties, it was hypothesized that injection of mepacrine or eugenol into the horn buds will destroy the horn producing cells with minimum pain and prevent the horn growth.

Based on the reported analgesic efficacy and the safety of subanaesthetic doses of methoxyflurane, it was hypothesized that methoxyflurane is an effective analgesic for disbudding in goat kids.

4) Formulation and evaluation of novel topical local anaesthetic formulation for anaesthesia of horn buds in goat kids.

   It was hypothesized that topical formulations with high concentrations of local anaesthetics formulated with chemical permeation enhancers will rapidly penetrate goat skin and anaesthetize horn buds for disbudding.

5) Articaine hydrochloride for velvet antler removal in deer

   a) Pharmacokinetics of articaine hydrochloride and its metabolite articainic acid after subcutaneous administration in red deer (*Cervus elaphus*).

   b) Analgesic efficacy of articaine hydrochloride for velvet antler removal in red deer (*Cervus elaphus*) and analysis of drug residues in the harvested velvet antlers.

   Hypothesis: Though articaine is an amide-type local anaesthetic like lignocaine, the metabolic pathway of articaine is different from lignocaine and does not produce 2,6-dimethylaniline (DMA). Neither articaine nor its metabolite has been reported to be carcinogenic. Considering the reported efficacy and safety of articaine, it was hypothesized that articaine hydrochloride will provide safe and effective analgesia for velvet antler removal in deer.

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CHAPTER 2

ARTICAINE HYDROCHLORIDE FOR DISBUDDING IN GOAT KIDS

This chapter consists of:

1) **A dose-ranging study of articaine hydrochloride in goat kids**

   Abstract presented at Science Week - *Australia and New Zealand College of Veterinary Scientists*, Gold Coast, Australia.
   Venkatachalam D, Chambers JP, Kongara K, Singh P (2017). A dose-ranging study and a pilot intravenous pharmacokinetic study of articaine hydrochloride in goat kids.

2) **Pharmacokinetics, toxicity, and efficacy of articaine hydrochloride in goat kids**

   Venkatachalam D, Chambers JP, Kongara K, Singh P. Pharmacokinetics, toxicity and efficacy of articaine hydrochloride in goat kids.
   The manuscript was submitted to *Veterinary Anaesthesia and Analgesia*.

Pharmacokinetics, toxicity, and efficacy of articaine hydrochloride in goat kids

1.1 Abstract

Objective To investigate the maximum tolerated dose, pharmacokinetics, toxicity, and efficacy of articaine hydrochloride in goat kids

Study design Experimental prospective study

Animals Total 22 male goat kids

Methods Four doses (5, 8, 10 and 15 mg kg^{-1} bodyweight) of articaine hydrochloride were intravenously administered over 60 seconds to determine the maximum tolerated dose. Articaine hydrochloride (8 mg kg^{-1}) was intravenously administered over 60 seconds and blood samples were collected at predetermined time points to study the intravenous pharmacokinetic parameters in goat kids. Cornual nerve block using articaine hydrochloride (1.5%) was carried out to investigate the analgesic efficacy and pharmacokinetics of articaine. Convulsive dose and plasma concentrations were determined following intravenous infusion of articaine hydrochloride (4 mg kg^{-1} $minute^{-1}$).

Results The maximum dose of articaine hydrochloride that did not produce any adverse effects was 8 mg kg^{-1}. The mean terminal half-life ($t_{½\lambda z}$) and the mean plasma clearance (CL) of articaine following intravenous administration were 0.66 hours and 5.33 L $hour^{-1}$ kg^{-1}, respectively. Following cornual nerve block, the mean maximum plasma concentration (C_{max}) of articaine was 586.58 ng mL^{-1} at 0.22 hour and its mean $t_{½\lambda z}$ was 1.26 hours. Anaesthesia of horn buds was observed within 4 minutes following the administration of articaine hydrochloride. The mean dose required to produce convulsions was 16.24 mg kg^{-1} and the mean convulsive plasma concentrations of articaine and articainic acid were 9.90 µg mL^{-1} and 1.52 µg mL^{-1}, respectively.

Conclusions Intravenous administration of 8 mg kg_{-1} was tolerated and therefore may be safe for perineural injections in goat kids. The pharmacokinetic data suggest that articaine was rapidly eliminated and cleared in goat kids. The plasma concentrations of articaine following cornual nerve block remained well below the convulsive plasma concentrations. Cornual nerve block using 1.5% articaine hydrochloride alleviated the acute pain during disbudding in goat kids. The pharmacokinetic and toxicity data suggest that articaine is a safe local anaesthetic in goat kids.

Clinical relevance: Articaine hydrochloride appears to be a safe and effective local anaesthetic for disbudding in goat kids.

Keywords Goat kids, articaine hydrochloride, disbudding, pharmacokinetics, toxicity, efficacy

1.2 Introduction

Most goats will develop horns which can cause injury to herd mates and animal keepers, and can also damage farm infrastructures like fences and pen partitions (Smith & Sherman 2009). Additionally, horned goats require more space at the feed barrier than hornless goats (Loretz et al. 2004). To avoid these problems, disbudding is commonly performed in goats (Nfor et al. 2016). Thermal cauterization is the most commonly used technique for disbudding in goat kids although it is stressful and painful without appropriate anaesthesia and analgesia (Smith & Sherman 2009; Hempstead et al. 2017). One of the effective ways to alleviate or minimize the pain during thermal cautery disbudding is by local anaesthetic nerve blockade (Smith & Sherman 2009). Lignocaine hydrochloride, the most commonly used local anaesthetic in veterinary medicine has been reported to produce toxicity in goat kids (Taylor 1991; Smith & Sherman 2009; Alvarez et al. 2015). The reasons for toxicity could be overdosing of kids by not considering their bodyweight and the increased risk of systemic absorption of the drug from the injection sites which are highly vascularized (Malavasi et al. 2016). The need for

blocking two nerves per horn bud (compared to a single nerve in calves) increases the chances of toxicity in goat kids (Harwood 2012). Therefore, a local anaesthetic with wider margin of safety would be preferred over lignocaine hydrochloride for disbudding in goat kids.

Articaine hydrochloride (4-methyl-3-(2-propylaminopropionamido) thiophene-2-carboxylic acid methyl ester hydrochloride) is one of the most recent local anaesthetics widely used in humans for local and regional anaesthesia (Yapp et al. 2011). It is a unique amide type local anaesthetic with a thiophene ring (instead of the usual benzene ring) that increases its lipid solubility and potency (Oertel et al. 1997). Another important feature of articaine hydrochloride is the presence of an ester group in its structure which allows it to be rapidly hydrolysed by plasma esterases to an inactive metabolite, articainic acid (Oertel et al. 1997; Snoeck 2012). Pharmacokinetic studies in humans and red deer reported that articaine hydrochloride was rapidly hydrolyzed and eliminated from systemic circulation (Oertel et al. 1997; Snoeck 2012; Venkatachalam et al. 2018a). Several studies in humans have suggested that articaine hydrochloride was a safe and effective local anaesthetic (Oertel et al. 1997; Malmed et al. 2001; Snoeck 2012). Few comparative studies reported that articaine was better than lignocaine in terms of safety and efficacy (Katyal 2010). Based on the reported pharmacological properties of articaine the authors hypothesize that articaine hydrochloride can be safer than lignocaine hydrochloride for cornual nerve block in goat kids. Since there were no data on the maximum tolerated dose (MTD) of articaine in goat kids, a dose-ranging study was conducted following intravenous administration of various doses of articaine hydrochloride over 60 seconds.

The objectives of this study were to determine the maximum tolerated dose, investigate the safety and describe the pharmacokinetics of articaine hydrochloride in goat kids following intravenous administration, evaluate the anaesthetic efficacy and pharmacokinetics of

articaine following cornual nerve block in goat kids, and to determine the convulsive dose of articaine, and its corresponding plasma concentrations in goat kids.

1.3 Materials and methods

Drugs and chemicals

Articaine hydrochloride (99.9%) and articainic acid (97%) were purchased from SCI Pharmtech (Taoyuan, Taiwan) and Toronto Research Chemicals (Toronto, Canada), respectively. Acetonitrile, methanol, water and formic acid were purchased from Fisher Scientific (Auckland, NZ). Reagent grade potassium dihydrogenphosphate, phosphoric acid and perchloric acid (70%) were obtained from standard vendors. Milli-Q water was produced by Milli-q PFplus system, (Millipore Cooperation, Billerica, MA, USA). Articaine hydrochloride (40 mg mL^{-1} and 15 mg mL^{-1}) for injection was prepared fresh each day by weighing an appropriate amount of the drug and dissolving in sterile normal saline. The solution was filtered through a syringe filter (0.45 μm, Phenomenex Inc, Auckland, NZ) prior to injection.

Experimental animals

Twenty two healthy male goat kids (one or two days old) were purchased from a commercial dairy farm (Opiki, New Zealand) and transported to the Massey University farm facility (Palmerston North, NZ). Kids received artificial colostrum (Excel Plus Colostrum, Farmlands) on the day of arrival and then were fed milk replacer (Milligans milk powder, Farmlands) thrice daily using milk feeding bottles and buckets. Kids were housed in groups of six in pens with clean and dry straw bedding. The experimental protocol was reviewed and approved by the Massey University animal ethics committee (Protocol 16/19).

Experimental design

This study consisted of four experiments.

Experiment 1: Dose-ranging study of articaine hydrochloride

Four male Saanen male goat kids (8 days old, 2.5 to 3 kg) were used in this study. Kids were gently restrained, hair around one of the cephalic veins was clipped, and a 20 gauge, 48 mm intravenous catheter (BD Insyte, Sandy, UT, USA) was placed. Four doses (5, 8, 10 and 15 mg kg^{-1} bodyweight) of articaine hydrochloride were administered by intravenous infusion for over 60 seconds in goat kids (one animal/dose). Animals were visually observed for adverse signs such as sedation, ataxia, and convulsion. Kids were euthanized by intravenous injection of pentobarbitone sodium (100 mg kg^{-1} bodyweight) when convulsions were observed.

Experiment 2: Pharmacokinetics of articaine hydrochloride following intravenous administration

Six, healthy male Saanen goat kids (2-3 weeks old) weighing 3.8 to 5.6 kg were used to study the intravenous disposition and the safety of articaine hydrochloride. Kids were gently restrained, hair around both the left and right cephalic veins were clipped and 20 gauge, 48 mm intravenous catheters (BD Insyte, Sandy, UT, USA) were placed in both the veins. Articaine hydrochloride (4%) was administered via one of the intravenous catheters at a dose of 8 mg kg^{-1} over a period of 60 seconds using a syringe pump (World Precision Instruments, Sarasota, FL, USA). Blood samples were collected from the other intravenous catheter in heparinized BD vacutainer tubes at 0 (blank sample), 1, 5, 10, 30 minutes and 1, 2, 4 and 8 hours following drug administration. The collected samples were placed on ice immediately after collection, and plasma was separated within 30 minutes of collection. The plasma samples were stored at -20℃ until analysis using a high-performance liquid chromatography (HPLC) method. Animals were observed for local anaesthetic associated toxicity signs such

as sedation, ataxia, and convulsion during drug administration and at 0.5, 1 and 2 hours following drug administration.

Experiment 3: Pharmacokinetics and efficacy of articaine hydrochloride following cornual nerve block

Six goat kids (2-3 weeks old, weighing 3.5 to 5.2 kg) were individually restrained and one of the cephalic veins was catheterized aseptically using a 20 gauge, 48 mm intravenous catheter (BD Insyte, Sandy, UT, USA). Articaine hydrochloride (1.5%) was subcutaneously injected (0.5 mL per site) to block the cornual branches of the lacrimal and infratrochlear nerves (Smith & Sherman 2009). Briefly, kids were gently restrained, using a 25 G needle the first injection (0.5 mL) was made by inserting the needle behind the caudal ridge of the supraorbital process halfway between the horn bud and lateral canthus of the eye, and the other injection (0.5 mL) was applied as a line block on the dorsomedial rim of the orbit (Smith & Sherman 2009). Following injection, the nerve blockade was evaluated by needle-prick or pin-prick test at 1-minute interval until no response was observed. This test involved gentle needle prick (20 G) on the skin around the horn buds and observation for withdrawal response (movement of the head away from the needle or head shaking). When no response to needle prick was observed, disbudding was carried out using a gas dehorner (Portasol, Elmira, OR, USA). Blood samples were collected at 0 (blank), 5, 10, 20, 30, 40 minutes and 1, 2, 4, 6 and 12 hours via the intravenous catheter following drug administration. Plasma was harvested from the blood samples and was stored at -20℃ until analysis using an HPLC method. Goat kids were observed in the pens for pain-related behaviours such as head shaking, head scratching, head rubbing and body shaking, and for local anaesthetic associated toxicity signs such as sedation, ataxia, and convulsion for 4 hours following drug administration.

Experiment 4: Determination of toxic dose of articaine hydrochloride and its corresponding plasma concentrations

Six goat kids (3-4 weeks old, weighing 5.8 to 7.4 kg) were used for this experiment. Both cephalic vein and saphenous vein were catheterized using intravenous catheters (20 gauge, 48 mm intravenous catheter, BD Insyte, Sandy, UT, USA). Via the catheter in the cephalic vein, articaine hydrochloride (4%) was administered at a dose rate of 4 mg kg^{-1} $minute^{-1}$ using a syringe pump (World Precision Instruments, Sarasota, FL, USA) until convulsions were seen. Convulsions were used as the endpoint of toxicity because it was difficult to determine the precise onset of other earlier toxicity signs like sedation or ataxia. Once convulsions were noticed drug administration was terminated and animals were euthanized by injecting pentobarbitone sodium (100 mg kg^{-1} bodyweight IV). Blood samples were collected from saphenous vein via the catheter prior to drug administration, at 1-minute intervals and at the time of onset of convulsion. The total dose administered and the time of onset of convulsions were recorded. Plasma was harvested from the collected blood samples and stored at -20℃ until analysis using a liquid chromatography-mass spectrometry (LC-MS) method.

Determination of plasma concentrations

The concentrations of articaine hydrochloride in the plasma samples collected for studying the pharmacokinetics were quantified using a modified HPLC method previously reported by Richter & Oertel (1999). The convulsive plasma concentrations of articaine and its metabolite articainic acid following intravenous administration were determined using an LC-MS/MS method.

Standard stock solutions (1 mg mL^{-1}) of articaine hydrochloride and articainic acid were prepared by dissolving the standards in water and methanol, respectively, and the working standard solutions were prepared by serially diluting the standard solutions in water.

Calibration standards and quality control samples were prepared freshly by adding working standard solutions to pooled goat plasma collected from untreated animals.

The HPLC (Shimadzu Corporation, Kyoto, Japan) analysis was carried out using C_{18} reversed-phase column (Synergi Hydro-RP Column 250 x 4.6 mm, 4 µm), and phosphate buffer (pH 3.0, adjusted using phosphoric acid) and acetonitrile (87:13%) as mobile phase. To 1 mL of plasma, 50 µL of perchloric acid was added and vortexed for 10 seconds. After 10 minutes, the samples were vortexed again and centrifuged at 4500 g for 10 minutes. The supernatant (800 µL) was collected and evaporated under a stream of air (70℃) and reconstituted in the mobile phase (100 µL). The volume of injection was 50 µL and the UV wavelength was set at 274 nm.

The LC-MS/MS analysis was carried out using an Ultra High Performance Liquid Chromatography coupled with a hybrid quadrupole orbitrap mass spectrometer (Thermo Scientific Q Exactive Focus Orbitrap system). Plasma samples (200 µL) were taken in 1.5 mL microcentrifuge tubes and methanol (600 µL) was added to precipitate the proteins. The samples were vortexed for 10 minutes and centrifuged at 4500 g. The supernatant was taken in phospholipid removal tubes (Phree tubes, Phenomenex) and centrifuged at 250 g for 5 minutes. The collected eluent was injected (10 µL) on to a C_{18} column (100 mm × 2.1 mm; Accucore, Auckland, NZ) maintained at a temperature of 25°C. The mobile phase consisted of acetonitrile (30%) and 0.1% formic acid in water (70%), delivered at a 0.3 mL $minute^{-1}$. Positive ion electrospray ionisation-mass spectrometry was used for the analysis of both articaine and articainic acid. Analytes were quantified using Parallel Reaction Monitoring (PRM) mode using the transitions of mass to charge ratio (m/Z) 285.12 → 86.1 for articaine, and m/Z 271.11 → 86.1 for articainic acid.

Please refer appendix for HPLC and LC-MS validation parameters.

Pharmacokinetic analysis

Pharmacokinetic parameters were non-compartmentally calculated using PKSolver add-on (Zhang et al. 2010) for Excel 2010 (Microsoft, Redmond, CA, USA). The maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were evaluated from the plasma concetration vs time curve. The elimination rate constant (λ_z) was estimated by linear regression of the logarithmic plasma concentration and the terminal half-life ($t_{1/2\ \lambda z}$) as calculated using the formula $t_{1/2\ \lambda z} = 0.693/\lambda_z$. The area under the curve (AUC) and the area under the first moment curve (AUMC) were determined by the linear trapezoidal rule. Mean residence time was obtained from AUMC/AUC and clearance was calculated as Dose/$AUC_{0-\infty}$.

1.4 Results

The standard curves were linear with correlation coefficients values (r^2) above 0.998 for both articaine and articainic acid. The lower limit of quantification (LLOQ) of articaine was 10 ng mL^{-1} and 1 ng mL^{-1} using HPLC and LC-MS/MS method, respectively. The LLOQ of articainic acid was 1 ng/mL. The lowest concentration of the analyte with less than 20% variation was considered as LLOQ. The intra- and inter-day co-efficient of variations (%CV) were ≤10.93% and ≤17.17%, respectively. Both precision and accuracy were within the acceptable criteria of <10% for inter-day and <15% for inter-day (except LLOQ where <20% is acceptable) assay (Jenkins et al 2015). The extraction recoveries were above 57.58% for articaine and 77.31% for articainic acid.

No adverse effects were observed following the intravenous administration of 5 or 8 mg kg^{-1} body weight of articaine hydrochloride but after the administration of 10 mg kg^{-1}, mild sedation was observed. During the administration of 15 mg kg^{-1}, sedation followed by ataxia and tonic-clonic convulsions were observed. The maximum dose of articaine hydrochloride

that did not produce any adverse effects was 8 mg kg^{-1}. Intravenous administration of 8 mg kg^{-1} articaine hydrochloride over 60 seconds and cornual nerve block using 1.5% articaine hydrochloride (0.5 mL per site) were tolerated by goat kids without any signs of local anaesthetic associated toxicity. The total dose of articaine hydrochloride administered for cornual nerve block was 7.13 ± 1.11 mg kg^{-1} (range 5.76 - 8.57 mg kg^{-1}). Semi-logarithmic plot of plasma concentration vs time of articaine after intravenous and cornual nerve block are shown in Figure. 2.1. The pharmacokinetic data following intravenous and cornual nerve block are listed in Table 2.1. Anaesthesia of horn buds was evident within 4 minutes following cornual nerve block (0.5 mL/site) using 1.5% articaine hydrochloride. Absence of withdrawal response and vocalization during disbudding confirmed the analgesic efficacy of articaine. Post-procedure observation of animals revealed pain-related behaviours such as head scratching and head shaking 25 minutes after cornual nerve block.

The toxicity signs observed during intravenous infusion of articaine hydrochloride (4 mg kg^{-1} $minute^{-1}$) include sedation, ataxia, and tonic-clonic convulsions. Two of the animals showed teeth grinding in addition to these signs. Individual animal doses and plasma concentrations of articaine and articainic acid that produced convulsions in goat kids are listed in Table 2.2. The average dose required to produce convulsion in goat kids was 16.24 ± 1.79 mg kg^{-1} and the average plasma concentrations of articaine and articainic acid at convulsions were 9.90 ± 2.38 µg mL^{-1} and 1.52 ± 0.91 µg mL^{-1}, respectively.

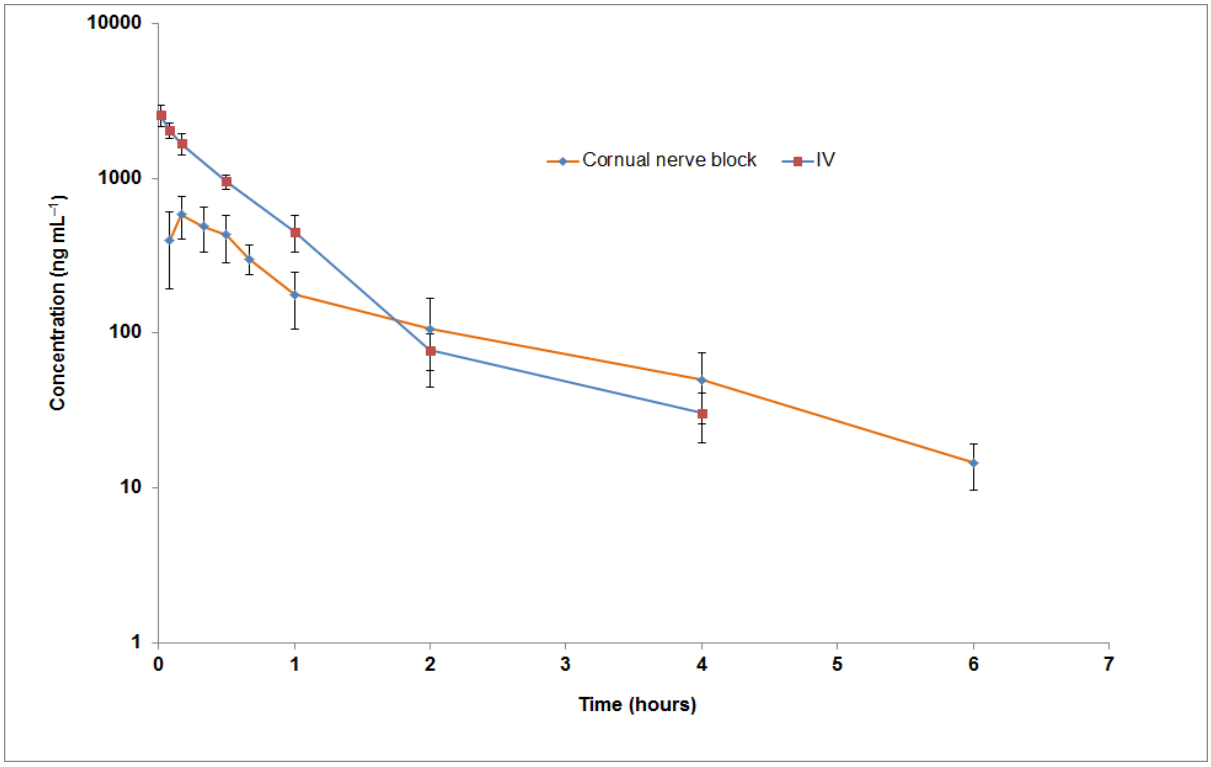

Fig 2.1. Semi-logarithmic plot of mean plasma articaine concentration vs time following intravenous administration of articaine hydrochloride (8 mg kg^{-1} over 60 seconds), and cornual nerve block using 1.5% articaine hydrochloride (0.5 mL per site) in goat kids

Table 2.1. Pharmacokinetic parameters (mean ± SD) of articaine hydrochloride following intravenous administration of articaine hydrochloride (8 mg kg^{-1} over 60 seconds), and cornual nerve block using 1.5% articaine hydrochloride (0.5 mL per site) in goat kids

Parameters	Intravenous (n=6)	Cornual nerve block (n=6)
C_{max} (ng mL^{-1})	-	586.58 ± 175.10
T_{max} (hours)	-	0.22 ± 0.09
AUC (hour ng mL^{-1})	1519.95 ± 190.59	749.28 ± 278.93
MRT (hour)	0.72 ± 0.08	1.62 ± 0.36
$t_{½\lambda z}$ (hour)	0.66 ± 0.14	1.26 ± 0.34
V_d (L kg^{-1})	5.14 ± 1.66	-
CL (L $hour^{-1}$ kg^{-1})	5.33 ± 0.66	-

C_{max}, maximum plasma concentration; T_{max}, time of C_{max}; AUC, area under the curve; MRT, mean residence time; $t_{½\lambda z}$, elimination half-life; V_d, apparent volume of distribution; CL, total body clearance

Table 2.2. Individual animal convulsive dose and its corresponding plasma concentration of articaine and articainic acid following intravenous infusion of articaine hydrochloride (4 mg kg^{-1} minute^{-1}) in goat kids

Animals	Total Dose (mg kg^{-1})	Plasma concentration of articaine (µg mL^{-1})	Plasma concentration of articainic acid (µg mL^{-1})
1	15.33	13.04	1.00
2	17.33	7.10	3.18
3	13.81	8.55	0.51
4	15.40	11.58	1.30
5	18.93	11.29	1.35
6	16.66	7.87	1.76
Mean ± SD	16.24 ± 1.79	9.90 ± 2.38	1.52 ± 0.91

1.5 Discussion

Both the analytical methods were sensitive, linear, accurate, and repeatable for the quantification of articaine and articainic acid in goat plasma. However, the LC-MS/MS method was simple to use and faster compared to the HPLC method for the analysis of analytes. The extraction recoveries (57%) of articaine from goat plasma using the sample preparation methods used in this study were low and variable. Low extraction recoveries (~40%) of articaine from human plasma have been reported following a liquid-liquid extraction method (Hoizey *et al.* 2009). Good extraction recoveries (72%) from human serum were obtained using an automated solid phase extraction (SPE) procedure (Richter & Oertel 1999). Solid phase extraction method described by Richter & Oertel (1999) was not used in this study because it was not possible to procure the special disk-cartridges for SPE. One of the major limitations of the analytical methods was that no internal standards were used in the analysis. An internal standard is a chemical substance that is added in a constant amount to

samples, blank and calibration standards to correct for variations in the instrument response, normalization of recovery differences and to correct for the loss of analyte during sample preparation, injection and ionization (Wang et al. 2017). Future studies should use internal standards to improve the accuracy of the analytical methods.

This is the first study to describe the maximum tolerated dose (MTD), pharmacokinetics, toxicity, and efficacy of articaine hydrochloride in goat kids. The results of the dose-ranging study suggest that articaine hydrochloride appears to be safe up to 8 mg kg^{-1}. Since the sample size used in the dose-ranging study was low (one animal per dose), the present study was conducted to confirm the safety of the MTD (8 mg kg^{-1}) of articaine was studied in six more animals. Similar to the results of the dose-ranging study no local anaesthetic associated toxicity signs were observed in any of the goat kids following intravenous administration of 8 mg kg^{-1} over 60 seconds. In adult dogs, repeated intravenous injection of 10 mg kg^{-1} day^{-1} articaine hydrochloride for 30 days was reported to be tolerated. The no-observed-adverse-effect-level (NOAEL) following subcutaneous administration was determined to be 40 mg kg^{-1} in dogs (FDA 2000). The maximum recommended dose of articaine hydrochloride in humans is 7 mg kg^{-1} which is three times less than the NOAEL value observed in dogs (FDA 2000; Scully 2014). The results of the present study and the dose-ranging study suggest that doses up to 8 mg kg^{-1} should be safe for perineural injections in goat kids.

Following cornual nerve block using 1.5% articaine hydrochloride (0.5 mL/site), rapid absorption of articaine was observed with a mean C_{max} 586.58 ± 175.10 ng mL^{-1} occurring at 0.22 ± 0.09 h (T_{max}). Rapid absorption of articaine has also been reported in red deer (Venkatachalam et al. 2018a) and humans (Snoeck 2012). This feature could be because of the vasolidatory property, similar to most of the local anaesthetics (Becker & Reed 2006). Rapid absorption has also been reported for lignocaine hydrochloride following cornual nerve

block in goat kids (Venkatachalam et al. 2018b). The C_{max} (586.58 ± 175.10 ng mL^{-1}) following cornual nerve block was around four times less than the concentration (2558.82 ± 412.85 ng mL^{-1}) observed at one minute following intravenous administration of 8 mg kg^{-1} for over 60 seconds (the maximum dose that did not show any toxicity signs).

The short elimination half-lives ($t_{½\lambda z}$) of articaine following intravenous administration (0.66 ± 0.14 h) and cornual nerve block (1.26 ± 0.34 h) indicate that articaine was rapidly eliminated following systemic absorption. Rapid elimination has also been reported in deer following subcutaneous administration (Venkatachalam et al. 2018a) and following various routes of administration in humans (Oertel et al. 1997). In comparison, the half-lives of lignocaine (1.71 ± 0.51 h) and its active metabolite, monoethylglycinexylidide (3.22 ± 1.21 h) following cornual nerve block of 1% lignocaine hydrochloride in goat kids were longer than articaine (Venkatachalam et al. 2018b). Rapid elimination of articaine can be attributed to its property to undergo rapid hydrolysis by plasma esterases to articainic acid (Oertel et al. 1997; Snoeck 2012). Articainic acid is an inactive metabolite whereas the primary metabolites of lignocaine are active and can increase the risk of toxicity during accidental intravenous administration or overdosage (Strong et al. 1975; Yapp et al. 2011). Post hoc power analysis using the data from the toxicity and pharmacokinetic studies suggest that the sample size used in these studies were sufficient with >95% power.

Plasma clearance of articaine was rapid in goat kids with a mean CLss of 5.33 ± 0.66 L kg^{-1}. High clearance of articaine has also been reported in humans (Jakobs et al. 1995). In a comparative study, the clearance of articaine was found to be 10 times greater than that of lignocaine in humans (Simon et al. 1998). Pharmacokinetics properties of articaine hydrochloride such as rapid hydrolysis to an inactive metabolite, and rapid elimination indicate that articaine may be safer than lignocaine for cornual nerve block in goat kids.

Because of its wider margin of safety articaine is clinically used as 4% solution whereas lignocaine is used as 2% solution (Oertel et al. 1997).

The needle-prick test suggests that analgesia was produced within 4 minutes following cornual nerve block using 1.5% articaine hydrochloride (0.5 mL per site) in goat kids. The pH of the articaine hydrochloride solution used in this study was 5.0. A more rapid onset of action would have been obtained if the pH of the drug solution was adjusted to neutral pH (7.4). Absence of pain-related behaviours such as head shaking, vocalization or struggles during the placement of disbudder or the destruction of horn buds confirms the analgesic efficacy of articaine. However, it must be noted that the injection of the drug at four sites to desensitize both the horn buds was stressful and painful to the animals as vocalization and struggles were observed during the injection. Sedation of animals prior to the injection of local anaesthetics may reduce this stress and pain.

The expression of pain-related behaviours such as head scratching and rubbing following disbudding indicate that 1.5% articaine could provide analgesia for only up to 25 minutes. Similar results were observed following cornual nerve block using 1% lignocaine hydrochloride in goat kids (Venkatachalam et al. 2018b). Therefore, post-operative analgesia is required in addition to local anaesthetic nerve blockade for disbudding in goat kids as behavioural and cortisol responses suggest that animals experience pain for at least 2 hours following thermal cautery disbudding in goat kids (Alvarz et al. 2015, Hempstead et al. 2017). Increasing the concentration and injection volume of articaine hydrochloride may increase the analgesic duration however, further studies are required to evaluate the safety and duration of analgesia of the dose.

The toxic dose of articaine hydrochloride and its corresponding plasma concentrations were determined to assess the margin of safety of the dose used for cornual nerve block in this

study. Following continuous intravenous infusion of 4% articaine hydrochloride at 4 mg kg^{-1} $minute^{-1}$, an identical sequence of toxicity was exhibited by all the animals. Initially, sedation, followed by ataxia, and tonic-clonic convulsions were observed. The mean convulsive plasma concentration of articaine (9.90 ± 2.38 µg mL^{-1}) was less than that reported for lignocaine (13.59 ±2.34 µg mL^{-1}), although the total dose required to produce convulsions was higher for articaine (16.24 ± 1.79 mg kg^{-1}) than lignocaine (13.59 ± 2.34 mg kg^{-1}) (Venkatachalam et al. 2018b). The mean convulsive plasma concentration of articaine (9.90 ± 2.38 µg mL^{-1}) was almost 16 times less than the C_{max} (586.58 ± 175.10 ng mL^{-1}) observed following cornual nerve block. This suggests that injection of 1.5% articaine hydrochloride (0.5 mL per site) for cornual nerve block should be safe in goat kids.

This study has determined the plasma concentrations of both bound and unbound articaine. Since at steady state, the toxic effects of local anaesthetics are directly related to the unbound drug concentration, it is important to measure the concentrations of the unbound drug to interpret the toxicity data. Future studies should investigate the in vitro and in vivo protein binding of articaine in goat kids to determine the unbound drug concentration. One of the limitations of this study was that the respiratory and cardiovascular parameters (respiratory rate, oxygen saturation of arterial blood and heart rate) were not monitored. A pulse oximeter was used during intravenous infusion of articaine hydrochloride but due to the movement of the animals during convulsions, accurate readings could not be recorded. However, it must be noted that during local anaesthetic toxicity, central nervous system (CNS) toxicity signs appear prior to cardiovascular toxicity signs as CNS is more sensitive than cardiovascular system to local anaesthetics (Malavasi et al. 2016).

In summary, the results of the present study suggest that articaine hydrochloride has favourable pharmacokinetic properties such as rapid hydrolysis to inactive metabolite, rapid

elimination, and high plasma clearance compared to lignocaine in goat kids. These pharmacokinetic properties and the requirement of high convulsive dose compared to lignocaine indicate that articaine could be a safer alternative to lignocaine in goat kids. Cornual nerve block (0.5 mL/site) using 1.5% articaine hydrochloride appears to be safe and effective in alleviating the acute pain during disbudding in goat kids. Even though articaine hydrochloride is commonly used in people it has not yet been approved for use in veterinary medicine. Further studies in a larger population to investigate the safety and efficacy of different doses of articaine are required before recommending the drug for cornual nerve block in goat kids.

1.6 Acknowledgement

This study was supported by Massey University Post Graduate research fund. The authors wish to thank Neil Ward and Anne Tunnicliffe for animal care and support.

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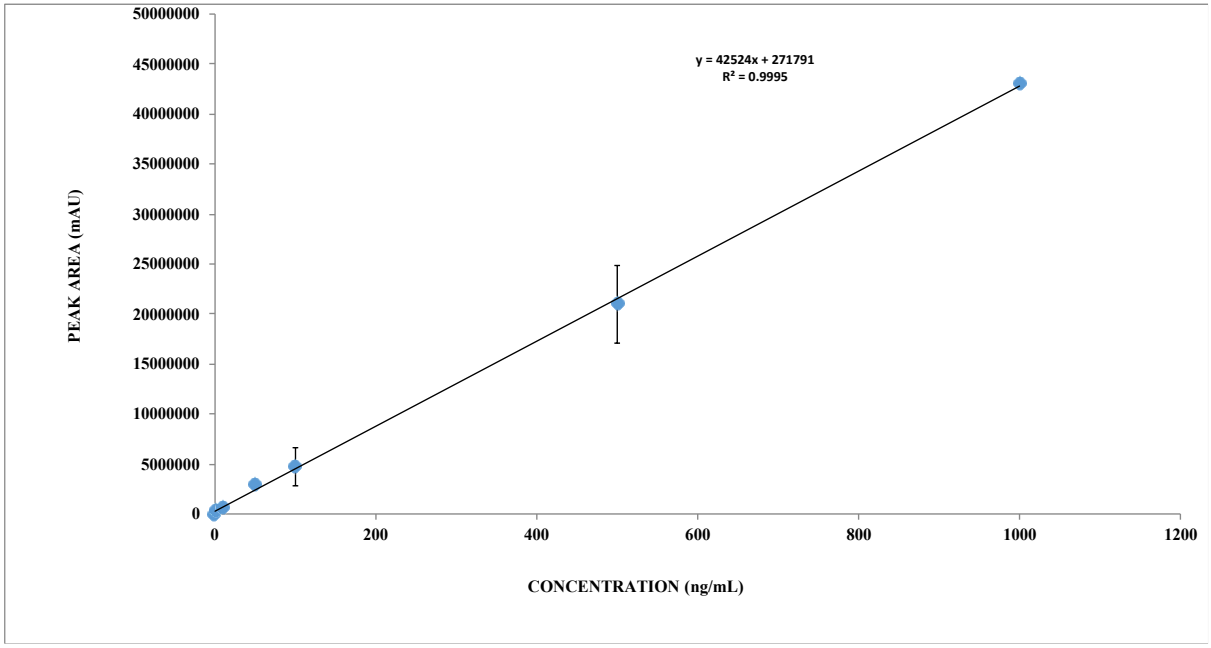
1.8 Appendix

LC-MS method validation

Figure 2.2: LC-MS calibration curves (n=3) of (a) articaine hydrochloride and (b) articainic acid constructed by spiking pooled plasma from untreated goat kids with different concentrations of each analyte.

a) Articaine



b) Articainic acid

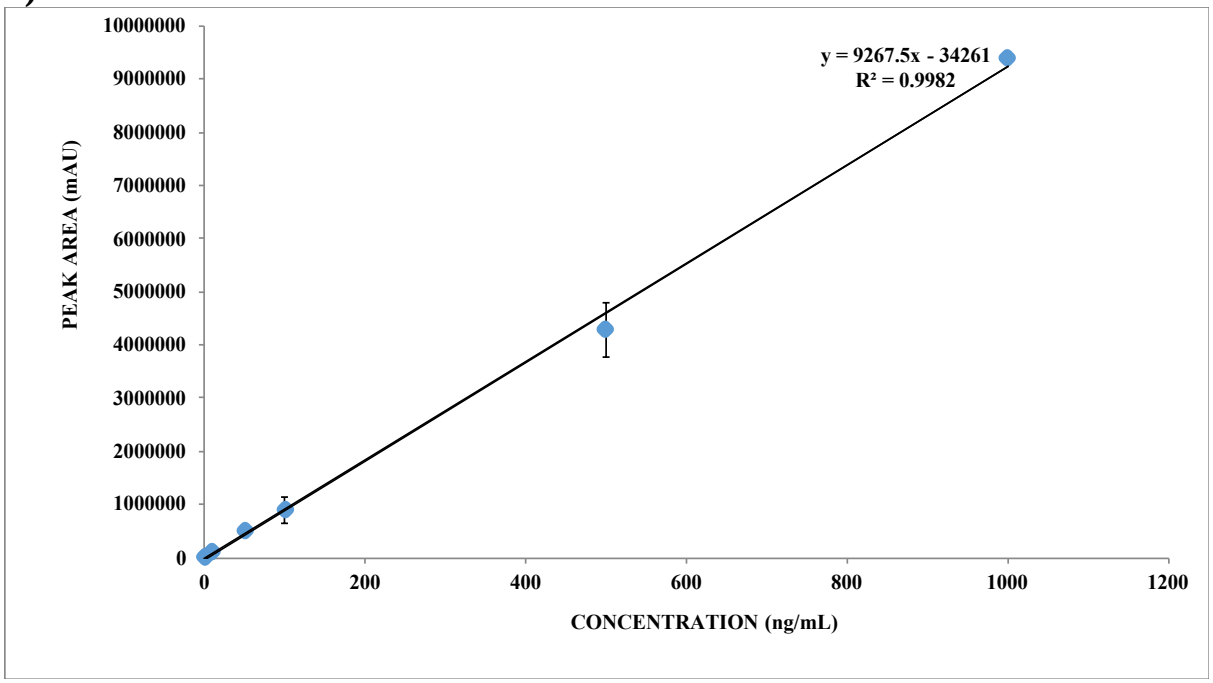


Table 2.3. Intra- and inter-day accuracy and precision of articaine in goat plasma.

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-day	1	0.99 ± 0.09	9.54	-0.60
	10	10.85 ± 1.19	10.93	8.55
	100	102.62 ± 4.90	4.78	2.62
	1000	959.00 ± 81.78	8.53	-4.10
Inter-day	1	1.03 ± 0.14	13.29	3.34
	10	11.29 ± 1.39	12.31	12.91
	100	106.46 ± 11.97	11.24	6.46
	1000	1103.85 ± 167.11	15.14	10.38

CV% - Percent coefficient of variation = (Standard deviation/measured mean concentration) × 100 (%).

RE% - Percent relative error = (Measured mean concentration - theoretical concentration/theoretical concentration) × 100 (%).

Table 2.4. Intra and inter-day accuracy and precision of articainic acid in goat plasma.

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-day	1	1.19 ± 0.06	4.93	18.83
	10	9.69 ± 0.64	6.59	-3.08
	100	102.09 ± 5.36	5.25	2.09
	1000	925.80 ± 39.40	4.26	-7.42
Inter-day	1	0.97 ± 0.17	17.17	-2.80
	10	7.75 ± 1.01	4.64	-16.94
	100	104.68 ± 9.00	8.60	4.68
	1000	1082.82 ± 146.52	13.53	8.28

Table 2.5. Extraction recoveries of articaine and articainic acid from goat plasma.

Concentration (ng/mL)	Articaine (%)	Articainic acid (%)
10	57.58 ± 1.70	77.31 ± 2.90
100	82.46 ± 0.40	84.59 ± 5.78
1000	78.02 ± 0.29	94.69 ± 1.11

HPLC method validation

Figure 2.3: HPLC calibration curve (n=3) of articaine hydrochloride constructed by spiking pooled plasma from untreated goat kids with different concentrations of articaine hydrochloride.

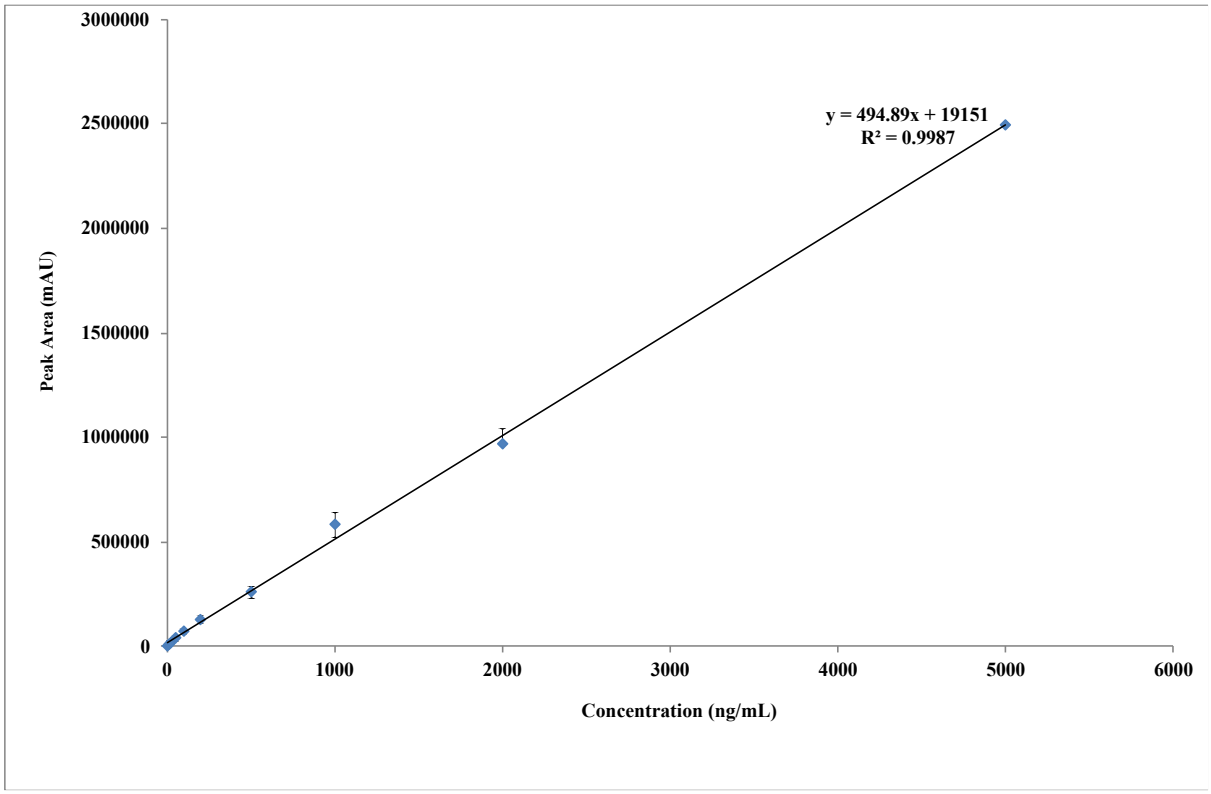


Table 2.6. Intra- and inter-day accuracy and precision of articaine in goat plasma.

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	50	55.69 ± 2.99	5.36	11.39
	500	557.98 ± 24.91	4.46	11.60
	1000	927.56 ± 29.56	3.19	-7.24
	2000	2120.00 ± 153.26	7.23	6.00
Inter-assay	50	49.98 ± 1.87	3.74	-0.05
	500	536.44 ± 27.60	5.15	7.29
	1000	929.26 ± 30.71	3.31	-7.07
	2000	2091.05 ± 107.42	5.14	4.55

CV% - Percent coefficient of variation = (Standard deviation/measured mean concentration) × 100 (%).
RE% - Percent relative error = (Measured mean concentration - theoretical concentration/theoretical concentration) × 100 (%).

Table 2.7. Extraction recovery (%) of articaine from goat kids.

Articaine concentration	Recovery % (Mean ± SD)
10	60.96 ± 10.87
50	79.35 ± 5.60
500	81.60 ± 0.22
1000	78.94 ± 5.12

Plasma concentrations and pharmacokinetic parameters

Table 2.8. Plasma concentrations of articaine following cornual nerve block using 1.5% articaine hydrochloride (0.5 mL per site) in six goat kids.

Time points (minutes)	1	2	3	4	5	6	Mean ± SD
5	122.23	614.73	666.43	284.58	391.10	315.43	399.08 ± 207.27
10	704.41	732.04	776.44	356.14	496.65	417.66	580.56 ± 179.22
20	307.90	636.05	701.23	341.70	521.64	428.82	489.56 ± 158.65
30	285.78	509.71	664.28	313.30	480.35	336.21	431.61 ± 146.27
40	276.01	344.69	414.83	244.36	273.67	259.54	302.18 ± 65.05
60	178.48	207.57	299.23	159.26	122.00	98.65	177.53 ± 71.21
120	88.85	128.99	216.93	97.21	75.17	34.03	106.86 ± 62.18
240	57.15	56.48	86.44	50.43	33.10	15.81	49.90 ± 23.98
360	9.92	10.90	18.06	19.16	BQL	BQL	14.51 ± 4.77

BQL –Below quantification limit

Table 2.9. Plasma concentrations of articaine following intravenous administration of articaine hydrochloride (8 mg kg^{-1} over 60 seconds) in six goat kids.

Time points (min-utes)	1	2	3	4	5	6	Mean ± SD
1	2214.47	2039.07	2580.14	2600.06	2694.86	3224.30	2558.82 ± 412.85
5	1808.36	-*	1956.82	2004.05	2010.39	2402.45	2036.42 ± 220.25
10	1681.77	-*	1297.74	1853.91	1586.95	1969.18	1677.91 ± 259.15
30	951.43	975.59	838.42	832.96	1045.89	1065.37	951.61 ± 99.2
60	467.94	552.30	261.89	490.68	366.37	573.54	452.12 ± 118.37
120	63.31	69.09	88.82	103.23	49.31	92.37	77.69 ± 20.38
240	22.12	29.07	26.16	40.42	18.51	46.47	30.46 ± 10.85

-* - Outlier (concentrations were too high)

Table 2.10. Pharmacokinetic parameters of articaine following cornual nerve block using 1.5% articaine hydrochloride (0.5 mL per site) in goat kids (n=6).

Parameters	1	2	3	4	5	6	Mean ± SD
C_{max} (ng mL^{-1})	704.41	732.04	776.44	356.14	521.64	428.82	586.58 ± 175.10
T_{max} (minute)	0.17	0.17	0.17	0.17	0.33	0.33	0.22 ± 0.09
$t_{½\lambda z}$ (hour)	1.22	1.16	1.14	1.68	1.61	0.77	1.62 ± 0.36
AUC (hour ng mL^{-1})	660.08	893.89	1229.53	657.08	634.03	421.07	749.28 ± 278.93
AUMC (hour ng mL^{-1})	1139.88	1337.82	2062.49	1404.03	1051.95	426.84	1237.17 ± 532.83
MRT (hour)	1.73	1.50	1.68	2.14	1.66	1.01	1.26 ± 0.34

Table 2.11. Pharmacokinetic parameters of articaine following intravenous administration of articaine hydrochloride (8 mg kg^{-1} over 60 seconds) in goat kids (n=6).

Parameters	1	2	3	4	5	6	Mean ± SD
$t_{½\lambda z}$ (hour)	0.57	0.63	0.93	0.65	0.54	0.64	0.66 ± 0.14
AUC (hour ng mL^{-1})	1461.02	1562.90	1264.88	1593.28	1411.05	1826.55	1519.95 ± 190.59
AUMC (hour ng mL^{-1})	987.07	1116.49	988.75	1279.75	852.57	1410.72	1105.90 ± 207.49
MRT (hour)	0.67	0.71	0.77	0.79	0.60	0.76	0.72 ± 0.08
Vd (L kg^{-1})	4.53	4.67	8.48	4.73	4.39	4.02	5.14 ± 1.66
CL (L $hour^{-1}$ kg^{-1})	5.48	5.12	6.32	5.02	5.67	4.38	5.33 ± 0.66

HPLC and LC-MS chromatograms

Figure 2.4: Overlay of HPLC chromatograms obtained following injection of untreated goat kid plasma sample (Green), untreated plasma spiked with 50 ng/mL of articaine hydrochloride (blue) and plasma sample collected at 4 hours following cornual nerve block in a goat kid (golden).

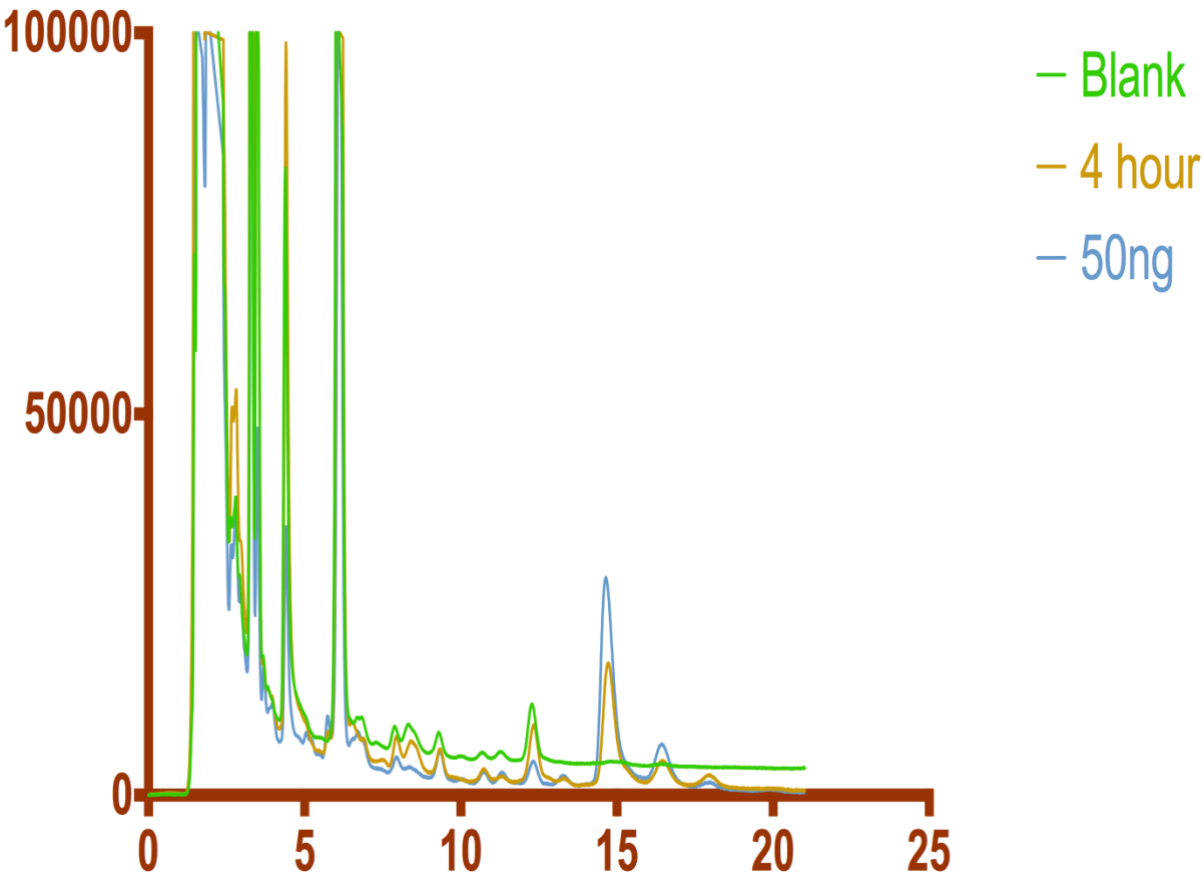
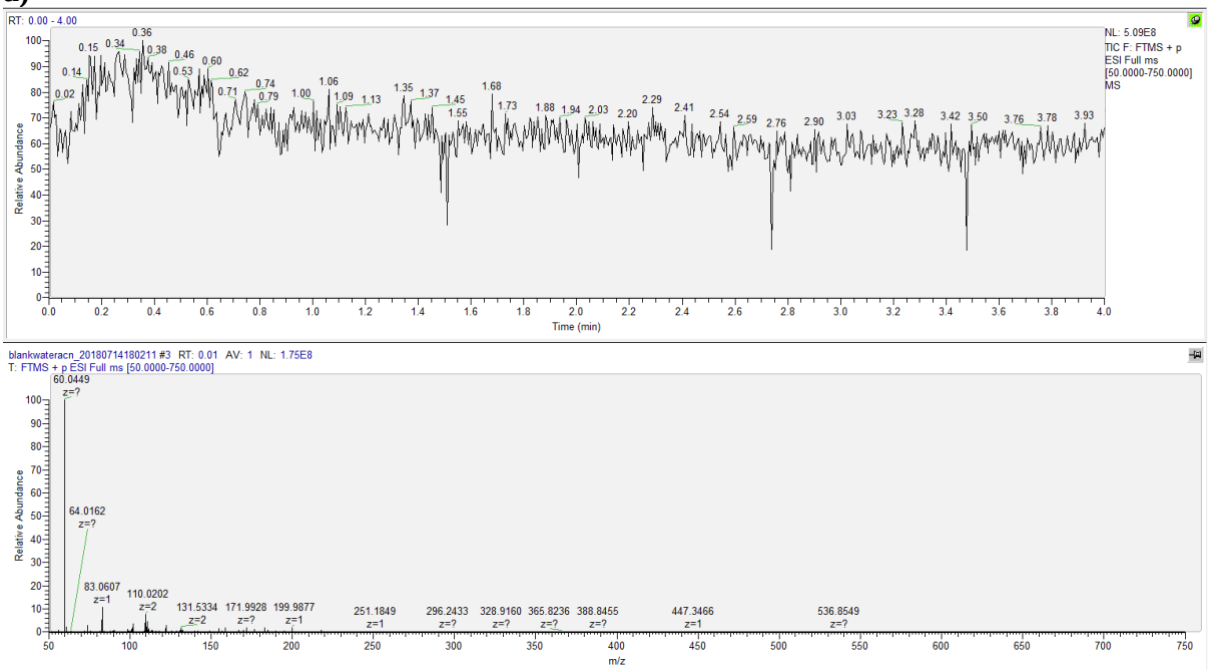
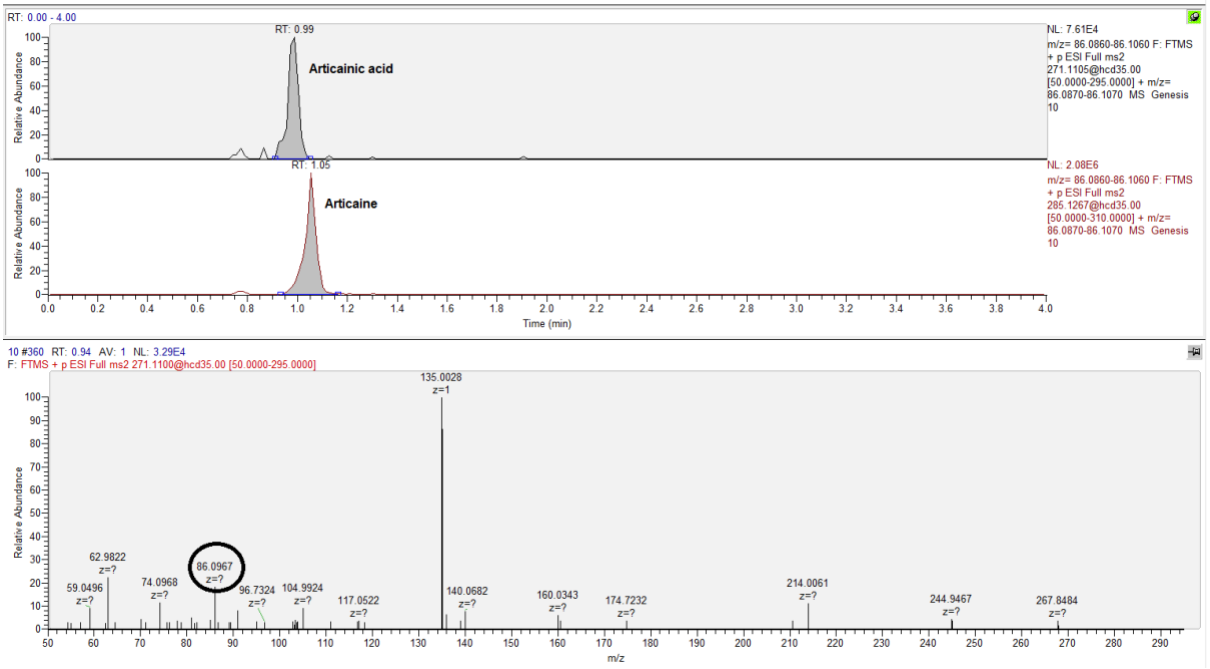


Figure 2.5: LC-MS chromatograms and electrospray ionization (PRM mode) of (a) plasma from untreated goat kids, (b) untreated plasma spiked with 10 ng mL^{-1} articaine and articainic acid added, and (c) plasma from a goat kid following intravenous infusion of articaine hydrochloride (4 mg kg^{-1} minute^{-1}).

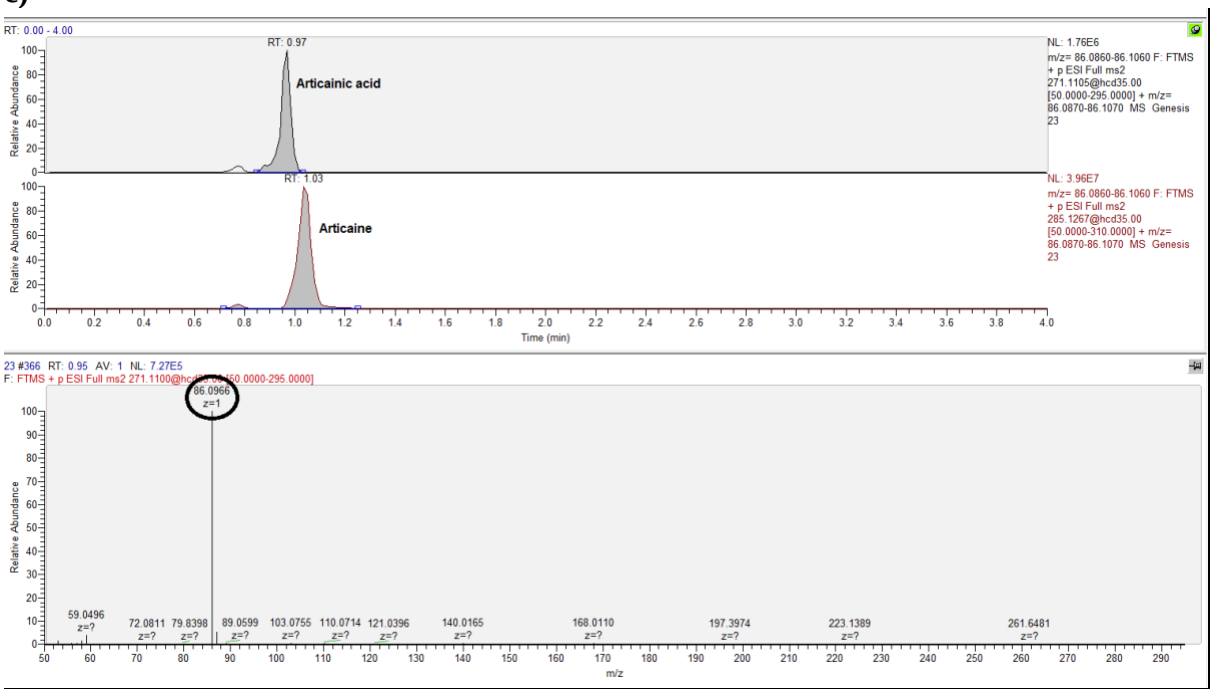
a)



b)



c)


RT: 0.00 - 4.00
RT: 0.97
Articainic acid
NL: 1.76E6
m/z= 86.0860-86.1060 F: FTMS + p ESI Full ms2 271.1105@hcd35.00 [50.0000-295.0000] + m/z= 86.0870-86.1070 MS Genesis 23
RT: 1.03
Articaine
NL: 3.96E7
m/z= 86.0860-86.1060 F: FTMS + p ESI Full ms2 285.1267@hcd35.00 [50.0000-310.0000] + m/z= 86.0870-86.1070 MS Genesis 23
Relative Abundance
Time (min)
23 #366 RT: 0.95 AV: 1 NL: 7.27E5
F: FTMS + p ESI Full ms2 271.1100@hcd35.00 [50.0000-295.0000]
86.0966 z=1
59.0496 z=?
72.0811 z=?
79.8398 z=?
89.0599 z=?
103.0755 z=?
110.0714 z=?
121.0396 z=?
140.0165 z=?
168.0110 z=?
197.3974 z=?
223.1389 z=?
261.6481 z=?
m/z

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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:	DINAKARAN VENKATACHALAM
Name/title of Primary Supervisor:	Dr. PREET SINGH
Name of Research Output and full reference:	
Venkatachalam D, Chambers JP, Kongara K, Singh P. Pharmacokinetics, toxicity, and efficacy of articaine hydrochloride in goat kids.	
In which Chapter is the Manuscript /Published work:	Chapter 2
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:	
Dinakaran had a primary role in study design, data collection, analysis, interpretation, and writing of the manuscript, with guidance from supervisors.	
For manuscripts intended for publication please indicate target journal:	
Veterinary Anaesthesia and Analgesia	
Candidate's Signature:	V. Dinakaran
Date:	26-04-2019
Primary Supervisor's Signature:	
Date:	26/April/2019

(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)

GRS Version 4– January 2019

Name of research output and full reference: Venkatachalam D, Chambers JP, Kongara K, Singh P. Pharmacokinetics, toxicity, and efficacy of articaine hydrochloride in goat kids.

CHAPTER 3

LIGNOCAINE HYDROCHLORIDE FOR DISBUDDING IN GOAT KIDS

This chapter was published in the journal "*Animals"*

Venkatachalam D, Chambers JP, Kongara K, Singh P (2018). Toxicity and pharmacokinetic studies of lidocaine and its active metabolite, monoethylglycinexylidide, in goat kids. *Animals*, 8(8): 142.

Toxicity and pharmacokinetic studies of lidocaine and its active metabolite, monoethylglycinexylidide, in goat kids

3.1 Abstract

This study determined the convulsant plasma concentrations and pharmacokinetic parameters of lidocaine and compared the results to recommend a safe dose of lidocaine hydrochloride for goat kids. The plasma concentrations of lidocaine and monoethylglycinexylidide (MGX) were quantified using liquid chromatography-mass spectrometry. A total dose of 7 mg/kg body weight (BW) was tolerated and should therefore be safe for local and regional anesthesia in goat kids. The mean plasma concentration and mean total dose that produced convulsions in goat kids were 13.59 ± 2.34 µg/mL and 12.31 ± 1.42 mg/kg BW (mean ± S.D.), respectively. The absorption of lidocaine following subcutaneous administration was rapid with C_{max} and T_{max} of 2.12 ± 0.81 µg/mL and 0.33 ± 0.11 h, respectively. The elimination half-lives ($t_{1/2\,\lambda z}$) of lidocaine hydrochloride and MGX were 1.71 ± 0.51 h and 3.19 ± 1.21 h, respectively. Injection of 1% lidocaine hydrochloride (0.5 mL/site) was safe and effective in blocking the nerves supplying horn buds in goat kids.

Keywords: disbudding; goat kids; lidocaine hydrochloride; toxicity; pharmacokinetics

3.2 Introduction

Disbudding is performed in domestic ruminants to prevent injuries among handlers or herd mates, to avoid damage to farm facilities, and to facilitate the use of head bails [1–4]. Additionally, hornless animals require less feeding trough space and are easier to handle and transport than horned animals [2,5]. Disbudding in dairy goats is becoming a routine husbandry procedure even though it is a very stressful and painful procedure when performed without appropriate pain relief [6,7]. Selective breeding for polledness can eliminate the need for disbudding, but in certain breeds of goats (Saanen, Alpine, and Toggenburg), the polled

condition is associated with serious reproductive disorders in both sexes. Therefore, disbudding is inevitable in such goat breeds [8]. Thermal cauterization is the most commonly used technique but it is painful and stressful without appropriate anesthesia and analgesia [8,9]. Therefore, it is recommended to provide pain relief to improve the welfare of the animals undergoing disbudding [3,6].

Effective local anesthesia is one of the ways to alleviate or minimize the pain associated with disbudding [6,7]. Lidocaine is the most commonly used local anesthetics in veterinary medicine but it has a historical reputation of being toxic to goat kids [8,10]. The reasons for toxicity include overdosing of the goat kids by not considering their body weight and the increased chances of systemic absorption of the drug from the highly vascularized injection sites [11]. The requirement for injecting local anesthetics at two nerve sites per horn bud (only one site in cattle) for cornual nerve block increases the chances of toxicity in goat kids. Even though goat kids have been reported to be sensitive to lidocaine, there are no data on the plasma concentrations of lidocaine that caused toxicity [8,10]. To determine the toxic dose of the local anesthetics, it is important to determine its toxic plasma/serum concentrations [11]. Therefore, the objectives of this study were to determine the toxic dose, its corresponding plasma concentrations, and compare the results with pharmacokinetic parameters following cornual nerve block in goat kids to recommend a safe dose for cornual nerve block.

3.3 Materials and methods

Reagents and drugs

Reference standards of lidocaine hydrochloride and monoethylglycinexylidide (≥95%) were purchased from Sigma Aldrich, Auckland, New Zealand. Lidocaine hydrochloride for injection was purchased from Ethical agents Ltd., Auckland, New Zealand. Acetonitrile,

methanol, water, and formic acid were mass spectrometry grade and were purchased from Fisher Scientific, Auckland, New Zealand. Heparin sodium and normal saline were purchased from Pfizer New Zealand Limited, Auckland, New Zealand, and Baxter Healthcare Pty Ltd., Old Toongabbie, NSW, Australia, respectively. Artificial colostrum and milk replacer were purchased from Farmlands Co-Operative Society Ltd., Palmerston North, New Zealand.

Experimental Animals

The study was conducted on healthy male Saanen goat kids collected from a commercial dairy goat farm. Kids were separated from their dams after receiving colostrum and were transported to the Massey University research facility. Animals were housed in pens with clean, dry straw bedding, and heating lamps to keep the pens warm. All animals received artificial colostrum on the day of arrival using feeding bottles and then fed with milk replacers via milk feeding buckets. Milk feeding buckets remained in the pens for ad libitum access to milk replacer. The study procedures were approved by the Massey University Animal Ethics Committee (Protocol number-MUAEC Protocol 17/41 and 17/54).

Study Design

Dose-Ranging Study

The aim of this experiment was to determine the maximum dose that can be safely used in goat kids without any adverse effects. This experiment was conducted on three male Saanen goat kids (7–10 days old, weighing 6.4 kg to 6.9 kg). Both right and left cephalic veins were catheterized using 20 gauge, 48 mm intravenous (I/V) catheter (BD Insyte, Sandy, UT, USA), for the administration of 2% lidocaine hydrochloride and pentobarbitone sodium. Three doses (7 mg/kg body weight (BW), 9 mg/kg BW, and 10 mg/kg BW) of lidocaine hydrochloride were administered (one animal/dose) by intravenous infusion over 60 s using a syringe pump (World Precision Instruments, Sarasota, FL, USA) and observed for toxicity signs (sedation

and convulsions). The infusion was stopped when convulsions appeared and animals were immediately euthanized by intravenous injection of pentobarbitone sodium (100 mg/kg BW).

Determination of Toxic Dose of Lidocaine and Its Corresponding Plasma Concentrations

Six male Saanen goat kids (seven to 10 days old, weighing 6.1 to 7.5 kg BW) were used to determine the convulsive dose and its corresponding plasma concentrations. The left cephalic vein was catheterized (20 gauge, 48 mm I/V catheter (BD Insyte, Sandy, UT, USA)) for intravenous infusion of 2% lidocaine hydrochloride (2 mg/kg/min) using a syringe pump and the right cephalic vein was catheterized for the collection of blood samples. Blood samples were collected prior to drug administration, at 1-min intervals and at the time of onset of convulsions. The total dose required to produce convulsions was calculated using the data from the infusion pump, and the plasma concentrations of lidocaine and its metabolite, monoethylglycinexylidide (MGX), in the collected samples were analyzed using a sensitive and simple liquid chromatography-mass spectrometry (LC-MS/MS) method described later. Drug administration was terminated when convulsions were observed and animals were euthanized immediately by intravenous injection of pentobarbitone sodium (100 mg/kg BW).

Pharmacokinetics of Lidocaine and Its Metabolite, MGX, Following Cornual Nerve Block in Goat Kids

This experiment included 10 male Saanen goat kids (less than a week old) with a body weight range of 3.3 kg to 4.7 kg. Animals were restrained gently and 1% lidocaine hydrochloride (commercially available 2% lidocaine hydrochloride solution was diluted in normal saline) was injected (0.5 mL per site) subcutaneously within 2 min around the cornual branches of the lacrimal and infratrochlear nerves of both the horn buds based on the procedure described by Sherman and Smith, 2009. The syringe plunger was pulled back prior to injection to ensure the needle was not in a blood vessel. After confirming the effect of the nerve blockade by pricking with a needle, horn buds were disbudded using a gas dehorner (Portasol, Elmira,

OR, USA). Although this is not a proper behavioral study, animals were still monitored for pain-associated behaviors such as head scratching, head shaking, vocalization, body shaking, and toxicity signs for 3 h after drug administration. Blood samples (1 mL) were collected via the catheter in the cephalic vein prior to drug administration (0 min) and at 10 min, 20 min, 30 min, and 40 min and 1 h, 2 h, 4 h, 6 h, 8 h, and 12 h following drug administration. Immediately after collection, blood samples were cooled on ice and plasma was separated and stored at −20 ◦C until analysis.

Analytical Procedure

Liquid Chromatography-Mass Spectrometry

A sensitive and simple LC-MS/MS method using Parallel reaction monitoring (PRM) mode was developed and validated to quantify the plasma concentrations of lidocaine and MGX.

Instrumentation and Conditions

The Ultra High Performance Liquid Chromatography system (Thermo Scientific™ Dionex UltiMate™ 3000 System, Germering, Germany) was equipped with a quaternary pump (Dionex Ultimate 3000 RS pump), a vacuum degasser, a column compartment (Dionex Ultimate 3000 RS Column Compartment), and an auto-sampler (Dionex Ultimate 3000 RS Autosampler). The analytes were separated using a 2.6 µm particle size C-18 column (Accucore 100 mm × 2.1 mm, Auckland, New Zealand) coupled with a security guard column (Accucore Defender Guard Column, Auckland, New Zealand) maintained at a temperature of 25 ◦C. The mobile phase consisted of 0.1% formic acid and acetonitrile (70:30, V/V) and was delivered at a flow rate of 0.3 mL/minute. The PRM analyses were carried out on a hybrid quadrupole orbitrap mass spectrometer (Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer, Thermo Scientific™, Bremen, Germany) with an electrospray-ionization interface. The precursor ions of lidocaine (m/z 235.180) and MGX (m/z 207.148) were included in the target list and were fragmented into their respective

daughter ions using collision energy of 35 eV, which were detected using a resolution of 35,000 FWHM. Data processing was performed using the Thermo Scientific™ Xcalibur® data system and quantitation was performed using peak-area ratios of the daughter ions of lidocaine (m/z 86.096) and MGX (m/z 58.065). Samples that exceeded the calibration limit were appropriately diluted with blank drug-free plasma and re-analyzed.

Sample Preparation

An aliquot of 240 µL plasma was taken in a 1.5 mL Eppendorf centrifuge tube and mixed with 480 µL of ice-cold methanol and vortexed for 10 s. After 10 min, the samples were vortexed again and centrifuged at 4,500 *g* for 10 min. The clear supernatant (200 µL) was mixed with 0.1% Formic acid (200 µL) and centrifuged at 4,500 *g* for 10 min. Then the supernatant was taken into the autosampler vials and 10 µL was injected into the column.

Preparation of Standards and Quality Control Samples

Standard stock solutions (1 mg/mL) of lidocaine hydrochloride and MGX were prepared by dissolving in methanol. Equal volumes of both the standard solutions were mixed and working solutions were then serially diluted using methanol. Calibration standards and quality control samples (0.0125 µg/mL, 0.125 µg/mL, and 1.250 µg/mL) were prepared freshly by spiking ice cold pooled blank goat plasma with working solutions.

LC-MS/MS Method Validation

Specificity of the method was determined by analyzing blank goat plasma samples and samples spiked with lidocaine hydrochloride and MGX. The linearity of the method was determined by linear regression analysis calculated using the least square regression method. Calibration curves (0.00125 µg/mL to 2.50 µg/mL) were built using pooled plasma obtained from untreated goat kids. The lower limit of detection and quantification of the compounds were determined by signal-to-noise ratios of 3:1 and 10:1, respectively. Recoveries (0.0125 µg/mL, 0.125 µg/mL, and 1.250 µg/mL) from goat plasma were calculated by comparing the

peak areas of spiked samples with control standards following the same sample preparation procedure described above. Intraday and interday precision and accuracy of the method were determined by running different concentrations of an independently prepared spiked goat plasma sample on the same day and for six different days, respectively. Carryover from the system was assessed by injecting blank plasma sample after an injection of the spiked plasma sample containing 2.50 µg/mL of compounds.

Pharmacokinetic Analysis

Pharmacokinetic parameters following subcutaneous injection were determined using noncompartmental analysis. PKSolver 'add-on' for Excel 2010 was used to calculate pharmacokinetic parameters using individual plasma concentration data [12]. The maximum plasma concentration (C_{max}) and time to achieve C_{max} (T_{max}) were determined directly from the plasma concentration data. The rate constant of the terminal phase (λ_z) was calculated by linear regression of the logarithmic plasma concentration. Half-life of the terminal phase ($t_{1/2\,\lambda z}$) was calculated as $\ln 2/\lambda_z$. The area under the curve (AUC) and the area under the first moment (AUMC) were determined using the linear trapezoidal method. Mean residence time (MRT) was calculated as AUMC/AUC. Data are reported in mean ± S.D.

3.4 Results

LC-MS/MS Method Validation

Representative chromatograms and mass spectra (obtained in PRM mode) of blank goat plasma, blank plasma spiked with 0.00125 µg/mL of analytes, and the plasma sample from an experimental animal are shown in Supplementary Material Figure 3.1. The retention times of lidocaine and MGX were 0.97 min and 0.93 min, respectively, and the total run time was 4 min. The calibration curves (Supplementary Material Figure 3.2) were linear over the concentration range of 0.00125 µg/mL to 2.50 µg/mL with a correlation coefficient (r^2) of

0.9972 for lidocaine and with r^2 of 0.9997 for MGX. The lower limit of quantification and detection for both lidocaine and MGX were 0.00125 µg/mL and 0.0005 µg/mL, respectively. The relative standard deviation of intraday assay for lidocaine and MGX were ≤10.31% and ≤9.50%, respectively. The relative standard deviation for interday assay for lidocaine and MGX were ≤12.69% and ≤15.64%, respectively. Both interday and intraday assay were within the acceptable criteria of <10% and <15%, respectively. The recoveries for lidocaine ranged from 78% to 84% and for MGX were from 65% to 79%. No carry-over effect was found after the injection of spiked samples containing the upper limit of quantification.

Animal Experiments

All animals were healthy prior to drug administration. In the dose-ranging experiment, no adverse effects were noted after the infusion of 7 mg/kg BW over 60 s but in the animals that received 9 mg/kg and 10 mg/kg, sedation followed by ataxia and convulsions were observed even before the completion of the infusion. Individual animal doses and plasma concentrations of lidocaine and its metabolite, MGX required to produce convulsions in goat kids after intravenous infusion of lidocaine hydrochloride were presented in Table 3.1. The average dosage required to produce convulsions in goat kids was 12.31 ± 1.42 mg/kg and the average convulsive plasma concentrations of lidocaine and MGX required to produce were 13.59 µg/mL and 0.39 µg/mL, respectively. An identical sequence of toxicity signs was exhibited by all the animals. Initially, sedation, followed by ataxia, and tonic-clonic convulsions were observed.

The mean pharmacokinetic parameters of lidocaine and its metabolite are presented in Table 3.2. The plasma concentrations of lidocaine and MGX vs. time following subcutaneous administration are shown in Figure 3.1. The plasma concentrations of lidocaine and MGX were below the limit of detection in the samples collected prior to drug administration and were above the limit of quantification for up to 12 h post drug administration. The average

dose of lidocaine administered for cornual nerve block was 5.7 ± 0.3 mg/kg. The mean peak plasma concentration 2.12 ± 0.81 µg/mL (range 1.32–4.05 µg/mL) of lidocaine was reached at around 0.33 ± 0.11 h. The C_{max} and T_{max} of MGX were 0.31 ± 0.32 µg/mL and 1.53 ± 0.61 h, respectively. The elimination half-lives of lidocaine and MGX were 1.71 ± 0.51 h and 3.22 ± 1.21 h, respectively. Following subcutaneous administration of lidocaine (0.5 mL/site) at the nerve sites, effective nerve blockade was produced and no pain-related behaviours were observed during disbudding but head scratching and head shaking were observed after 20 min. However, no lidocaine-related toxic signs were observed in any of the animals.

Table 3.1. Individual animal doses and plasma concentrations of lidocaine and its metabolite, monoethylglycinexylidide, which produced convulsions in goat kids following intravenous infusion of 2% lidocaine hydrochloride (2 mg/kg/min).

Animal Number	Total Dose (mg/kg)	Plasma Concentrations (µg/mL)	
		Lidocaine	Monoethylglycinexylidide
1	10.23	12.68	0.31
2	11.70	12.64	0.49
3	13.84	15.01	0.62
4	13.98	17.35	0.39
5	11.76	13.33	0.26
6	12.33	10.53	0.26
Mean ± S.D. (standard deviation)	**12.31 ± 1.42**	**13.59 ± 2.34**	**0.39 ± 0.14**

Table 3.2. Mean ± S.D. pharmacokinetic parameters of lidocaine and monoethylglycinexylidide following subcutaneous administration of 1% lidocaine hydrochloride (0.5 mL per site) around the cornual branches of the lacrimal and infratrochlear nerves of both the horn buds in goat kids (*n* = 10).

Parameter	Lidocaine	Monoethylglycinexylidide
C_{max} [1] (µg/mL)	2.12 ± 0.81	0.31 ± 0.32
T_{max} [2] (h)	0.33 ± 0.11	1.53 ± 0.61
AUC (µg/mL h) [3]	3.12 ± 1.09	1.34 ± 1.20
Terminal half-life (h)	1.71 ± 0.51	3.22 ± 1.21
Mean residence time (h)	1.59 ± 0.36	4.67 ± 1.34

[1] Maximum plasma concentration, [2] time to reach maximum plasma concentration, and [3] area under the curve.

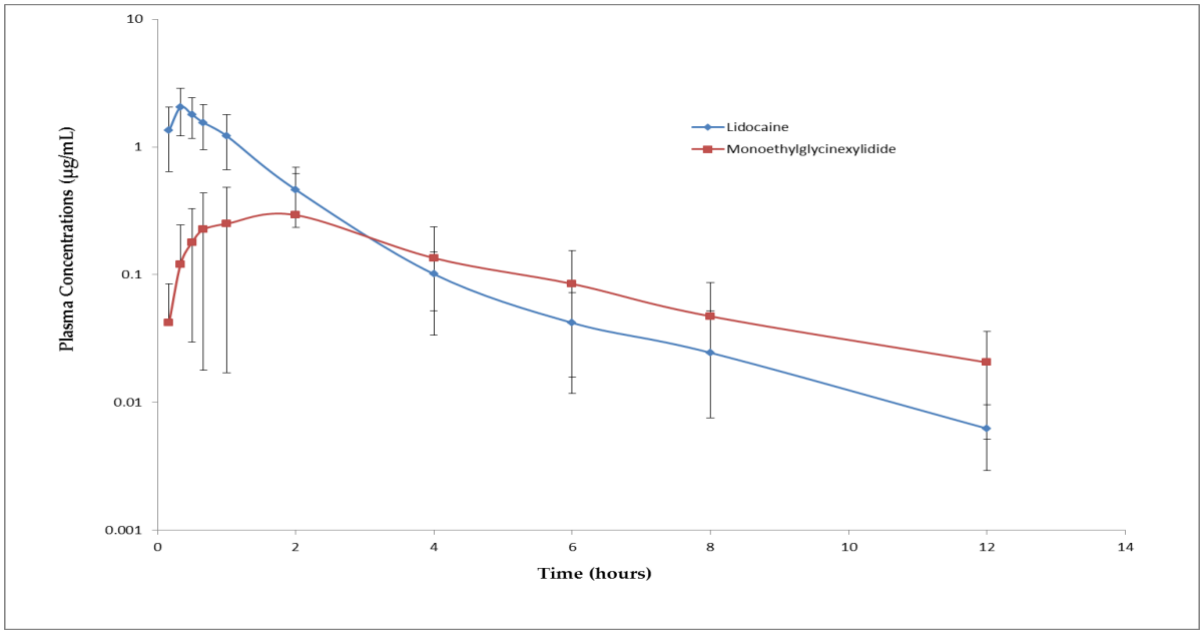

Figure 3.1. Mean (±S.D.) plasma concentrations of lidocaine (red line) and its metabolite, monoethylglycinexylidide (blue line), in goat kids following cornual nerve block using 1% lidocaine (0.5 mL per site). Note the log scale of the *y*-axis.

3.5 Discussion

A simple and sensitive LC-MS/MS method using PRM mode has been developed and validated for the simultaneous quantification of lidocaine and its metabolite MGX in goat plasma. The validation parameters were within the acceptable range (Appendix). One of the limitations of the LC-MS/MS method was that no internal standards were used. Future studies should consider using internal standards to improve the precision of the assay.

Lidocaine hydrochloride has been widely used as a local anesthetic in both veterinary and human medicine. Toxicity can occur if excessive drug doses are administered or because of accidental intravenous injection [13]. In goats, lidocaine has been reported to have historical reputation of being toxic especially during cornual nerve block in young animals [8,11]. However, there is no data on the toxic dose and the plasma concentration at which toxicity was observed. Only one study reported that convulsions were observed in a goat kid following intramuscular injection of lidocaine at approximately 10 mg/kg but there is no

report on the plasma concentration at which toxicity occurred [10]. The toxic concentrations and pharmacokinetics of lidocaine have been studied in humans, sheep, dogs, and horses, but, to our knowledge, there are no reports in goats [14–17]. This is the first study that reports the pharmacokinetics, and convulsive toxic doses and its concentrations in goat kids. Since the metabolite MGX can contribute to the toxicity of lidocaine, this study has also quantified the plasma concentration of MGX.

In the dose-ranging study, a dose of 7 mg/kg BW administered intravenously over a period of 60 seconds did not produce any observable toxicity signs. Therefore, this dose should be safe for cornual nerve block in goat kids as toxicity is unlikely to occur even if this dose is accidentally injected into veins.

The mean toxic dose required to produce convulsions in the goat kids (12.42 mg/kg) was less than that reported in newborn sheep (18.40 mg/kg) [15]. The mean plasma concentration required to produce convulsions in the present study was (13.59 ± 2.34 µg/mL) similar to that reported in newborn sheep (16.6 ± 1.2 µg/mL) [15]. In dogs, the mean concentration of lidocaine that produced toxicity was 8.21 ± 1.69 µg/mL, which is lower than that found in the present study (13.59 ± 2.34 µg/mL) [16]. The serum concentration (3.24 ± 0.74 µg/mL) that produced intoxication in horses was significantly lower than that found in goat kids and other species [17]. The differences could be because of the different end points used to determine the toxicity in various species. In dogs, the tonic extension phase was considered as the toxic sign while in horses, skeletal muscle fasciculation was used [16,17]. In the present study, convulsions were used as the end point. Another possible reason for the differences could be the rate of drug administration used in different species [16,17]. Species and age differences have been reported to occur in the toxicity of lidocaine [15–17]. Young animals are less sensitive to lidocaine toxicity than adult animals because of higher volumes of distribution in young animals [11–15]. The limitation of our study was that cardiovascular parameters were

not recorded. However, it should be noted that the central nervous system is more sensitive than the cardiovascular system. Two to four times higher concentrations are required to produce cardiovascular toxicity signs than central nervous system signs [11–15].

Post-hoc power analysis of the data from this study revealed that the sample size (n=10) used in both the pharmacokinetic and toxicity studies were sufficient (>95% power). The absorption of lidocaine following subcutaneous administration was rapid with an average T_{max} of 0.33 ± 0.11 h. The rate of elimination of lidocaine and MGX was moderate with a mean $t_{1/2\lambda z}$ of 2.28 h and 3.20 h, respectively. The mean peak plasma concentration of lidocaine (2.12 ± 0.81 µg/mL) observed after subcutaneous administration was almost 6.5 times less than the mean plasma concentration that produced convulsions (13.59 ± 2.34 µg/mL). The toxic dose of local anesthetics depends on the peak plasma concentration of the drug. Lower peak plasma concentrations reduce the chances of developing toxicity [18]. Since the C_{max} following injection of 1% lidocaine hydrochloride (0.5 mL/site) was significantly lower than the toxic plasma concentration, this dose may be safe for cornual nerve block in goat kids. The dose used in this study is not only safe but also effective in blocking the nerves supplying horn buds since no pain-related behavioral signs were observed during disbudding. However, most of the goat kids started showing signs like head scratching and head shaking 20 min after drug administration, which means that the anesthetic effect lasted only for 20 min. Increasing the concentration of lidocaine may increase the duration of the anesthetic effect. However, studies have shown that administration of lidocaine alone is not sufficient to provide post-operative pain relief for disbudding [6]. Injection of lidocaine at four sites to desensitize both the horn buds was stressful and painful to the animals as vocalization and struggles were observed during the injection. Therefore, there is a need for a better pain management protocol for goats undergoing disbudding.

3.6 Conclusions

Based on the results of the present study, 7 mg/kg bodyweight may be safe to use in goat kids for local and regional nerve blocks. Yet, further studies using more animals are warranted before clinical use of this dose. The toxic conclusive concentrations determined in this study may be used as a standard to compare the peak plasma concentrations following various routes of administration to determine the safe dose in goat kids. Injection of 0.5 mL per site of 1% lidocaine is safe and effective to anaesthetize horn buds in goat kids. Significant differences observed in the peak plasma concentration and the convulsant plasma concentration suggests that the use of higher lidocaine hydrochloride concentration (1.5%) for cornual nerve block in goat kids could be safe and may increase the duration of anesthesia. However, further pharmacokinetic and efficacy studies to determine the peak plasma concentrations and duration of analgesia using those doses are required to assess the safety of the dose before clinical use.

3.7 Acknowledgments

We acknowledge the technical support provided by Scott Fraser, Farzin Sahebjam, Erin Wilson, and Sandeep Karna.

3.8 References

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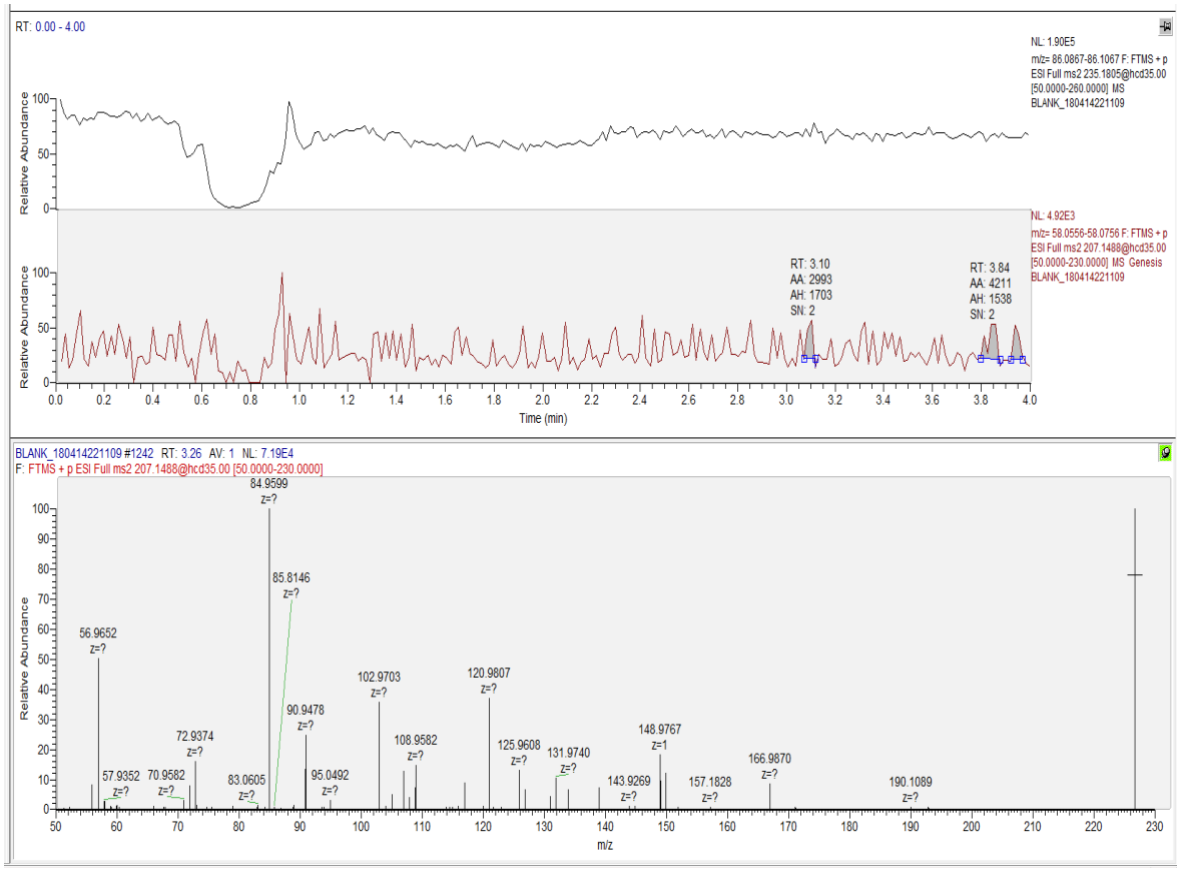
3.9 Supplementary Materials

Supplementary Figure 3.1. Chromatograms and mass spectra (obtained in PRM mode) of (a) blank goat plasma, (b) blank plasma spiked with 0.00125 µg/mL of lidocaine and monoethylglycinexylidide, and (c) plasma from an experimental animal after cornual nerve block.

a.



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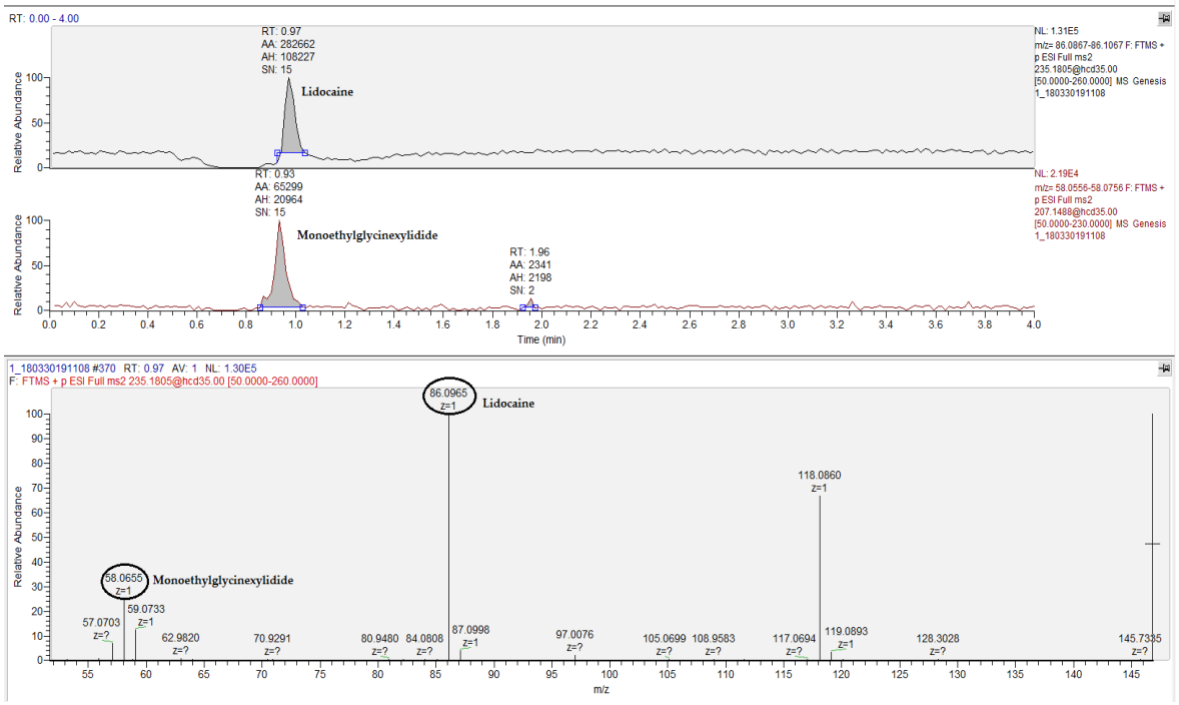
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SN: 15
Monoethylglycinexylidide
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AA: 2341
AH: 2198
SN: 2
NL: 2.19E4
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Relative Abundance
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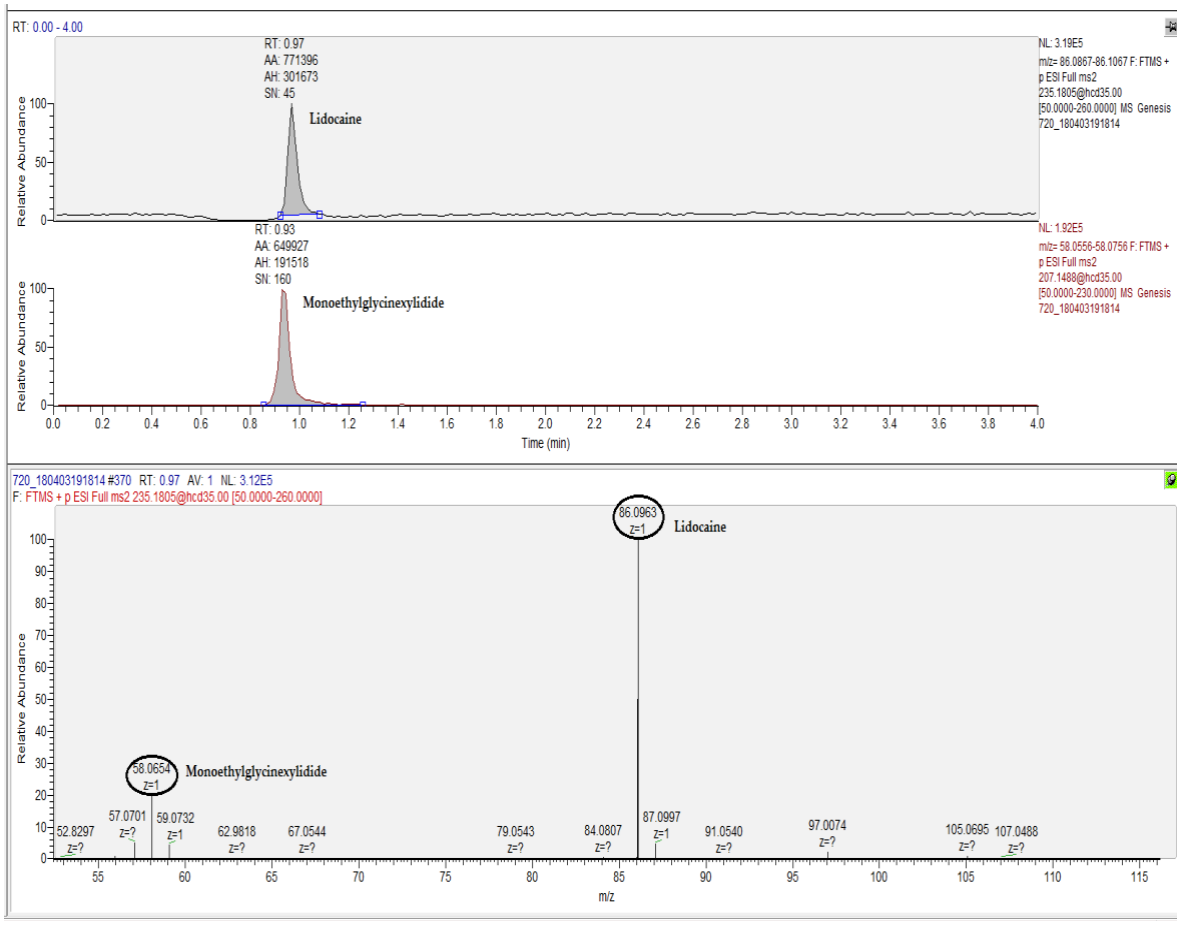
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Relative Abundance
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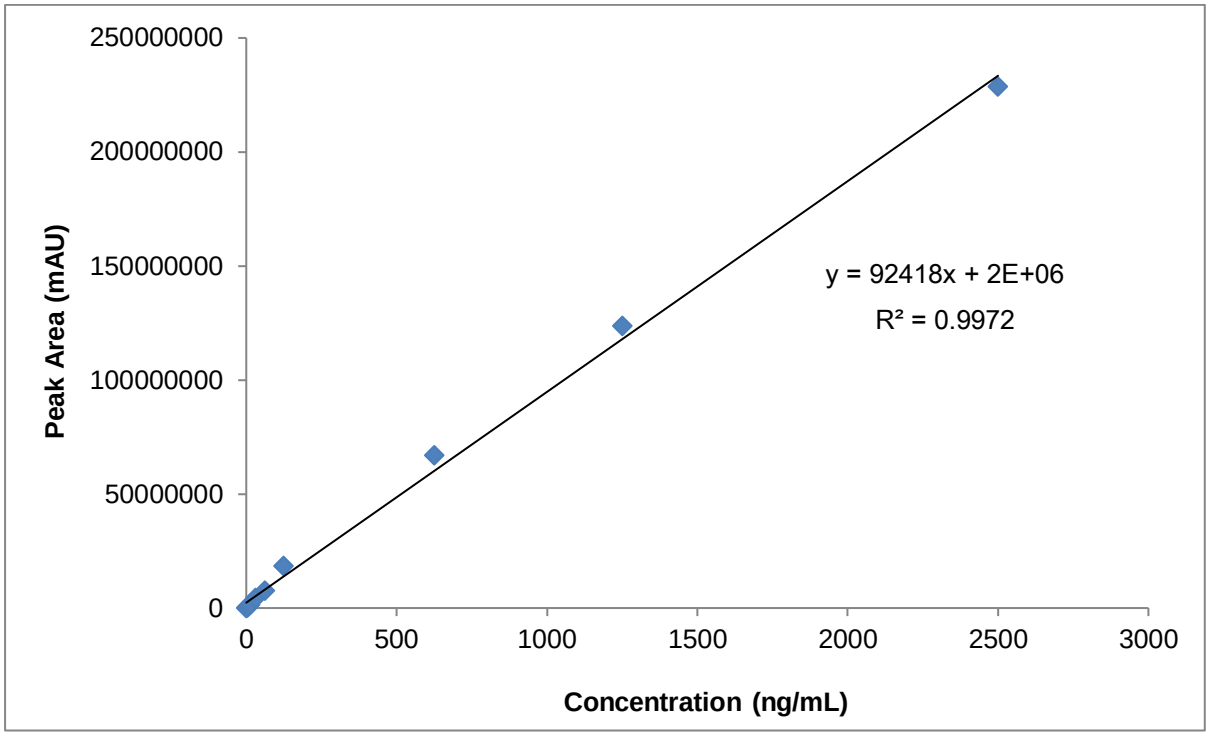
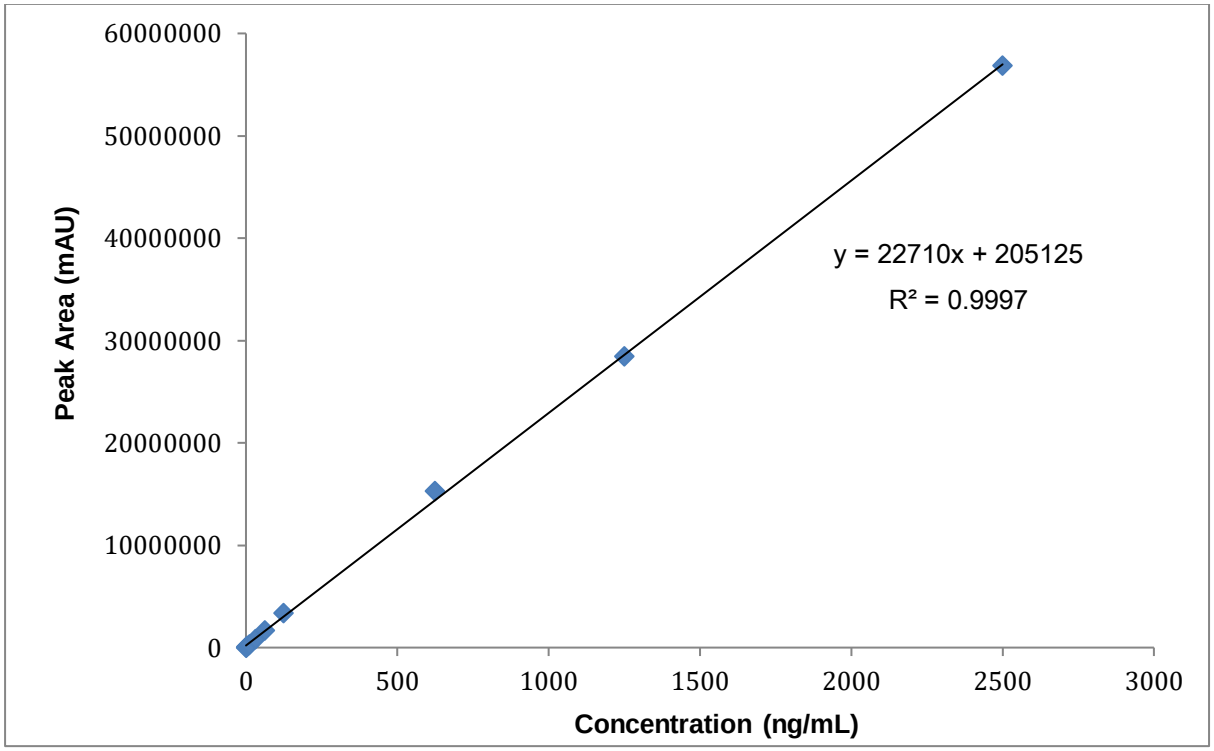
m/z

Supplementary Figure 3.2. Calibration curves (0.00125 – 2.50 µg/mL) of lidocaine (a) and monoethylglycinexylidide; (b) constructed by spiking pooled plasma from untreated goat kids.

a. Lidocaine



b. Monoethylglycinexylidide



3.10 Appendix

Table 3.3. Intra-assay and inter-assay accuracy and precision of lidocaine in goat plasma.

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	12.5	12.37±1.12	9.02	-1.07
	125	117.21±12.09	10.31	-6.23
	1250	1294.99±45.32	3.50	3.60
Inter-assay	12.5	14.00±1.73	12.34	12.04
	125	124.56±15.81	12.69	-0.35
	1250	1292.61±147.37	11.40	3.41

Table 3.4. Intra-assay and inter-assay accuracy and precision of monoethylglycinexylidide in goat plasma.

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	12.5	12.80±0.23	1.80	2.42
	125	126.82±12.04	9.50	1.46
	1250	1321.80±68.12	5.15	5.74
Inter-assay	12.5	12.20±1.76	14.42	-2.39
	125	130.25±20.04	15.39	4.20
	1250	1238.01±193.64	15.64	-0.96

Table 3.5. Extraction recoveries of lidocaine and monoethylglycinexylidide in goat plasma

Concentrations (ng/mL)	Lidocaine (%)	Monoethylglycinexylidide (%)
12.5	77.55±1.25	78.86±2.98
125	78.74±11.70	64.75±7.12
1250	83.88±21.54	68.22±10.82

Table 3.6. Individual animal plasma concentrations (ng/mL) of lidocaine following subcutaneous administration of 1% lidocaine hydrochloride (0.5 mL per site) around the cornual branches of the lacrimal and infratrochlear nerves of both the horn buds in goat kids (n=10).

Time Points (minutes)	1	2	3	4	5	6	7	8	9	10	Mean ± SD
10	1433.8	1042.7	1342.6	1294.0	1171.2	2493.4	1886.3	2404.5	595.1	957.8	1462.1±618.4
20	4058.3	1361.5	1503.3	1983.8	1566.2	2227.3	1742.7	2748.4	1328.0	1941.1	2046.1±829.4
30	3275.1	1537.7	1659.5	1967.5	1103.0	2179.7	1556.9	1677.4	1076.5	1859.0	1789.2±625.6
40	2761.8	1127.1	1163.0	1739.0	*	2174.0	1236.3	1549.2	847.3	1334.9	1548.1±598.0
60	2310.4	1050.3	1034.7	1543.7	775.4	1565.0	894.6	1417.9	391.8	*	1220.4±559.5
120	733.9	777.4	412.8	605.8	251.3	458.1	420.9	662.2	179.9	126.9	462.9±229.6
240	93.8	168.1	50.6	161.8	44.9	112.1	75.4	137.6	133.6	33.2	101.1±49.1
360	32.6	122.2	23.2	43.7	19.3	59.0	31.8	28.6	29.2	30.6	42.0±30.3
480	10.0	101.8	6.6	22.6	10.9	29.5	19.7	16.6	11.7	15.7	24.5±28.0
720	8.3	8.2	3.5	2.1	2.6	7.8	4.9	13.2	5.5	6.5	6.3±3.3

*– samples were not collected

Table 3.7. Pharmacokinetic parameters of lidocaine following subcutaneous administration of 1% lidocaine hydrochloride (0.5 mL per site) around the cornual branches of the lacrimal and infratrochlear nerves of both the horn buds in goat kids (n=10).

Parameters	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Mean ± SD**
C_{max} (µg mL^{-1})	4.06	1.54	1.66	1.98	1.57	2.49	1.89	2.75	1.33	1.94	2.12±0.81
T_{max} (hour)	0.33	0.50	0.50	0.33	0.33	0.17	0.17	0.33	0.33	0.33	0.33±0.11
$t_{½\lambda z}$ (hour)	1.16	1.65	1.21	1.16	1.97	2.07	2.08	1.38	1.73	2.74	1.71±0.51
AUC (hour µg mL^{-1})	5.09	3.71	2.53	3.73	1.95	3.87	2.68	3.87	1.61	2.19	3.12±1.09
AUMC (hour µg mL^{-1})	6.51	9.19	3.39	5.91	2.66	6.00	4.14	6.08	3.15	2.95	5.00±2.09
MRT (hour)	1.27	2.46	1.34	1.58	1.36	1.54	1.53	1.56	1.94	1.33	1.59±0.36

Table 3.8. Individual animal plasma concentrations (ng mL^{-1}) of monoethylglycinexylidide following subcutaneous administration of 1% lidocaine hydrochloride (0.5 mL per site) around the cornual branches of the lacrimal and infratrochlear nerves of both the horn buds in goat kids (n=10).

Time Points (minutes)	1	2	3	4	5	6	7	8	9	10	Mean ± SD
10	42.2	25.9	34.4	13.6	20.7	20.3	46.6	28.3	10.9	51.5	29.4±13.9
20	460.6	59.0	164.1	66.9	71.5	71.7	115.7	66.9	55.6	77.1	120.9±123.8
30	582.8	121.7	185.5	111.3	98.9	114.8	208.0	85.4	82.1	199.8	179.0±149.4
40	763.0	124.4	218.5	132.1	*	139.9	221.5	104.4	94.1	237.1	226.1±208.3
60	855.9	160.2	211.6	141.7	154.1	186.8	291.3	164.5	89.9	*	250.7±233.5
120	1204.0	254.1	248.3	186.4	114.5	157.1	341.8	172.1	90.1	163.6	293.2±328.3
240	396.0	158.6	123.0	112.5	51.6	68.5	192.0	98.6	62.1	87.4	135.0±101.6
360	255.7	126.6	82.0	61.9	23.1	43.9	118.2	41.1	45.3	49.9	84.8±68.9
480	143.9	52.9	31.9	43.7	14.5	25.9	83.7	27.0	12.8	36.8	47.3±39.7
720	52.5	28.5	11.4	31.5	4.6	9.6	33.5	13.1	5.6	15.6	20.6±15.4

*– samples were not collected

Table 3.9. Pharmacokinetic parameters of monoethylglycinexylidide following subcutaneous administration of 1% lidocaine hydrochloride (0.5 mL per site) around the cornual branches of the lacrimal and infratrochlear nerves of both the horn buds in goat kids.

Parameters	1	2	3	4	5	6	7	8	9	10	Mean ± SD
C_{max} (µg mL^{-1})	1.20	0.25	0.25	0.19	0.15	0.19	0.34	0.17	0.09	0.24	0.31±0.32
T_{max} (hour)	2.00	2.00	2.00	2.00	1.00	1.00	2.00	2.00	0.67	0.67	1.53±0.61
$t_{½\lambda z}$ (hour)	2.71	3.07	2.23	6.42	2.55	2.75	3.22	3.67	2.34	3.30	3.22±1.21
AUC (hour µg mL^{-1})	4.59	1.34	1.16	0.98	0.54	0.75	1.76	0.81	0.51	0.92	1.34±1.20
AUMC (hour µg mL^{-1})	18.60	7.72	4.51	10.33	1.89	3.17	9.64	4.10	2.13	4.48	6.66±5.14
MRT (hour)	3.88	5.27	3.77	8.12	3.41	4.01	5.02	4.66	4.04	4.50	4.67±1.34

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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:	DINAKARAN VENKATACHALAM
Name/title of Primary Supervisor:	Dr. PREET SINGH
Name of Research Output and full reference:	
[redacted]	
In which Chapter is the Manuscript /Published work:	Chapter 3
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and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
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GRS Version 4– January 2019

Name of research output and full reference: Venkatachalam D, Chambers JP, Kongara K, Singh P (2018). Toxicity and pharmacokinetic studies of lidocaine and its active metabolite, monoethylglycinexylidide, in goat kids. *Animals*, 8(8): 142.

CHAPTER 4

NOVEL DISBUDDING AND ANALGESIC TECHNIQUES IN GOAT KIDS

This chapter consists of:

1) **A pilot study on the efficacy of novel disbudding and analgesic methods in goat kids.**

   Manuscript prepared as per the format of *New Zealand Veterinary Journal.*

2) **Formulation and evaluation of a novel topical local anaesthetic formulation for anaesthesia of horn buds in goat kids.**
   Manuscript prepared as per the format of *New Zealand Veterinary Journal.*

Both the manuscripts have not been submitted for review yet because one of the pharma companies is interested in filing a patent application for the novel disbudding techniques.

1.1 A pilot study on the efficacy of novel disbudding and analgesic methods in goat kids

4.1.1 Abstract

AIM: The objective of this study was to evaluate novel disbudding and analgesic techniques in goat kids.

METHODS: Eighteen male Saanen goat kids (<10 days old, 3.4–4.6 kg) were used in this study. Goat kids were randomly allocated to receive one of the treatments: i) eugenol (n=6), ii) mepacrine (n=6) and iii) methoxyflurane (n=6). After clipping the hair around the horn buds, eugenol (0.2 mL) or mepacrine (0.2 mL) was injected into the centre of one of the horn buds using a needle and syringe. Pain related behaviours were observed during and following injection. The effects of these compounds on the horn buds were observed, and animals were euthanized for the gross pathological examination at the end of six weeks. Methoxyflurane was administered via a modified hand-held inhaler and analgesia of the horn buds was evaluated.

RESULTS: Injection of eugenol or mepacrine using needle and syringe was painful. Both eugenol and mepacrine produced painless necrosis of horn buds but was not effective in completely preventing the growth of horn buds. Methoxyflurane produced cutaneous analgesia but did not provide sufficient analgesia for disbudding.

CONCLUSIONS AND CLINICAL RELEVANCE: This pilot study has provided basic information on these novel techniques. Future studies are required to understand the suitability of these novel techniques for disbudding and analgesia in goat kids.

KEY WORDS: *Eugenol, mepacrine, methoxyflurane, disbudding, goat kids*

4.1.2 Introduction

Disbudding is commonly performed on dairy cattle and goat farms to avoid injuries to herd mates and handlers (Smith and Sherman 2009). Hornless animals are easy to transport, require less trough space than horned animals, and can adapt to the modern farm facilities such as automatic robotic feeder (Stookey and Goonewardene 1996; Staněk *et al.* 2018;

Loretz *et al.* 2004). Thermal cautery disbudding (hot-iron disbudding) is the most commonly used disbudding method in goat kids though it is painful and stressful without appropriate anaesthesia and analgesia (Smith and Sherman 2009; Alvarez *et al.* 2015). In addition to pain and stress, thermal cautery disbudding has been reported to cause damage to the skull and brain, and even mortality (Thompson *et al.* 2005; Hempstead *et al.* 2018b). Goat kids have a much thinner skull, and undeveloped frontal bone and frontal sinuses compared to calves, hence the risk of skull and brain damage is high in goat kids compared to calves (Smith and Sherman 2009; Thompson *et al*. 2005). Because of the risks associated with disbudding, in the United Kingdom and some European countries, disbudding in goat kids must be performed only by a veterinarian (Wagman *et al.* 2018). However, in New Zealand and other countries, there are no such restrictions. Therefore, a safe and less painful disbudding method is required to improve the welfare of goat kids.

Alternative methods to thermal cautery disbudding such as cryosurgical and chemical disbudding have been evaluated in goat kids (Hempstead *et al.* 2018a). The cryosurgical technique involves the application of liquid nitrogen while the chemical method uses caustic paste (sodium, calcium, or potassium hydroxide paste) to destroy the horn buds. Physiological and behavioural changes indicated that application of both liquid nitrogen and caustic paste were more painful than cautery disbudding (Hempstead *et al.* 2018a). Additionally, caustic paste can damage the eyes of goat kids and the udder of does (Thomas *et al.* 2005). Recently, clove oil injection has been reported to be effective in destroying horn buds (Molaei *et al.* 2015). However, the authors did not provide much information on the degree of distress observed during or after the procedure. One of the objectives of this study was to evaluate the efficacy and the pain associated with the injection of eugenol (the active ingredient of clove oil) for disbudding in goat kids.

Mepacrine, also known as quinacrine (4-N-(6-chloro-2-methoxyacridin-9-yl)-1-N,1-N-diethylpentane-1,4-diamine) was widely used in humans for the treatment of malaria, giardia, lupus erythematosus, rheumatoid arthritis, pleural effusion or pneumothorax, cancer, and for sterilisation in women (Lippes 2002; Ugarte *et al.* 2018). It possesses sclerotic, anti-inflammatory, anti-cancer, analgesic, and local anaesthetic-like properties (Volpi *et al.* 1981; Abdel-Latif *et al.* 1983; Lippes 2015). When injected into tumours, it produces local necrosis and subsequent fibrosis (Sotelo *et al.* 2004). Considering the pharmacological properties of mepacrine particularly asymptomatic sclerosis, necrosis, and local anaesthetic like-properties, we propose that injection of mepacrine into the horn buds may destroy the horn producing cells with minimum pain and prevent the growth of horns.

Another objective of this study was to evaluate the analgesic efficacy of methoxyflurane for disbudding in goat kids. Methoxyflurane is an old inhalation anaesthetic which was used in people and animals (Dayan 2016). It had many disadvantages as an anaesthetic but was a potent analgesic and the analgesic effects lasted for many hours after inhalation (Gaskell *et al.* 2016; Dayan 2016). It has been widely used as a self-administered inhalation analgesic for trauma and minor procedures such as burn dressings and drainage of abscess (Gaskel *et al.* 2016). Methoxyflurane is commercially available as a small hand-held inhaler (Penthrox) that can be easily self-administered by people. Using the commercially available human inhalation device (Penthrox) the analgesic efficacy of methoxyflurane was investigated in goat kids for disbudding.

Please refer to **Chapter 1 (General introduction)** for more details on eugenol, mepacrine and methoxyflurane.

4.1.3 Materials and Methods

Chemicals and reagents

Eugenol and mepacrine dihydrochloride were purchased from Sigma Aldrich (Auckland, New Zealand). Methoxyflurane and Penthrox inhaler were purchased from Medical Developments International Limited (Victoria, Australia). Aqueous mepacrine dihydrochloride solution (150 mg/mL) for injection was prepared by weighing appropriate quantity of mepacrine dihydrochloride and dissolving in sterile water.

Animals

Two days old male Saanen goat kids were purchased from a private dairy goat farm (Opiki, New Zealand) and transported to Massey University research facility (Palmerston North, New Zealand). Kids were housed in groups of six in pens with clean and dry straw bedding. They received artificial colostrum on the day of arrival and were then provided with milk replacers using plastic milk feeders thrice daily. The feeders remained in the pens for *ad libitum* access to milk. Animals were identified using coloured stock marking paint. The experimental procedures were approved by the Massey University Animal ethics committee (MUAEC protocol 17/68).

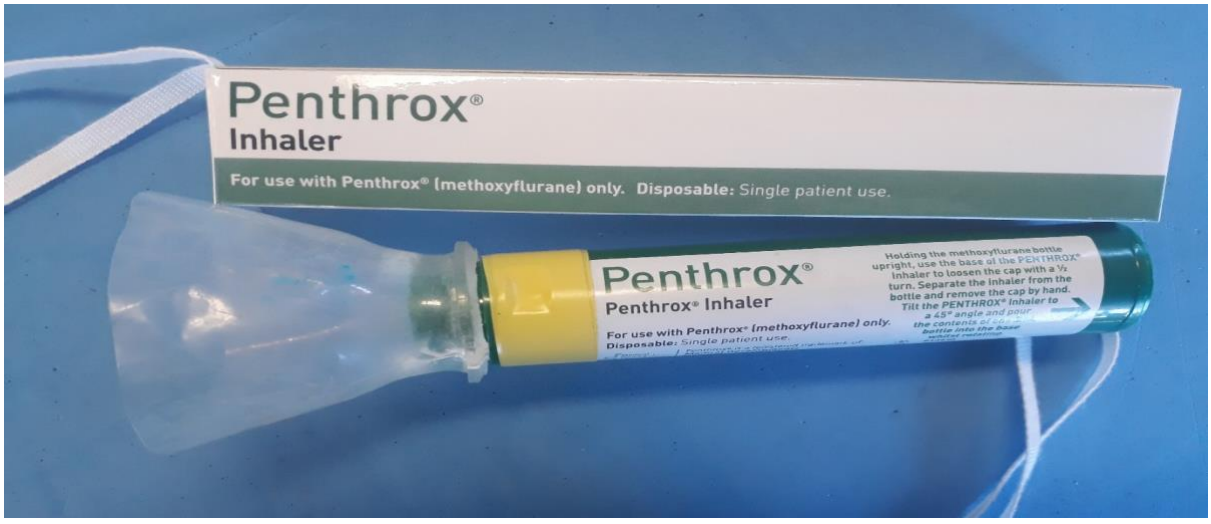
Study design

A total of 18 goat kids (less than 10 days old, weighing 3.4 to 4.6 kg) were enrolled in this study. Animals were randomly allocated to receive one of the three treatments: (i) eugenol (n = 6), (ii) mepacrine (n= 6) and (iii) methoxyflurane (n = 6). On the day of treatment, kids were manually restrained and hair covering the horn bud region was clipped using a hair clipper. Using a 26 G needle attached to a 1 mL syringe, 0.2 mL of eugenol or mepacrine (150 mg mL^{-1}) was injected into the centre of either right or left horn bud (Hempstead *et al.* 2018b). The other horn bud acted as a control and did not receive any treatment. Animals

were observed for vocalization and struggles (rapid movement of head) during injection, and for head scratching, head rubbing and body shaking for 2 hours after the injections. The general clinical assessment of the animals was made daily for 6 weeks. Kids were euthanized after six weeks using pentobarbitone sodium (100 mg kg^{-1} IV) and observed for the gross pathological changes in the skull and brain.

For the administration of methoxyflurane, the neck of a coke plastic bottle (250 mL) was modified as a face mask, and attached to the mouthpiece of the Penthrox inhaler (Figure 4.1). Methoxyflurane (1.5 mL) was added into the Penthrox inhaler using a syringe and the mask was placed on the face covering mouth and nose of goat kids for inhalation. After 2 minutes, the analgesic effect was assessed by gently placing a hot disbudding iron on the horn buds and observing for withdrawal response which is indicated by head shaking or movement of head away from the hot iron. If no response was observed, hot iron was held tightly over the horn buds till a response was observed or till the destruction of horn buds, whichever was earlier. If there was a response, further 1.5 mL of methoxyflurane was administered and analgesia was assessed again. If the additional dose was ineffective, kids were allowed to recover and no further experiment was carried out on them.

Figure 4.1. Image of the Penthrox inhaler used for the administration of methoxyflurane.

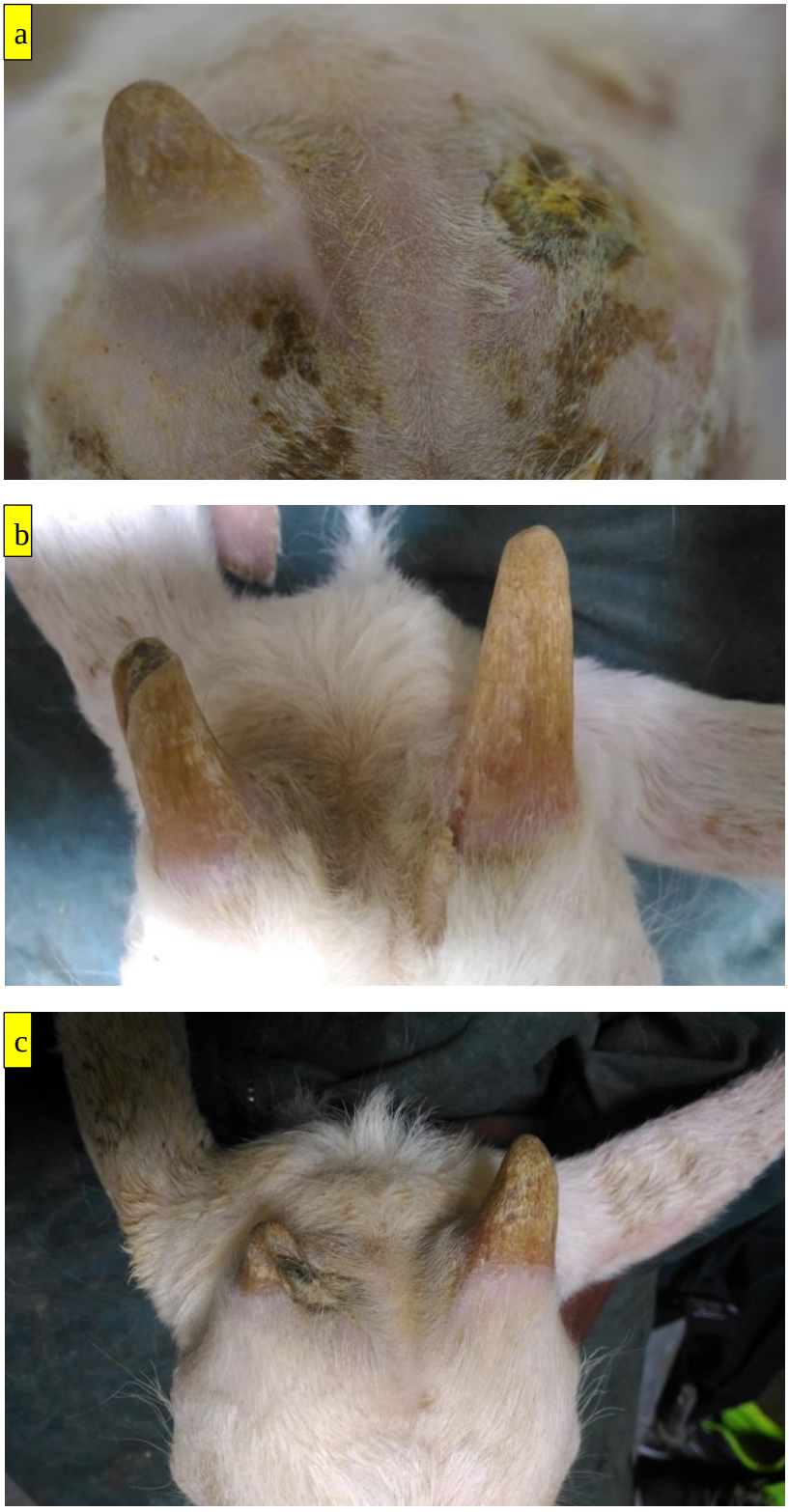


4.1.4 Results

Vocalization and struggles were observed in all the animals during the injection of eugenol or mepacrine. No pain-related behaviours were observed after the injection of eugenol or mepacrine. Swelling around the horn bud region was observed in all the animals following the injection of eugenol. The swelling resolved after few days and was not painful to touch. Necrosis (dark coloured) of horn bud tissues was seen in goat kids within two days of injection of eugenol (Figure 4.2). No swelling was observed in the animals that received mepacrine, and necrosis of the horn bud tissues was obvious after five to seven days following the injection of mepacrine (Figure 4.3). At the end of six weeks, horn growth was completely arrested in only one goat kid that received eugenol (Figure 4.2a). Scurs (distorted regrowth of horns) and localized areas of necrosis were observed in the rest of the animals that received eugenol or mepacrine. The height of horn buds was not measured in this study which is a limitation. On visual examination, the height of the horn buds that received eugenol or mepacrine appeared smaller than the height of the control horn buds. The gross pathological examination did not show any damage to skull or brain.

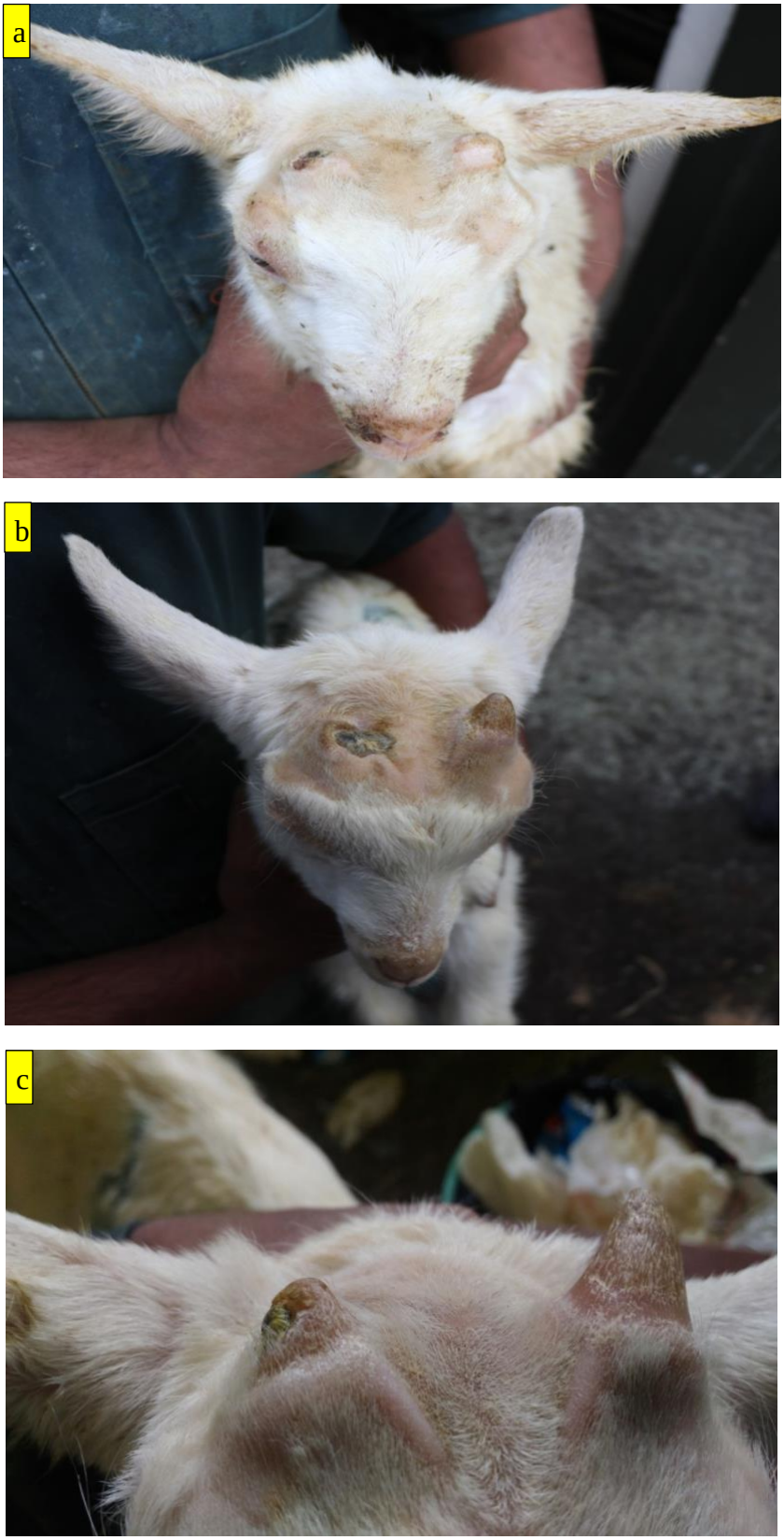
Following the administration of methoxyflurane, no response was observed to the placement of hot iron but when the hot iron was held firmly to the horn buds, withdrawal response was shown by all the animals. A similar response was seen even after the administration of additional dose (1.5 mL) of methoxyflurane.

Figure 4.2: Images of horn buds of different goat kids treated with eugenol.



Images show the dorsal view of head of different goat kids treated with eugenol a) Three weeks post-treatment, note the complete necrosis of the treated horn bud (left), b) Six weeks and c) Five weeks post-treatment. Partial growth of horn buds can be seen on the treated horn buds (right).

Figure 4.3: Images of horn buds of different goat kids treated with mepacrine.



Images show the dorsal view of head of different goat kids treated with mepacrine a) Three weeks post-treatment, b) and c) Four weeks post-treatment. Note the partial growth of horn buds on the treated horn buds (right).

4.1.5 Discussion

This pilot study evaluated novel disbudding methods and a novel analgesic method for disbudding in goat kids. Several studies on disbudding using clove oil and eugenol have been reported in goat kids and calves after the completion of the experiments reported in this study (Hempstead *et al.* 2018 b; Abbasi *et al.* 2018; Sutherland *et al.* 2019). In this study, injection of both eugenol and mepacrine into the horn buds produced necrosis of the horn bud tissues and reduced the growth of horn buds. However, complete destruction of horn buds was noticed only in one animal. Necrosis of horn buds and formation of scurs in most of the animals suggest that injection of 0.2 mL of eugenol or mepacrine was not sufficient to cover and destroy the entire horn buds. The injection volume (0.2 mL) reported by Molaei *et al* (2015) in goat kids was used in this study. The concentration of mepacrine used in this study was based on the results of a previous study in rats that reported that single injection of 150 mg can cause tissue necrosis (Sotelo *et al.* 2004). Molaei *et al* (2015) reported that clove oil injection (0.2 mL) was effective in preventing the horn growth in all the study animals whereas Hempstead *et al* (2018b) reported that clove oil injection was not completely effective in preventing the growth of horn buds as scurs were seen in most of the goat kids. The effectiveness of eugenol and mepacrine for disbudding may be increased by increasing the injection volume as larger volume will cover the entire horn bud and destroy all the horn producing cells. Also, spreading agents such as hyaluronidases may help in dispersal of the compounds and effectively destroy the horn buds.

Vocalization and struggles during the injection of eugenol and mepacrine indicate that pain was experienced during the injection of the compounds. It was not clear whether the pain was due to the needle stick or/and due to the compounds. Injection of normal saline into the horn buds may provide information on whether the pain was due to the chemicals or because of needle insertion. Hempstead *et al* (2018a b) reported that injection of clove oil into the horn

buds of goat kids produced similar acute pain as thermal cautery disbudding. Eugenol is a local anaesthetic that can penetrate the nerve fibres quickly and inhibit the voltage-gated sodium channels to produce anaesthesia (Park *et al.* 2011). Mepacrine produces painless sclerosis and possesses local anaesthetic like properties (Ehsanian *et al.* 2011; Abdel-Latif *et al.* 1983). Therefore, injection of these compounds may not be painful. Further research is needed to investigate if injection of these compounds induce pain. The needle stick pain may be avoided by using needless jet injector or microneedle assisted jet injector. The absence of pain-related behaviours after the injections suggests that these compounds produced necrosis of horn buds without pain. Painless necrosis is advantageous for disbudding as this will eliminate the need for post-operative analgesia.

Swelling around the horn buds was seen in the goat kid that received eugenol. Similar adverse effects have been reported following the injection of clove oil in goat kids and calves (Hempstead *et al.* 2018b; Sutherland *et al.* 2019). This was likely because of the hypersensitivity reaction caused by eugenol. Subcutaneous injection of eugenol in rats has been reported to produce necrosis and inflammation (Webb and Bussel 1981). In humans, few reports on irritation and hypersensitivity reactions of mucosa following the application of eugenol containing dental materials have been described (Sarrami *et al.* 2002). Unlike eugenol, swelling was not observed in the animals that received mepacrine, and there are no reports on the hypersensitivity reactions to mepacrine.

Absence of skull or brain damage suggests that these novel techniques may be safer than thermal cautery disbudding. Both eugenol and mepacrine appear to destroy the horn buds hence both the compounds should be further evaluated for disbudding in goat kids using minimally invasive administration techniques such as needless jet injector or needle assisted jet injector.

Absence of withdrawal response to the placement of hot-iron on horn buds following the administration of methoxyflurane suggests that some degree of cutaneous analgesia was produced by methoxyflurane. However, complete analgesia of horn buds was not produced by this technique. Methoxyflurane is an effective analgesic even at sub anaesthetic doses. In humans, inhalation of methoxyflurane via Penthrox inhaler produces effective analgesia for acute trauma associated pain and for minor surgical procedures (Gaskel *et al.* 2016). It has been used as a battlefield analgesic by the New Zealand and Australian army for many years and starting to be used in public hospitals for procedures such as wound dressing, prostate and bone marrow biopsy (McLennan 2007; Gaskel *et al.* 2016; Jephcott *et al.* 2018). Methoxyflurane failed to produce effective analgesia in goat kids possibly because the minimum alveolar concentration required to produce effective analgesia was not achieved using the Penthrox inhaler and modified face mask. Hempstead *et al.* (2018c) reported that administration of isoflurane using a vaporiser, anaesthetized goat kids and reduced the disbudding pain. This technique may be effective but the need for costly equipment to administer isoflurane and the time required for the recovery of the animals are not favourable for commercial farms. Design of a portable device for the proper administration of methoxyflurane in goat kids would improve the efficacy of this novel technique. Further studies using proper administration techniques are required to understand the analgesic efficacy of methoxyflurane in goat kids.

4.1.6 Conclusion

The results of the pilot study suggest that injection of eugenol and mepacrine over the horn buds appear to destroy the horn producing cells in goat kids. These novel disbudding techniques appear to be simple, safe and may eliminate the need for post-operative analgesia. Further investigation of these compounds using non-invasive/minimally invasive administration techniques and using different injection volumes for effective prevention of

horn growth are required. Administration of methoxyflurane via Penthrox inhaler produced cutaneous analgesia but failed to produce effective analgesia for disbudding in goat kids. Future studies using proper administration technique are required to understand the analgesic efficacy of methoxyflurane for disbudding in goat kids.

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1.2 Formulation and evaluation of a novel topical local anaesthetic formulation for anaesthesia of horn buds in goat kids

4.2.1 Abstract

AIM: To formulate and evaluate a novel topical local anaesthetic cream for anaesthesia of horn buds in goat kids.

Methods: An Oil-in-water emulsion containing articaine, tetracaine and bupivacaine and chemical penetration enhancers was prepared. The percutaneous permeation of the local anaesthetics from the novel formulation and EMLA cream was determined *in vitro* using Franz diffusion system. The *in vivo* efficacy of the novel formulation and EMLA cream was investigated in goat kids by applying the formulations around the skin of horn buds and evaluating the anaesthetic effect using a needle prick test. A liquid chromatography-mass spectrometry (LC-MS) method was developed and validated for the simultaneous quantification of the local anaesthetics in the samples collected from the *in vitro* and *in vivo* studies.

Results: The LC-MS method was simple, sensitive and accurate for the simultaneous detection and quantification of local anaesthetics. The results of the *in vitro* study suggest that the rate of permeation of articaine was greater than the permeation of other local anaesthetics through goat skin. *In vivo* results showed that neither the novel formulation nor EMLA cream produced effective anaesthesia of horn buds in goat kids. However, fast and long lasting cutaneous analgesia was produced by the novel formulation. The plasma concentrations of the local anaesthetics after the application of the novel formulation and EMLA cream remained well below the toxic plasma concentrations of respective local anaesthetics.

CONCLUSIONS AND CLINICAL RELEVANCE: The novel formulation did not produce effective anaesthesia of horn buds for disbudding in goat kids. Future studies are required to evaluate the efficacy of the novel formulation. The novel formulation can be evaluated for analgesia of wounds caused by various painful husbandry procedures such as tail docking, dehorning and castration.

KEY WORDS: Novel topical local anaesthetic formulation, EMLA, disbudding, goat kids, liquid chromatography-mass spectrometry

%CV	Percent coefficient of variation
%RE	Percent relative error
CPEs	Chemical penetration enhancers
Jss	Steady-state flux
K_p	Apparent permeability coefficients
LC-MS	Liquid chromatography-mass spectrometry
PRM	Parallel reaction monitoring

4.2.2 Introduction

Horned goats can be a potential danger to other animals and handlers (Smith and Sherman 2009). Additionally, horned goats can damage farm facilities, pose difficulties in transportation, and require more feeding trough space than hornless goats (Loretz *et al.* 2004; Al-Sobayil 2007). To avoid these problems, disbudding is routinely performed on dairy goat farms. Several disbudding techniques have been described in goats including thermal cautery disbudding (Smith and Sherman 2009), cryosurgical technique (Hempstead *et al.* 2018), application of caustic paste (Hempstead *et al.* 2017), and injection of clove oil (Molaei *et al.* 2015). Irrespective of the technique used, the procedure causes pain and distress in goat kids if performed without the administration of appropriate anaesthesia and analgesia (Hempstead *et al.* 2018; Hempstead *et al.* 2017). Cornual nerve block using local anaesthetics is one of the effective ways to alleviate acute pain during disbudding. However, the injection of local anaesthetics at four sites to desensitize the horn buds in goat kids requires skill and experience. Moreover, the injection of local anaesthetics at four sites (for both the horn buds) causes distress and pain in goat kids. A simple and less stressful analgesic technique that can be performed by a farmer would be preferred in commercial farms.

Topical local anaesthetic formulations have been used in humans for a variety of indications including anaesthesia for intravenous catheterization, vaccinations, skin graft harvesting, and removal of genital warts (Oni *et al.* 2012; Menter *et al.* 1997; Bagshaw *et al.* 2015). EMLA, a eutectic mixture of lidocaine and prilocaine is the most widely used topical local anaesthetic formulation in humans (Schreiber *et al.* 2013). Fierheller *et al.* (2012) investigated the efficacy of EMLA for anaesthesia of horn buds in calves. The authors reported that EMLA was ineffective in providing anaesthesia for disbudding because of the poor penetration of the local anaesthetics through thick calf skin. EMLA is an emulsion composed of low concentrations of lidocaine (2.5 %) and prilocaine (2.5 %). These low concentrations of local anaesthetics may not effectively penetrate the thick skin of calves to attain the concentration required to block the nerves supplying the horn buds. Chemical penetration enhancers (also known as sorption promoter or accelerants) are commonly used in topical and transdermal formulations to enhance the transport of drugs across the skin (Dragicevic and Maibach 2016). We propose that topical local anaesthetic formulations containing high concentrations of local anaesthetics and potent penetration enhancers may effectively penetrate goat skin to block the nerves supplying the horn buds.

4.2.3 Materials and methods

Drugs, chemicals, and apparatus

Articaine hydrochloride (99.9%) was purchased from SCI Pharmtech (Taoyuan, Taiwan), and articaine free base was synthesized from the hydrochloride salt using the procedure outlined in Annexure 1. Prilocaine, lidocaine, tetracaine, and bupivacaine free bases were procured from Wuhan Hezhong Biochemical Manufacturing Co, ltd (China). Eucalyptus oil, menthol, poly hydrogenated castor Oil (PEG 40), and Carbopol (Ultrez 21) polymer were purchased from PureNature (Auckland, New Zealand). EMLA cream was purchased from Aspen (Auckland, NZ). Sodium hydroxide and Tegaderm transparent adhesive film for occlusion

were purchased from Merck (Auckland, NZ) and 3M (Auckland, NZ), respectively. LC-MS grade acetonitrile, methanol, water, and formic acid were purchased from Fisher Scientific (Auckland, NZ). Franz cell apparatus and Franz cells were purchased from Permeagear (Hellertown Pennsylvania, USA).

Preparation of topical local anaesthetic cream

An Oil-in-water emulsion containing articaine, tetracaine, and bupivacaine was prepared. The emulsion composed of an oil phase (local anaesthetics, eucalyptus and menthol) suspended in an aqueous phase with poly hydrogenated castor oil as an emulsifier. The composition of the local anaesthetic cream is presented in table 4.1.

Preparation of aqueous phase (0.7% carbopol gel): Appropriate quantity of carbopol Ultrez 21 polymer (7 mg in 100 g of water) was taken in a flask and mixed thoroughly in water. The pH of the solution was adjusted to 9 using 1 N sodium hydroxide to form a gel.

Preparation of Oil phase: Local anaesthetics (articaine (8%), tetracaine (8%) and bupivacaine (4%)) were taken in a small flask and gently heated on a heating pad (50 ^{0}C) to convert the crystals to liquid. To this mixture, eucalyptus oil (8%), menthol (7%), and poly hydrogenated castor Oil (PEG 40) (7%) were added, gently heated (50 ^{0}C) and mixed.

Appropriate quantities of oil phase (42%) and carbopol gel (58%) were taken in a flask and homogenised at 25,000 rpm for 10 minutes at room temperature using a homogenizer (Ultra-Turrax T25, IKA, Malaysia). The prepared topical formulation was aliquoted into three portions in plastic tubes and stored at 4℃ until use. Two portions were used for *in vitro* and *in vivo* studies, and the third portion was stored at 4° C for six months to assess the stability of the emulsion.

Table 4.1. Composition of the novel local anaesthetic formulation

Ingredients	Per 100 grams of cream
Articaine (8%)	8 g
Tetracaine (8%)	8 g
Bupivacaine (4%)	4 g
Eucalyptus oil (8%)	8 g
Menthol (7%)	7 g
Poly hydrogenated castor oil (PEG 40) (7%)	7 g
Carbopol gel (0.7%)	0.7 g
Water (57.3%)	57.3 g

1N Sodium hydroxide to adjust pH to 9

In vitro permeation study

The percutaneous permeation of drugs and chemicals can be evaluated *in vitro* using Franz diffusion cells. In vitro models correlate well with the *in vivo* conditions for the percutaneous permeation of drugs (Franz *et al.* 2009; Hill 2015). Franz diffusion cells are widely used for the screening of drug formulations and chemical penetration enhancers prior to *in vivo* experiments.

The *in vitro* permeation of local anaesthetics from various formulations in the present study was investigated using a multi-station franz diffusion cell system (PermeGear, USA) (Figure 4.4). Franz cells are glass diffusion cells containing a donor chamber (diameter 1 cm) and a jacketed receptor chamber (8 mL), connected using a screw clamp. The multi-station franz diffusion cell system consisted of nine franz cell stations placed on a magnetic stirrer. The jacket of the receptor chambers was connected to a water bath to maintain the temperature of skin and receptor fluid (approx. 32° C) during the experiment. The percutaneous permeation of local anaesthetics from the novel topical formulation (n=3) and EMLA cream (n=3) were evaluated using goat skin.

Figure 4.4: Multi-station Franz diffusion cell system used for *in vitro* permeation study

Preparation of skin samples

Male Sannen goat kids were euthanized by injecting pentobarbitone sodium (100 mg/kg) into the cephalic vein and skin samples from the thoracic region were collected. Hair covering the skin was clipped using a hair clipper, and subcutaneous fat was carefully removed using a forceps and scissors. The skin samples were then stored at -20° C until use.

Permeation experiment

The frozen skin samples were thawed to room temperature, cut into circular discs (approximately 2 cm in diameter) and mounted between the donor and receptor chamber with the stratum corneum layer facing uppermost (Mills *et al*. 2005). After adding magnetic stirring beads to the receptor chamber, 8 mL of LC-MS grade water was added using a syringe and air bubbles were removed by tilting the Franz cells. The cells were then placed on the stations and the outer jacket of the receptor chamber was connected to the water bath maintained at 32° C. Water (1 mL) was added to the donor chamber to hydrate the skin samples for 60 minutes. After removing the water from the donor chamber using a pipette,

100 mg of the novel topical formulation (n=3) or EMLA cream (n=3) was added on to the skin using 1 mL syringes. The formulations were spread across the skin using glass rods, and the donor chamber was occluded with paraffin films. Samples (500 µL) were collected from the receptor chamber via the sampling ports using syringes and sampling tubes at 0, 15, 30 minutes and 1, 2, 4, 6, 8, 12, and 24 hours. LC-MS grade water (500 µL) was replaced into the receptor chamber immediately after the collection of each sample. The collected samples were stored at -20℃ until analysis using LC-MS/MS method.

In vivo study

Experimental animals

Twenty healthy male goat kids (one or two days old) were obtained from a goat farm (Opiki, New Zealand). Artificial colostrum (Excel Plus Colostrum, Farmlands, New Zealand) was fed to all the goat kids on the first day, and then were given milk replacer (Milligans milk powder, Farmlands) thrice daily using milk feeding buckets. Kids were housed on straw bedding in groups of ten. The experimental protocol was approved by the Massey University animal ethics committee (MUAEC Protocol 17/41).

Application of formulation, evaluation of anaesthesia and blood collection

Animals were randomly assigned to receive either the novel topical formulation (n = 10) or EMLA cream (n = 10). Kids were gently restrained and hair around one of the cephalic veins was clipped using a hair clipper. After preparing the site aseptically, an intravenous catheter (BD Insyte, Sandy, UT, USA) was placed into one of the cephalic veins. The hair around the horn bud region was clipped and one gram of EMLA or the novel topical cream was applied on the skin around each horn bud (approx. 10 cm^2) and occluded with tegaderm transparent adhesive to enhance drug absorption. The site of application was secured using bandages and animals were kept individually in feeding compartments for 90 minutes to avoid the removal of the occlusion. After 90 minutes, bandages, tegaderm and the cream were removed, and the

animals were housed in pens. Anaesthesia of horn buds and skin around the horn buds was assessed by needle prick at 30 minutes and 1, 1.5, 2, 4, 6, 8, and 12 hours following the application of the creams. Blood samples were collected at 0, 15, 30, 45 minutes and 1, 1.5, 2, 4, 6, 8 and 12 hours in heparinized vacutainers. Plasma was separated from the collected blood samples and stored at -20℃ until analysis using LC-MS/MS.

Analytical method

An LC-MS/MS method was developed and validated for the simultaneous quantification of articaine, lidocaine, prilocaine, bupivacaine and tetracaine in water and goat plasma.

Instrumentation

HPLC conditions

The ultra-high performance liquid chromatography system was equipped with a quaternary pump, a vacuum degasser, a column compartment and an autosampler (Dionex Ultimate 3000 System; Thermo Scientific, Germering, Germany). Chromatography was carried out using C_{18} column (100 mm × 2.1 mm; Accucore, Auckland, NZ) maintained at 25°C, and isocratic mobile phase consisting of 0.1% formic acid and acetonitrile (75:25, v/v). The mobile phase was delivered at a flow rate of 0.3 mL/minute into the mass spectrometer.

Mass Spectrometry conditions

Mass spectrometric detection was carried out on a hybrid quadrupole orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) with an electrospray-ionisation interface, positive ion mode. Quantification of analytes was performed in parallel reaction monitoring (PRM) mode using the transitions of m/Z 285.126→86.096, m/z 235.0→86.096, 220.311→86.096, 265.363→176.107 and 289.0→140.133 for articaine, lidocaine, prilocaine, tetracaine, and bupivacaine, respectively.

Preparation of standard solutions and quality control sample

Primary stock solutions (5 mg/mL) of each local anaesthetic (articaine, lidocaine, tetracaine, bupivacaine and prilocaine) were prepared by weighing appropriate quantities of standards and dissolving in methanol. Equal quantities of each primary stock solution of local anaesthetics were mixed to obtain an intermediate-stock solution (1 mg/mL of each local anaesthetic). The intermediate-stock was serially diluted in methanol to prepare working standards ranging from 10 ng/mL to 5000 ng/mL. Quality control samples and calibration standards were prepared by spiking 20 µL of working standard solutions into 180 µL of LCMS water or blank goat plasma. The concentrations of calibration standards ranged from 1 ng/mL to 500 ng/mL, and quality control samples were prepared at low (1 ng/mL), medium (10 ng/mL) and high (100 ng/mL) concentrations.

Sample preparation

Plasma samples (200 µL), including calibration standards, quality control samples, and samples obtained from *in vivo* study were precipitated with methanol (600 µL) and vortexed for 10 s. After 10 minutes, the samples were centrifuged at 3000g for 10 minutes. The supernatant (400 µL) was transferred to a phospholipid removal tube and centrifuged at 200 g for 2 minutes. The eluants were collected in a glass tube, transferred into autosampler vials and a 5 µL was injected onto the column. The samples collected from the Franz cells were simply diluted with appropriate quantities of LCMS grade water and injected (5 µL) onto the column.

Method Validation

The LC-MS/MS method was validated for specificity, linearity, precision, accuracy, recovery, and carry-over effect.

Specificity

The specificity was determined by running blank plasma samples and plasma samples spiked with low concentrations (10 ng/mL) of local anaesthetics, and comparing their chromatograms for any interfering peaks at the retention times of the respective local anaesthetics.

Linearity and lower limit of quantification

The linearity of the method was evaluated by linear regression analysis. The known concentrations of the local anaesthetics (x-axis) were plotted against the corresponding peak areas (y-axis) and regression equations ($y = mx + c$) were obtained. The calibration curves were constructed for concentrations ranging from 1 ng/mL to 500 ng/mL. The lower limit of quantification is defined as the lowest concentration on the calibration curve with a minimum signal to noise ratio of 10:1.

Intra-day and inter-day precision and accuracy

The intra- and inter-day precision and accuracy of the method was evaluated by running quality control samples in triplicates on a single day and on six different days, respectively. The values were expressed as percent coefficient of variation (%CV) and percent relative error (%RE).

Extraction recovery and matrix effect

The extraction recovery of local anaesthetics from plasma was determined by comparing the peak areas of local anaesthetics in the quality control samples prepared by spiking the standards in blank plasma samples prior to extraction with the peak areas of local anaesthetics spiked after extraction. The matrix effect was determined by comparing the peak areas of the local anaesthetics in the quality control samples spiked in the blank plasma samples after extraction with the peak areas of the same concentrations prepared in the mobile phase.

Carry-over effect

The carry-over effect from the system was assessed by injecting a drug-free plasma sample after the injection of a plasma sample containing the highest concentration of the calibration standard (500 ng/mL). Similarly, the carry-over effect was also assessed for samples prepared in water by injecting the highest concentration of the calibration standard followed by injection of blank water.

In vitro data analysis

The rate of permeation of the local anaesthetics through the skin was calculated by dividing the concentrations in the receptor solution at each collection time point with the duration of the collection period (Mills *et al.* 2004). The cumulative drug permeation was calculated using the following formula:

$$Q_t = V_r C_t + \sum_{i=0}^{t-1} V_s C_i$$

Where, V_r and C_t are the volumes of the receptor solution and the concentration of the local anaesthetics in the receptor solution at each time point, respectively. Where, *Vs* is the volume of the sample, and C_i is the concentration of the local anaesthetic of the i_{th} sample (Wang *et al.* 2016). The cumulative drug permeation per unit surface area of the skin was expressed as Q_t/S (S, is the surface area of skin 1 cm^2). The steady state flux of local anaesthetics was estimated using linear regression equation of the concentration vs time data. The steady flux can be expressed using the following equation

$$J_{ss} = \frac{\Delta Q_t}{\Delta t \times S}$$

Where Qt is the total quantity of the local anaesthetic

Apparent permeability coefficients (K_p) were determined for each local anaesthetic using the formula

$$K_p = J_{ss}/C_d$$

Where C_d is the concentrations of the local anaesthetics in the donor chamber, it was assumed that the concentrations of the local anaesthetics in the receptor fluid is negligible compared to the concentration in the donor chamber (Sintov and Shafir 2005).

4.2.4 Results

Representative chromatograms and the mass spectra (PRM mode) of blank plasma, blank plasma spiked with 10 ng/mL of each local anaesthetic, and plasma sample obtained from *in vivo* experiment are shown in Supplementary Figure 4.1. No interfering peaks were seen at the retention times of any of the local anaesthetics. The calibration curves of local anaesthetics were linear over the concentration range of 1 ng/mL to 500 ng/mL, with correlation coefficients (r_2) values above 0.996. The standard curves of all the local anaesthetics constructed in water and goat plasma are shown in the Supplementary Figure 4.2 and 3, respectively. The lower limit of quantifications of the analytes was 1 ng/mL. The intra-day and inter-day variations (%CV) of quality control samples prepared in water were below 11.32% and 14.54%, respectively. The intra-day and inter-day variations (%CV) of quality control samples in plasma were below 10.12% and 17.30%, respectively. The extraction recoveries of different local anaesthetics from the quality control samples ranged from 43 ± 1.9 % to 105 ± 11.8 %. Carry-over effect was not noticed in the blank samples after the injection of the high standard concentration (500 ng/mL).

No phase separation was observed in the aliquot stored at 4℃ for 6 months. The cumulative permeation of local anaesthetics through goat skin from the novel formulation and EMLA cream are shown in Figure 4.5. Table 4.2 shows the steady-state fluxes and apparent

permeability coefficients of various local anaesthetics. All the animals responded to needle prick on their horn buds at all the time points. However, no reaction was observed to needle prick on the skin around the horn buds from 30 minutes to 8 hours following the application of novel formulation and from 60 minutes to 3 hours after the application of EMLA cream. The mean ± SD plasma concentrations of local anaesthetics following the application of EMLA and the novel topical local anaesthetic cream are shown in figure 4.6. Erythema of the skin around the horn buds was seen after the removal of the occlusion in all the animals. It was more prominent in the animals that received the novel topical cream than those received EMLA cream. No other adverse effects were observed following the application of EMLA or the novel cream.

Table 4.2. The steady-state fluxes and apparent permeability coefficients of various local anaesthetics

Local anaesthetics	**Steady-state flux (J_{ss})**	**Apparent permeability coefficients ($K_{p} * 103$) (cm h^{-1})**
Articaine (8%)	56.7 ± 3.5	7.1 ± 0.4
Tetracaine (8%)	10.2 ± 0.8	1.3 ± 0.1
Bupivacaine (4%)	3.9 ± 0.4	1.0 ± 0.1
Lidocaine (2.5%)	18.6 ± 3.1	7.4 ± 1.2
Prilocaine (2.5%)	16.9 ± 2.1	6.7 ± 0.9

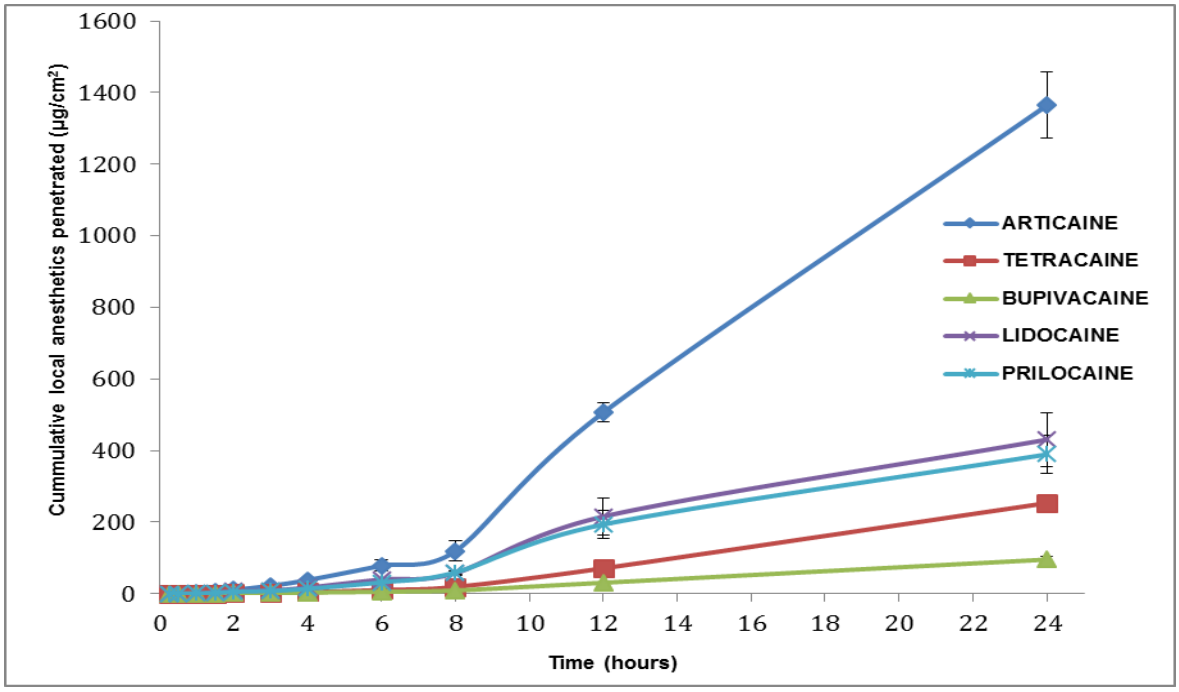

Figure 4.5: The cumulative permeation of local anaesthetics through goat skin from the novel formulation (articaine, tetracaine, and bupivacaine) and EMLA cream (lignocaine and prilocaine).

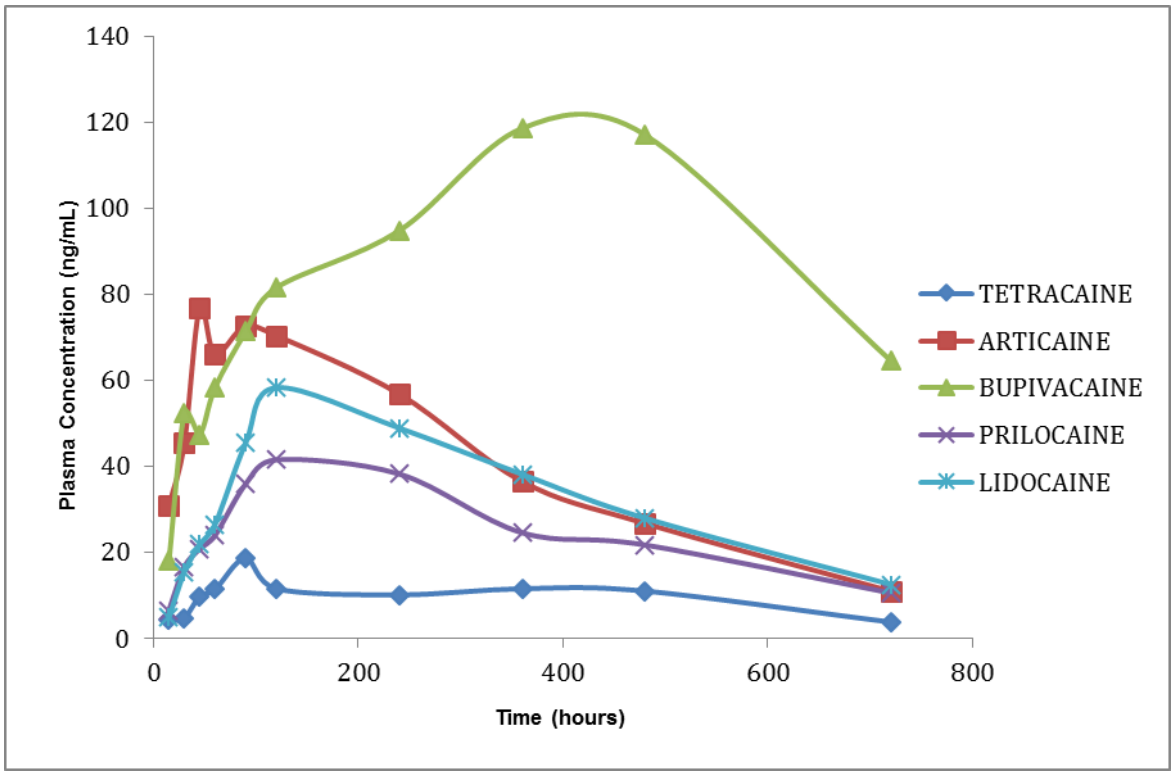

Figure 4.6: The mean ± SD plasma concentrations of local anaesthetics following the application of novel topical local anaesthetic cream (articaine, tetracaine, and bupivacaine) and EMLA (lignocaine and prilocaine).

4.2.5 Discussion

The LC-MS/MS method was simple, sensitive, accurate and rapid for the simultaneous quantification of the local anaesthetics. Initially, HPLC was used for the quantification of the local anaesthetics in the samples from the Franz cells but it was time consuming and was not suitable for the analysis of large quantities of samples. Phosphate-buffered saline (pH 7.4; 0.1 M) was initially used as the receptor solution in the Franz cell. The salts in the phosphate-buffered saline precipitated in the sweep cone of the ion source area and clogged the needle of the heated-electrospray ionization probe of the mass spectrometer. Hence, LCMS grade water was then used as the receptor solution. The experimental conditions used in this study did not mimic the in vivo conditions as addition of water in the Franz cell chambers can hydrate the skin and increase the permeability of drugs. Future studies should consider using biological buffers and desalt the samples prior to LC-MS analysis.

In a pilot study, several chemical penetration enhancers (CPEs) including D-limonene, eucalyptus oil, menthol, isopropyl myristate, dimethylformamide, transcutol-P, N-methyl-2-pyrrolidine and octylsalicylate were evaluated for the permeation of the local anaesthetics across the goat skin (results are not presented as the sample size was low). Among these penetration enhancers, the combination of eucalyptus oil and menthol was found to enhance the permeation of the local anaesthetics (articaine, tetracaine and bupivacaine) better than other CPEs. Different types of formulations including macroemulsions, microemulsion, hydroethanolic gel, liposomes, and ethosomes were prepared and evaluated. The local anaesthetic loading capacity in microemulsions, hydroethanolic gel, liposomes, or ethosomes were less compared to macroemulsions. In addition, the penetration of local anaesthetics through goat skin was greater from the novel formulation (macroemulsion) compared to other formulations.

The combination of articaine, tetracaine and bupivacaine were used in the novel formulation. Articaine was chosen as one of the components of the formulation because it is a new local anaesthetic drug and no previous studies on its topical use have been reported. Tetracaine was added to the mixture because it formed a eutectic mixture with articaine, and bupivacaine was chosen to increase the duration of the anaesthetic effect. The absence of phase separation at refrigerated temperature suggests that the novel formulation may be stable for up to six months. However, further studies to evaluate the particle size and stability at different temperatures are required to characterize the formulation. This is the first study to investigate the topical local anaesthetic effect of articaine. One interesting finding was that when equal quantities of articaine and tetracaine were mixed, eutectic mixture was formed. At room temperature, both articaine (melting point - 172℃) and tetracaine (melting point - 46℃) existed as crystals but when they were mixed, the crystals melted and existed as liquid oil. However, the precise melting point of the eutectic mixture was not determined in this study. Eutectic mixtures are advantageous in topical and transdermal formulations as they possess high thermodynamic activities that enhance the permeation of drugs through the skin (Kang and Jun 2003). The common examples of eutectic mixtures are EMLA cream (eutectic mixture of lidocaine and prilocaine), and Pliaglis (eutectic mixture of lidocaine and tetracaine).

The *in vitro* study results suggested that the permeation of articaine through goat skin was greater than other local anaesthetics. The cumulative concentrations of local anaesthetics were greater in the novel formulation than EMLA cream. This was likely because high concentrations of local anaesthetics were present in the novel formulation than EMLA cream. EMLA cream was used for comparison because previous studies have used EMLA cream to study its efficacy for the anaesthesia of horn buds (Fierheller *et al.* 2012; Winder *et al.* 2016). Other commercially available topical local anesthetic formulations containing tetracaine

(Ametop) or high concentrations of lignocaine (LMX) can also be used to compare the efficacy of the novel formulations.

The order of flux through goat skin was articaine>lidocaine>prilocaine>tetracaine>bupivacaine. Several factors including molecular weight, dissociation constant (pK_a), lipophilicity (log p), and melting point of the drug determine the flux of the drug across the skin (Marwah *et al.* 2016). Though articaine has higher molecular weight (284 g/mol) and lower lipophilicity (logP - 1.9) than tetracaine (264 g/mol; logP – 3.5), the flux of articaine was higher than tetracaine because of its lower pK_a value (7.8) than tetracaine (8.5). Most of the local anaesthetics are weak bases that exist in equilibrium between the ionized, water-soluble, and unionized, lipid-soluble form. Only the unionized lipid-soluble forms can penetrate the lipid bilayer of skin and axoplasm of neuron. However, it must be noted that after entering the axoplasm, the unionized forms gain a hydrogen ion to become ionized forms and it is the ionized forms that bind to the sodium channels (Becker and Reed 2012). The ratio of unionized to ionized forms at a given pH (pH of the formulation was 9) is determined by the pKa of the drug. Even though the concentrations of tetracaine and articaine were the same in the novel formulation, the diffusion of articaine was better than tetracaine because of its lower pKa value (7.8) than tetracaine (8.5).

Plasma concentration profile of local anaesthetics following the application of the formulations indicates that the concentrations of local anaesthetics remained well below the reported toxic plasma concentrations of respective local anaesthetics (Munson *et al.* 1977, Becker and Reed 2012). Even though the plasma concentrations of bupivacaine were greater than articaine and other local anaesthetics this does not mean that the permeation of bupivacaine was higher than other local anaesthetics. The concentrations of bupivacaine were higher because of its relative slower metabolism and longer half- life than articaine and

other local anaesthetics. A post-hoc power analysis of the data from the in vivo study revealed that at least 14 animals would be needed to show a significant difference in drug permeation.

Neither the novel formulation nor EMLA cream was effective in providing effective anaesthesia of horn buds. The failure was likely because the formulations could not penetrate deep enough to achieve the concentrations required to block the nerve fibres supplying horn buds. Future studies to determine the depth of skin analgesia after applying the formulation on the skin around the horn bud region of goat kids are required to understand the efficacy of this novel formulation (Bjerring and Arendt-Nielsen 1990).

The novel formulation produced faster and long-lasting cutaneous analgesia compared to EMLA cream. The duration of action of the novel formulation was longer than EMLA cream because the novel formulation had high concentrations of long acting local anesthetics bupivacaine and tetracaine. The potencies of local anesthetics (bupivacaine, tetracaine and articaine) used in the novel formulation are greater than the potencies of the local anesthetics present in EMLA cream (lidocaine and prilocaine). Persistence of cutaneous analgesia for 8 hours indicates that the novel formulation may be useful for procedures that require prolonged topical analgesia. The novel formulation can be evaluated for analgesia of wounds following painful husbandry procedures such as scoop dehorning, mulesing, castration, branding, tail docking and velvet antler removal. Even though EMLA cream is a safe and widely used topical local anaesthetic cream, systemic toxicity has been reported following its application in humans, particularly when large quantities are applied for a prolonged period (Hahn *et al.* 2004; Tran and Koo 2014). The risk of systemic toxicity should be less when topical formulations containing only articaine as an active ingredient are used because articaine undergoes rapid hydrolysis in plasma following systemic absorption (Yapp *et al.* 2011; Oertel 1997). However, future studies to evaluate the efficacy and safety of the topical

local anaesthetic formulations containing articaine are required before recommending for clinical use.

4.2.6 References

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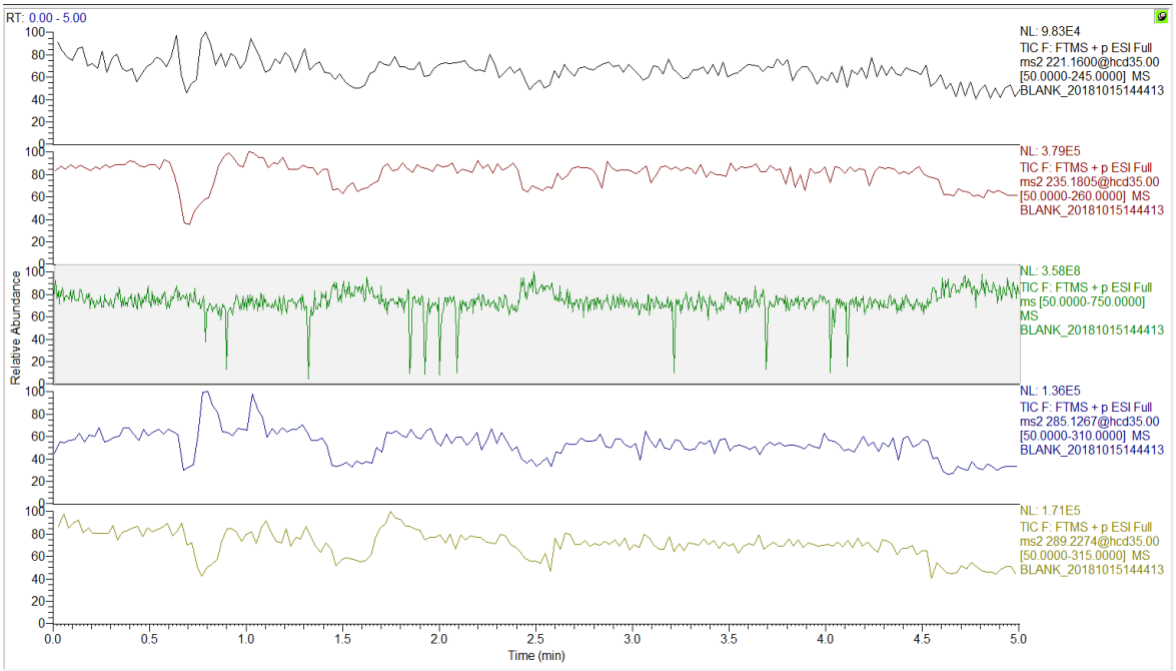
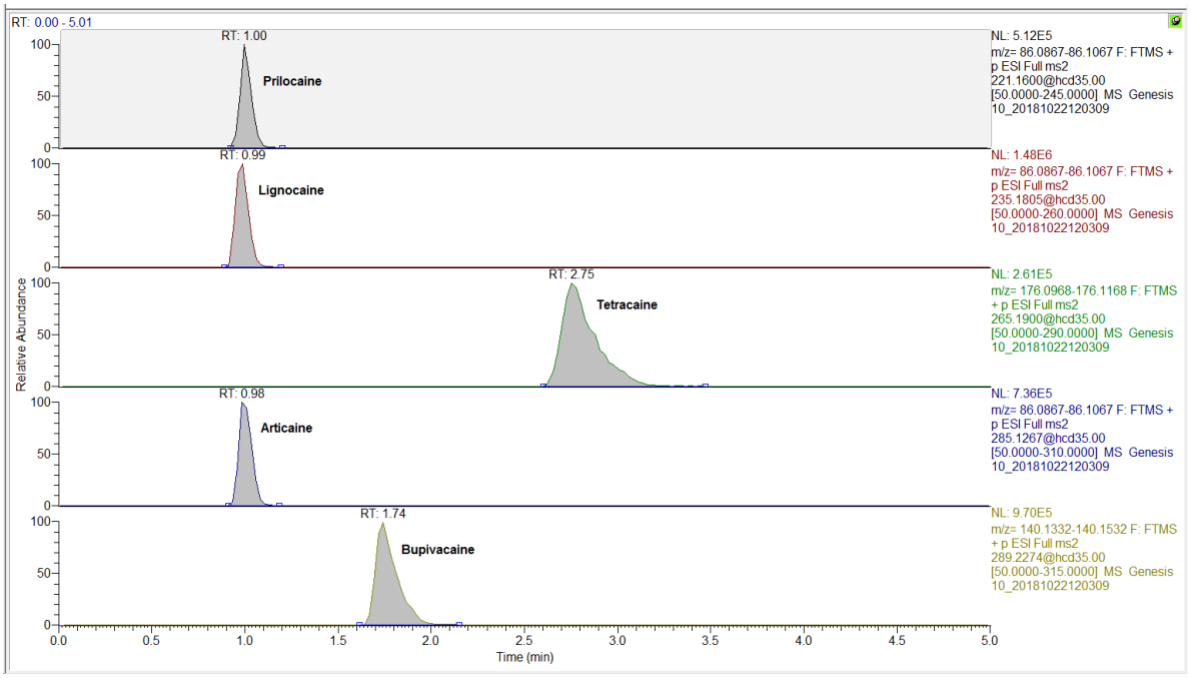
4.2.7 Supplementary materials

Supplementary Figure 4.1: Chromatograms of (a) plasma from untreated goat kids, (b) blank plasma with 10 ng mL^{-1} of local anaesthetics (prilocaine, lignocaine, tetracaine, articaine, and bupivacaine) added, and plasma from goat kids following the application of (c) EMLA cream and (d) novel topical local anaesthetic formulation.

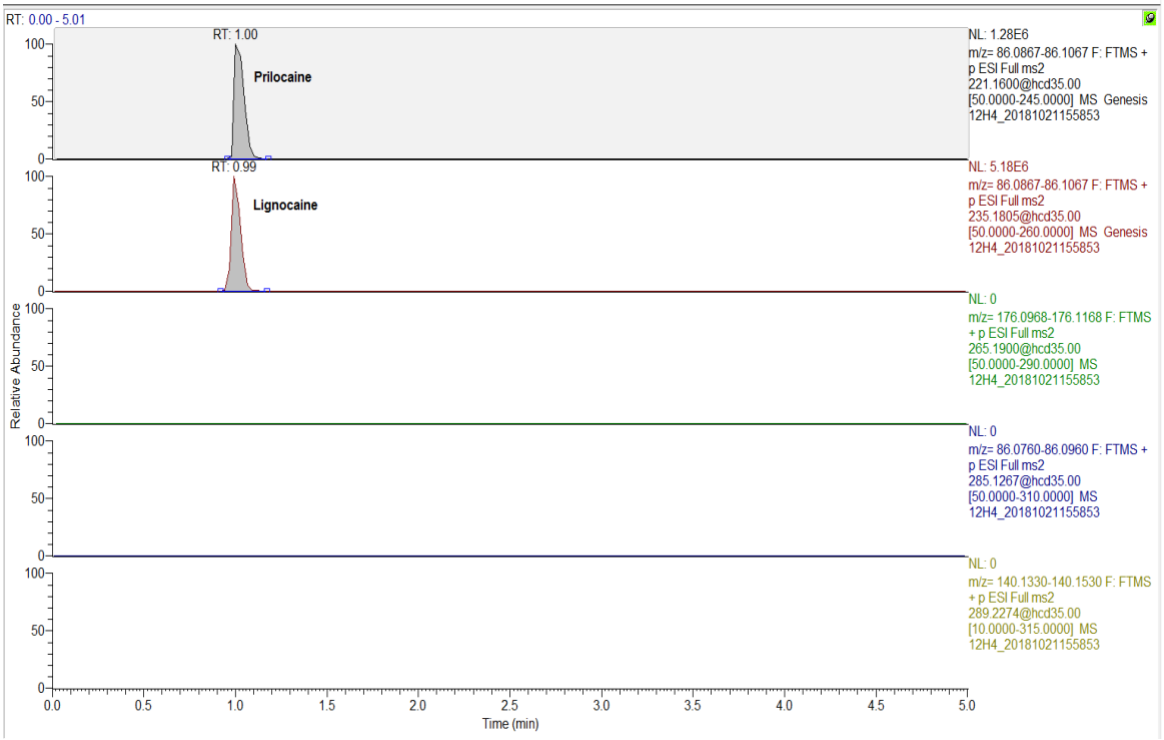
a) Blank plasma



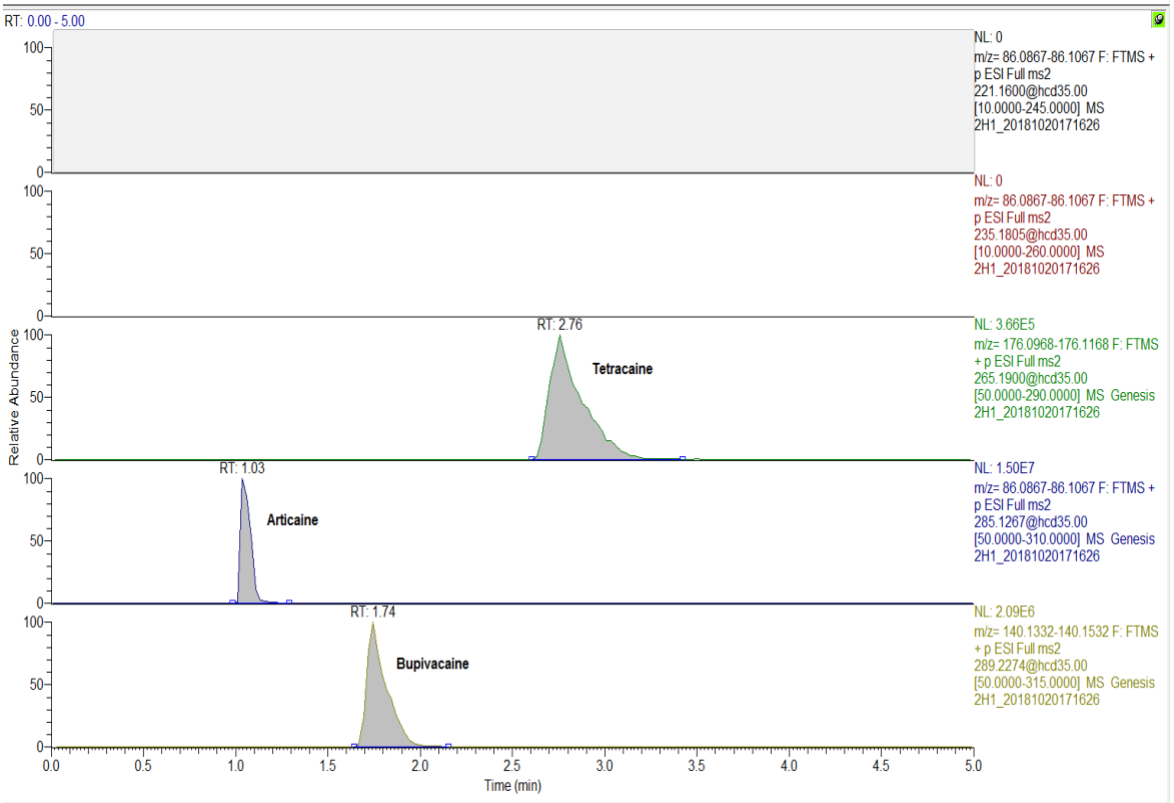
b) Plasma spiked with local anaesthetics



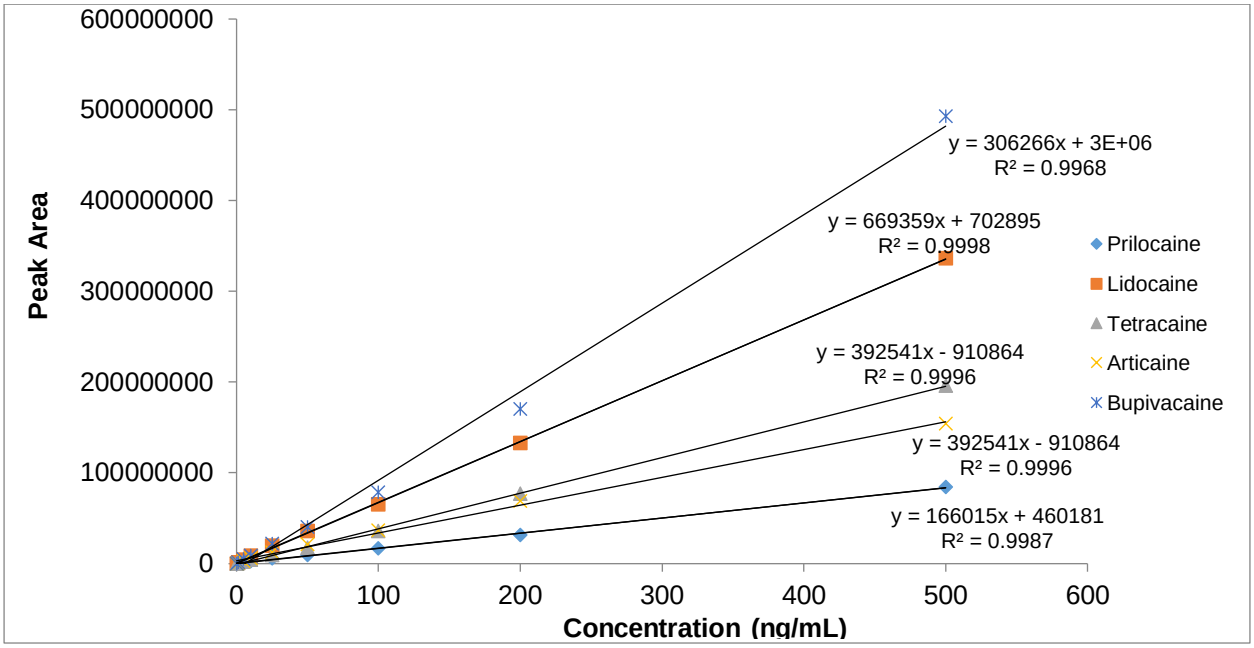
c) Plasma sample from a goat kid following the application of EMLA cream (Prilocaine and lignocaine)



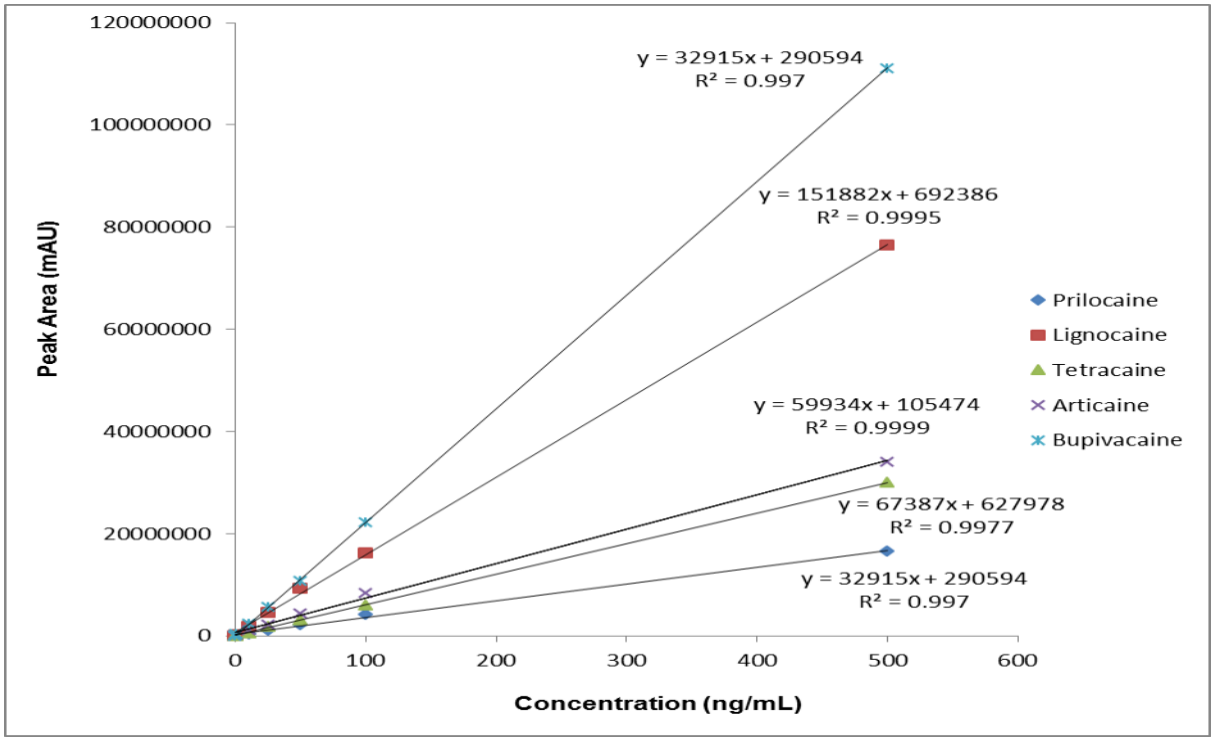
d) Plasma sample from a goat kid following the application of novel topical cream (Tetracaine, articaine, and bupivacaine)



Supplementary Figure 4.2: Representative calibration curves of prilocaine, lignocaine, tetracaine, articaine, and bupivacaine constructed by spiking LC-MS water with different concentrations of each analyte.



Supplementary Figure 4.3: Representative calibration curves of prilocaine, lignocaine, tetracaine, articaine, and bupivacaine constructed by spiking pooled plasma sample from untreated goat kids with different concentrations of each analyte.

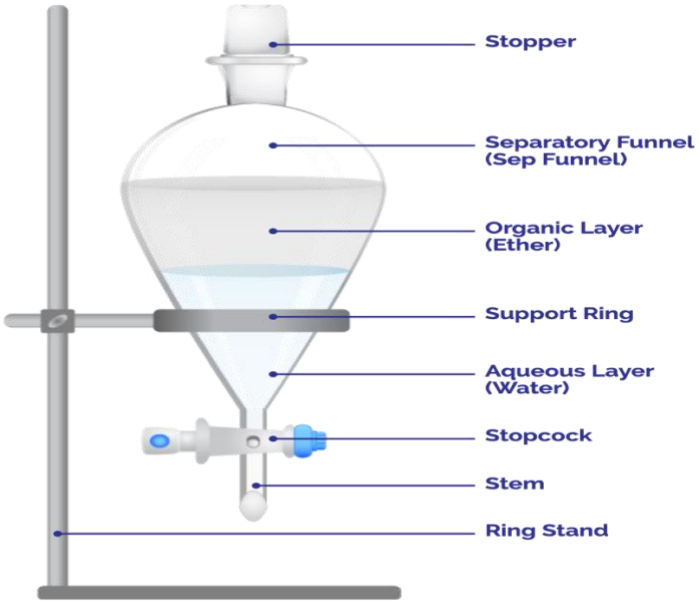


4.2.8 APPENDIX – 1

Preparation of articaine free base from articaine hydrochloride

Weigh 100 grams of articaine hydrochloride in a beaker and add 500 mL of Milli-Q water to dissolve the drug. To this solution, add 15 grams of solid sodium hydroxide (NaOH) pellets and stir for 5-10 minutes using a glas rod. Transfer the contents into a separation funnel (Supplementary Figure 4.4) and add 500 mL of ether. Add the stopper to the separation funnel to mix the contents. Using a smooth rocking motion, shake the contents for 20-30 seconds to achieve equilibrium. During shaking, periodically vent the funnel (open the stopcock) to release solvent vapors and relieve any built-up pressure. Remove the stopper and wait till the layers (aquenous and organic) are fully separated. Discard the aqueous layer by opening the stopcock. Collect the organic layer in a beaker and dry the collected ether solution over anhydrous magnesium sulphate ($MgSO_4$). Filter off the $MgSO_4$ using a Whatman filter paper into a round bottomed flask. Rotavap off the ether and place the flask on a freeze drier. Remove the flask from the freeze drier once the contents are dry and store in a refrigerator.

Supplementary Figure 4.4: Set up used for the preparation of articaine free base



4.2.9 APPENDIX – 2

Table 4.3. Intra- and inter-day accuracy and precision of prilocaine in LC-MS water

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	0.97 ± 0.10	10.12	-3.40
	10	9.33 ± 0.74	7.92	-6.72
	100	97.40 ± 8.89	9.13	-2.60
Inter-assay	1	0.98 ± 0.09	9.54	-2.01
	10	10.10 ± 1.13	11.23	1.03
	100	119.05 ± 7.70	6.47	15.38

Table 4.4. Intra- and inter-day accuracy and precision of lignocaine in LC-MS water

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	1.06 ± 0.08	7.25	5.52
	10	10.23 ± 1.10	10.75	2.26
	100	101.03 ± 11.44	11.32	1.03
Inter-assay	1	1.17 ± 0.20	12.55	-7.51
	10	11.58 ± 1.13	9.78	15.75
	100	115.92 ± 13.96	12.04	15.92

Table 4.5. Intra- and inter-day accuracy and precision of tetracaine in LC-MS water

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	0.96 ± 0.03	3.58	-3.92
	10	10.12 ± 0.92	9.05	1.18
	100	97.46 ± 3.98	4.08	-2.54
Inter-assay	1	0.93 ± 0.08	8.70	-7.26
	10	10.64 ± 1.13	10.64	6.44
	100	102.60 ± 13.19	12.86	2.60

Table 4.6. Intra- and inter-day accuracy and precision of articaine in LC-MS water

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	0.95 ± 0.06	6.45	-4.55
	10	9.60 ± 0.50	5.24	-4.03
	100	93.54 ± 6.80	7.27	-6.46
Inter-assay	1	0.89 ± 0.12	13.06	-10.71
	10	9.38 ± 1.36	14.54	-6.17
	100	99.33 ± 10.23	10.30	-0.67

Table 4.7. Intra- and inter-day accuracy and precision of bupivacaine in LC-MS water

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	0.89 ± 0.06	6.38	-10.04
	10	10.21 ± 0.98	9.60	2.08
	100	101.15 ± 7.82	7.73	1.15
Inter-assay	1	0.91 ± 0.08	8.36	-9.10
	10	11.03 ± 1.07	9.73	10.34
	100	112.40 ± 16.07	14.29	12.40

Table 4.8. Intra- and inter-day accuracy and precision of prilocaine in goat plasma

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	0.90 ± 0.09	10.12	-9.73
	10	10.02 ± 0.25	2.46	0.19
	100	94.19 ± 3.82	4.06	-5.81
Inter-assay	1	0.94 ± 0.06	6.93	-6.35
	10	9.85 ± 0.30	3.01	-1.47
	100	102.28 ± 5.67	5.54	2.28

Table 4.9. Intra- and inter-day accuracy and precision of lignocaine in goat plasma

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	1.01 ± 0.06	6.21	0.77
	10	10.59 ± 0.26	2.42	5.88
	100	103.49 ± 1.71	1.65	3.49
Inter-assay	1	0.88 ± 0.12	6.93	-12.38
	10	8.44 ± 1.49	3.01	-15.59
	100	87.63 ± 16.96	5.54	-12.37

Table 4.10. Intra- and inter-day accuracy and precision of tetracaine in goat plasma

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	1.05 ± 0.09	8.65	4.96
	10	9.89 ± 0.37	3.69	-1.09
	100	99.30 ± 1.60	1.61	-0.70
Inter-assay	1	1.19 ± 0.20	16.74	19.28
	10	11.55 ± 1.17	10.11	15.47
	100	112.43 ± 8.03	7.15	12.43

Table 4.11. Intra- and inter-day accuracy and precision of articaine in goat plasma

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	0.93 ± 0.09	9.81	-6.78
	10	9.80 ± 0.22	2.29	-1.95
	100	94.33 ± 0.59	0.63	-5.67
Inter-assay	1	0.77 ± 0.13	17.30	-22.65
	10	10.71 ± 1.57	14.68	7.13
	100	96.93 ± 3.72	3.84	-3.07

Table 4.12. Intra- and inter-day accuracy and precision of bupivacaine in goat plasma

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-assay	1	1.02 ± 0.09	8.61	1.51
	10	9.78 ± 0.36	3.70	-2.21
	100	99.41 ± 2.06	2.08	-0.59
Inter-assay	1	1.12 ± 0.10	8.98	12.18
	10	10.26 ± 0.35	3.44	2.58
	100	103.02 ± 6.33	6.14	3.02

Table 4.13. Extraction recoveries of various local anaesthetics from goat plasma

Concentration (ng/mL)	Mean extraction recovery (%)				
	Prilocaine	**Lidocaine**	**Tetracaine**	**Articaine**	**Bupivacaine**
1	55.0 ± 6.4	65.9 ± 8.6	43.1 ± 1.9	105.8 ± 11.8	80.9 ± 10.3
10	64.1 ± 4.2	70.3 ± 5.4	76.4 ± 8.6	44.3 ± 6.2	104.0 ± 7.1
100	94.7 ± 8.2	84.4 ± 10.8	98.8 ± 4.7	74.6 ± 2.3	101.3 ± 9.4

Table 4.14. Concentrations (mean ± SD) of various local anaesthetics in the samples collected from the receptor chamber of franz cells following the application of the novel formulation (articaine, tetracaine, and bupivacaine) and EMLA cream (lignocaine and prilocaine) on goat skin.

Time points (hours)	Novel topical cream			EMLA cream	
	Articaine	Tetracaine	Bupivacaine	Lignocaine	Prilocaine
0.25	6.0±2.5	BDL	BDL	BDL	BDL
0.5	12.6±3.2	BDL	3.6±1.0	8.5±6.4	4.9±0.4
1	142.8±51.0	10.4±2.8	13.9±6.7	113.0±5.7	79.1±14.2
1.5	469.6±131.1	31.2±10.4	47.5±13.7	382.7±127.8	274.8±98.7
2	1265.4±140.5	84.5±11.1	97.1±9.3	876.4±229.7	658.3±171.2
3	2643.8±946.0	255.9±66.0	213.4±58.2	1049.6±107.5	913.4±187.3
4	4447.5±592.1	493.8±117.1	349.2±56.7	1980.6±368.8	1608.8±179.6
6	9202.3±2045.1	1225.4±370.4	646.7±174.4	4673.9±356.8	3748.2±203.7
8	13746.7±3342.8	2235.0±378.9	1040.6±218.4	6612.5±659.7	6951.8±627.6
12	61251.9±3254.5	8579.5±514.5	3665.2±183.2	25936.7±6543.0	23263.4±4674.5
24	164720.6±11199.7	30837.6±2416.8	11538.0±1155.0	51181.3±9130.5	46305.0±6506.5

BDL –Below detection limit

Table 4.15. Plasma concentrations (mean ± SD) of local anaesthetics following the application of novel topical local anaesthetic cream and EMLA cream in goat kids.

Time points (minutes)	Novel topical cream			EMLA cream	
	Articaine	Tetracaine	Bupivacaine	Prilocaine	Lignocaine
15	30.77±39.95	4.26±3.48	18.05±22.06	6.58±7.89	4.91±5.78
30	45.41±46.72	4.76±3.23	52.22±58.23	16.57±16.76	15.39±21.31
45	76.82±62.05	9.80±8.71	47.44±40.21	20.79±21.68	21.81±29.51
60	66.12±41.62	11.37±6.19	58.29±30.61	23.94±21.33	26.50±31.46
90	72.78±41.64	18.70±14.35	71.30±40.78	35.92±18.49	45.47±28.55
120	70.39±43.50	11.56±6.19	81.54±53.13	41.52±17.02	58.29±27.80
240	56.82±34.09	10.14±4.55	94.78±58.83	38.22±22.24	48.91±21.89
360	36.48±14.54	11.58±4.67	118.58±75.23	24.56±7.92	38.15±14.38
480	26.74±5.70	11.00±9.90	117.05±53.44	21.73±14.77	27.96±16.37
720	10.97±2.29	3.81±1.75	64.54±42.57	10.58±7.05	12.53±11.66

CHAPTER 5

ARTICAINE HYDROCHLORIDE FOR VELVET ANTLER REMOVAL IN RED DEER

This Chapter consists of:

1) **Pharmacokinetics of articaine hydrochloride and its metabolite articainic acid after subcutaneous administration in red deer (*Cervus elaphus*).**

   This study was published as research article in the *New Zealand Veterinary Journal.*
   Venkatachalam D, Chambers JP, Kongara K, Singh P (2018). Pharmacokinetics of articaine hydrochloride and its metabolite articainic acid after subcutaneous administration in red deer (*Cervus elaphus*). *New Zealand Veterinary Journal*, 66(1):16-20.

2) **Analgesic efficacy of articaine hydrochloride for velvet antler removal in red deer (*Cervus elaphus*) and analysis of drug residues in the harvested velvet antlers.** This manuscript has been accepted for publication in the *New Zealand Veterinary Journal*.

   Venkatachalam D, Chambers JP, Kongara K, Singh P. Analgesic efficacy of articaine hydrochloride for velvet antler removal in red deer (*Cervus elaphus*) and analysis of drug residues in the harvested velvet antlers. https://doi.org/10.1080/00480169.2019.1611503.

5.1 Pharmacokinetics of articaine hydrochloride and its metabolite articainic acid after subcutaneous administration in red deer (*Cervus elaphus*)

5.1.1 Abstract

AIM: To develop and validate a simple and sensitive method using liquid chromatography-mass spectrometry (LC-MS) for quantification of articaine, and its major metabolite articainic acid, in plasma of red deer (*Cervus elaphus*), and to investigate the pharmacokinetics of articaine hydrochloride and articainic acid in red deer following S/C administration of articaine hydrochloride as a complete ring block around the antler pedicle.

METHODS: The LC-MS method was validated by determining linearity, sensitivity, recovery, carry-over and repeatability. Articaine hydrochloride (40 mg/mL) was administered S/C to six healthy male red deer, at a dose of 1 mL/cm of pedicle circumference, as a complete ring block around the base of each antler. Blood samples were collected at various times over the following 12 hours. Concentrations in plasma of articaine and articainic acid were quantified using the validated LC-MS method. Pharmacokinetic parameters of articaine and articainic acid were estimated using non-compartmental analysis.

RESULTS: Calibration curves were linear for both articaine and articainic acid. The limits of quantifications for articaine and articainic acid were 5 and 10 ng/mL, respectively. Extraction recoveries were >72% for articaine and >68% for articainic acid. After S/C administration as a ring block around the base of each antler, mean maximum concentrations in plasma (C_{max}) of articaine were 1,013.9 (SD 510.1) ng/mL, detected at 0.17 (SD 0.00) hours, and the C_{max} for articainic acid was 762.6 (SD 95.4) ng/mL at 0.50 (SD 0.00) hours. The elimination half-lives of articaine hydrochloride and articainic acid were 1.12 (SD 0.17) and 0.90 (SD 0.07) hours, respectively.

CONCLUSIONS AND CLINICAL RELEVANCE: The LC-MS method used for the quantification of articaine and its metabolite articainic acid in the plasma of red deer was simple, accurate and sensitive. Articaine hydrochloride was rapidly absorbed, hydrolysed to its inactive metabolite articainic acid, and eliminated following S/C administration as a ring block in red deer. These favourable pharmacokinetic properties suggest that articaine hydrochloride should be tested for efficacy as a local anaesthetic in red deer for removal of velvet antlers. Further studies to evaluate the safety and residues of articaine hydrochloride and articainic acid are required before articaine can be recommended for use as a local anaesthetic for this purpose.

KEY WORDS: *Articaine hydrochloride, local anaesthetic, liquid chromatography-mass spectrometry, pharmacokinetics, red deer*

C_{max}	Maximum concentration in plasma
DMA	2,6-dimethylaniline
LC-MS	Liquid chromatography-mass spectrometry
T_{max}	Time to reach maximum concentration in plasma

5.1.2 Introduction

Antlers are unique structures found in cervids which may be cast and regrown annually (Price and Allen 2004). Velvet antlers are highly innervated and vascularised cartilaginous structures that are harvested for commercial purpose in New Zealand, China, Canada and North America. In New Zealand, the removal of velvet antlers (velvetting) can only be carried out after desensitisation of the antlers (Anonymous 1992). A complete ring block around the antler pedicle, using the local anaesthetic lidocaine hydrochloride, is the most widely used and reliable technique to desensitise antlers before velvetting (Woodbury *et al.*

2002; Johnson *et al.* 2005). However, there are concerns regarding residues of lidocaine in the harvested velvet antlers (Woodbury *et al.* 2002; Bagonluri *et al.* 2005). In a limited survey of harvested velvet antlers, lidocaine was detected in 50% of samples at concentrations greater than the maximum permitted residue level of 0.1 mg/kg set by the New Zealand Food Safety Authority (Clear and Morris 2005). Lidocaine is metabolised by N-dealkylation in the liver to active metabolites, which are further metabolised to an aromatic amine, 2,6-dimethylaniline (DMA) (Duan *et al.* 2008). DMA is classified as potential carcinogen in humans (Anonymous 1993) and is hepatotoxic in rats (Duan *et al.* 2008). It has been reported to increase the incidence of various types of tumours in rats and humans (Anonymous 1990; Beland *et al.* 1997; Duan *et al.* 2008).

Articaine hydrochloride (4-methyl-3-(2-propylaminopropionamido) thiophene-2-carboxylic acid methyl ester hydrochloride) is a unique amide-type local anaesthetic. It is widely used in humans where it is considered to be safe and effective (Oertel *et al.* 1997; Vree and Gielen 2005; Su *et al.* 2016). It is rapidly metabolised to inactive metabolites (mostly articainic acid) by plasma cholinesterases, thus decreasing systemic toxicity to the cardiovascular and central nervous systems which is common to all local anaesthetics (Snoeck 2012). Toxicity studies of articaine in rats and dogs also showed no pathomorphological systemic changes, even after administration of systemically toxic doses, and during in vitro and in vivo studies no mutagenic properties were associated with articaine, even at cytotoxic concentrations or the maximum tolerated dose (Leuschner and Leblanc 1999).

Given concerns regarding lidocaine residue in harvested antlers and the reported advantages of articaine hydrochloride, we propose that articaine hydrochloride may be preferable to lidocaine as a local anaesthetic for velvetting in red deer (*Cervus elaphus*). However, it must be noted that articaine hydrochloride is not yet approved for use in veterinary medicine and

no studies have been conducted in animals to evaluate the carcinogenicity of articaine or its metabolites.

The objectives of this study were to develop and validate a simple and sensitive method using liquid chromatography-mass spectrometry (LC-MS) for the quantification of articaine and its metabolite articainic acid in plasma of red deer, and to determine the pharmacokinetic parameters of articaine hydrochloride following S/C administration as a complete ring block around the base of the antlers in red deer.

5.1.3 Materials and methods

Reagents and drugs

Articaine hydrochloride standard (99.9%) was purchased from SCI Pharmtech (Taoyuan, Taiwan) and the purity was confirmed using nuclear magnetic resonance crystallography at Massey University (Palmerston North, NZ). Articainic acid standard (97%) was obtained from Toronto Research Chemicals (Toronto, Canada). Acetonitrile, methanol, water and formic acid were LC-MS grade and were purchased from Fisher Scientific (Auckland, NZ). Reagent grade perchloric acid was obtained from BDH (Auckland, NZ). Articaine hydrochloride solution (40 mg/mL) was prepared freshly on the day of use by weighing an appropriate amount of standard and dissolving it in milliQ water. The solution was then filtered through a syringe filter (0.45 µm, Phenomenex Inc, Auckland, NZ) and made up to 40 mg/mL with sterile normal saline.

Preparation of standards and quality control samples

Standard stock solutions (1 mg/mL) of articaine hydrochloride and articainic acid were prepared by dissolving in water and methanol, respectively. Equal volumes of both stock solutions were mixed and working solutions were then prepared by dilution with water. Standard solutions for the construction of calibration curves and quality control samples were

prepared freshly by adding working solutions of either articaine or articainic acid to ice cold pooled plasma obtained from untreated red deer.

Sample preparation

A 0.5 mL aliquot of ice-cold plasma was added to a 1.5 mL microcentrifuge tube and 25 µL of perchloric acid was then added and mixed using a vortex mixer for 10 seconds to precipitate proteins. After 10 minutes, the samples were mixed again using the vortex mixer then centrifuged at 2,800g for 10 minutes and 5 µL of supernatant was injected on to the chromatography column.

Liquid chromatography-mass spectrometry

Liquid chromatography was carried out using an ultra high performance liquid chromatography system equipped with a quaternary pump, a vacuum degasser, a column compartment and an autosampler (Dionex Ultimate 3000 System; Thermo Scientific, Germering, Germany). Chromatographic separations were achieved using a 2.6 µm particle size C-18 column (100 mm × 2.1 mm; Accucore, Auckland, NZ) coupled with a security guard column (Defender Guard Column; Accucore) maintained at a temperature of 30°C. The mobile phase consisted of 0.1% formic acid and acetonitrile (70:30, v/v) and was delivered at a flow rate of 0.3 mL/minute.

Mass spectrometric detection was performed using a hybrid quadrupole orbitrap mass spectrometer (Q Exactive Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer; Thermo Scientific, Bremen, Germany) with an electrospray-ionisation interface. Positive ion electrospray ionisation-mass spectrometry was used for the analysis of both articaine and articainic acid. Mass spectrometry conditions are given in Table 5.1. Data processing was performed using the Xcalibur data system (Thermo Scientific) and quantitation was performed in the full scan MS mode using peak-area ratios of the target ion of articaine (mass

to charge ratio 285.16) and articainic acid (mass to charge ratio 272.11). Samples which exceeded the calibration limit were appropriately diluted with drug-free plasma and reanalysed.

Table 5.1. Mass spectrometry conditions for the quantification of articaine and articainic acid in the plasma of red deer.

Parameter	Value
Resolution setting	70,000
Mass range	100.0–500.0 m/z
Spray voltage	3.3 kV
Sheath gas flow rate	30.0 arbitrary units
Auxiliary gas flow rate	5.0 arbitrary units
Capillary temperature	320°C
Heater temperature	350°C
Radio frequency lens level	50.0
HCD collision energy	35 eV

HCD=higher energy collisional dissociation; m/z=mass-to-charge ratio

Validation

The linearity of the LC-MS method was determined by linear regression analysis. Calibration curves were constructed using three replicates of pooled plasma obtained from untreated deer spiked with working solutions of either articaine hydrochloride or articainic acid, at concentrations ranging between 5–2,000 ng/mL and 10–1,000 ng/mL, respectively. The lower limit of detection and quantification of the compounds were set at signal to noise ratios of 3:1 and 10:1, respectively.

Recoveries of articaine hydrochloride and articainic acid from red deer plasma after extraction were calculated by measuring the peak areas for three replicate samples of plasma from untreated deer containing 20, 200 and 1,000 ng/mL of quality control standards, following the same sample preparation procedure described above. Specificity of the method was determined by comparing the chromatograms of blank plasma samples obtained from six

untreated deer and a spiked plasma sample containing 20 ng/mL of articaine and articainic acid.

Intra-day and inter-day precision and accuracy of the method were determined by processing three replicates of plasma samples from untreated deer containing three concentrations (20, 200 and 1,000 ng/mL) of analytes six times on one day, and on six different days, respectively.

Carry-over from the system was assessed by injecting a drug-free plasma sample after injection of a plasma sample containing either articaine or articainic acid at concentrations equivalent to the respective upper limit of quantification.

Animals and experimental procedure

Six healthy 2-year-old male red deer weighing between 105– 116 kg were used for this study. The experimental protocol was approved by the Massey University Animal Ethics Committee (Palmerston North, NZ) and the study was conducted at Massey University Deer Research Unit (Palmerston North, NZ).

Each red deer was physically restrained in a hydraulic crush, the hair around the base of antlers was clipped and the pedicle circumference of each antler was measured using a measuring tape. The left or right facial vein was surgically prepared and a 20 gauge, 48 mm I/V catheter (BD Insyte, Sandy, UT, USA) was placed and secured with cyanoacrylate adhesive (Super Glue; Loctite, Auckland, NZ). The patency of the catheter was maintained using heparinised (10 USP/mL) saline.

Within 5 minutes of catheterisation, articaine hydrochloride (40 mg/mL) at a dose of 1 mL/cm of pedicle circumference was administered S/C as a complete ring block (4–5 injections) around the base of both the antlers using a 20 gauge needle attached to a 20 mL

syringe. The syringe plunger was pulled back prior to infiltration to ensure the needle was not in a blood vessel. The time taken for drug administration did not exceed 2 minutes in any of the animals. Blood samples were collected, via the catheter in the facial vein, into heparinised vacutainers (BD Vacutainer, Plymouth, UK) prior to drug administration (0 minutes), and 10, 20, 30, 40 minutes and 1, 2, 4, 6, 8, and 12 hours following drug administration. Immediately after collection, blood samples were cooled on ice and plasma was separated and stored at −20°C. LC-MS analysis was carried out within 1 month of sample collection.

For 2 hours after drug administration, animals were visually observed for adverse signs such as muscle tremor, sedation or convulsion while unrestrained in a pen.

Pharmacokinetic analysis

Pharmacokinetic parameters were determined using non-compartmental analysis. The PKSolver add-on (Zhang *et al.* 2010) for Excel 2010 (Microsoft, Redmond, CA, USA) was used to calculate pharmacokinetic parameters using individual concentrations of articaine and articainic acid in plasma. The maximum concentration in plasma (C_{max}) and time to achieve C_{max} (T_{max}) were determined directly from the curves of plasma concentrations over time. The rate constant of the terminal phase (λ_z) was calculated by linear regression of the logarithmic plasma concentration and the terminal half life was calculated as:

$$t_{½\lambda z} = \ln 2/\lambda_z$$

The area under the curve (AUC) and the area under the first moment curve (AUMC) were determined using the linear trapezoidal method. Mean residence time was calculated as:

$$MRT = AUMC/AUC$$

5.1.4 Results

Calibration curves (Supplementary Figure 5.1) were linear with correlation coefficients of 0.997 for articaine and 0.998 for articainic acid. The lower limits of quantification were 5 ng/mL and 10 ng/mL, and the lower limits of detection were 1 ng/mL and 5 ng/mL, for articaine and articainic acid, respectively. The assay intra-day CV for articaine and articainic acid were <4.1 and 5.3%, respectively, and the inter-day CV for articaine and articainic acid were <10.9 and 10.7%, respectively. Both inter-day and intra-day variations were within the acceptable values of <10% and <15%, respectively. The extraction recoveries for articaine ranged from 72–79% and for articainic acid from 68–75% (Table 5.2). No carry-over effect was found when a drug-free plasma sample was injected after a sample containing concentrations of the analytes equivalent to the upper limit of quantification. Analysis of six blank plasma samples from untreated deer showed no interfering peak on the chromatograms at the retention times of articaine or articainic acid.

Representative chromatograms and electrospray ionisation full scan MS mode are shown in Supplementary Figure 5.2 for plasma samples that were drug-free, or spiked with 20 ng/mL of articaine and articainic acid, or from a red deer following S/C administration of 40 mg/mL articaine hydrochloride as a complete ring block around the antlers.

Pharmacokinetics

No signs of adverse effects were observed in any animal following administration of articaine hydrochloride. Mean concentrations of articaine and its metabolite articainic acid in plasma following S/C administration of articaine as a ring block are shown in Figure 5.1. Peak concentrations of articaine in plasma were detected in the sample collected 10 minutes after drug administration, which was the first sample collected. Concentrations in plasma of articaine hydrochloride and articainic acid were above the limit of quantification for 6 and 4

hours after administration, respectively. The pharmacokinetic parameters of both compounds following S/C administration of articaine hydrochloride as a ring block are shown in Table 5.3.

Table 5.2. Mean (±SD) recovery (%) of articaine and articainic acid spiked into drug-free plasma from red deer (*Cervus elaphus*) at three different concentrations, after extraction and detection using liquid chromatography-mass spectrometry.

Concentration (ng/mL)	Articaine	Articainic acid
20	75.7±1.3	75.6±3.1
200	79.0±4.9	68.9±1.4
1,000	72.4±3.9	72.2±4.0

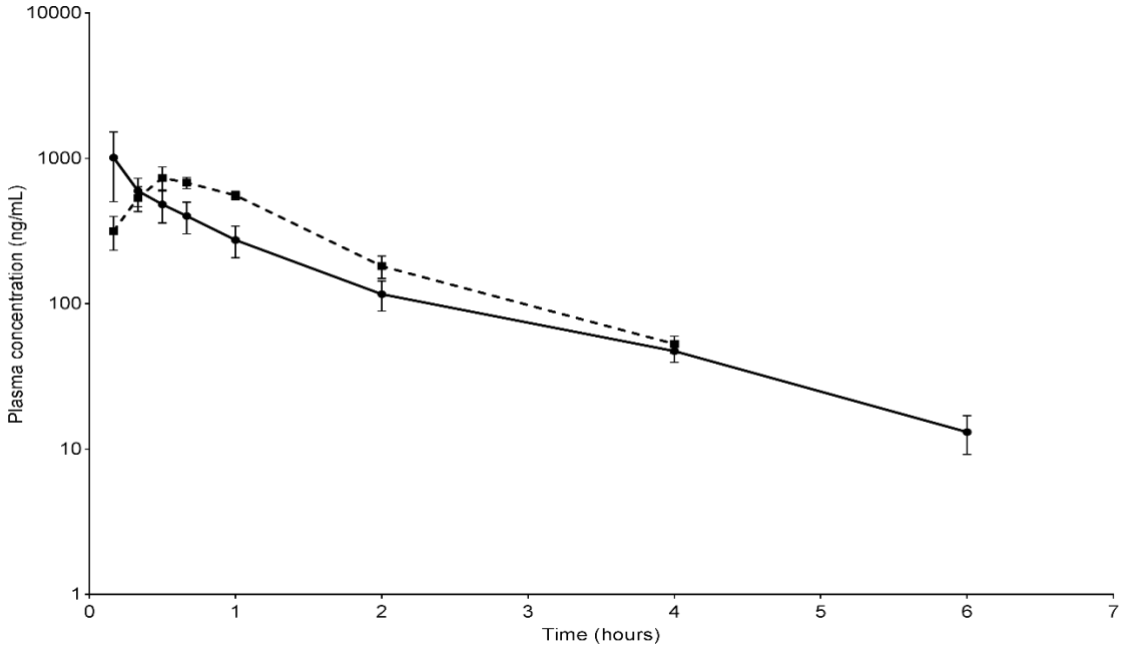


Figure 5.1. Mean (±SD) concentrations of articaine (solid line) and articainic acid (dotted line) in plasma of red deer (Cervus elaphus; n=6) following S/C administration of 40 mg/mL articaine hydrochloride (1 mL/cm pedicle circumference) as a complete ring block around the antler pedicle. Note the log scale of the y-axis.

Table 5.3. Mean (±SD) pharmacokinetic parameters of articaine and articainic acid following S/C administration of 40 mg/ml articaine hydrochloride (1 mL/cm pedicle circumference) as a complete ring block around the antler pedicle of red deer (*Cervus elaphus*; n=6).

Parameter	Articaine	Articainic acid
Maximum plasma concentration (ng/mL)	1013.9±510.1	762.6±95.4
T_{max} (hours)	0.17±0.00*	0.50±0.00*
AUC (ng/mL.hour)	936.9±157.1	1196.0±80.7
AUMC (ng/mL.hour)	1326.5±151.0	1701.7±93.1
Terminal half life (hours)	1.12±0.17	0.90±0.07
Mean residence time (hours)	1.45±0.29	1.43±0.08

AUC=Area under the curve; AUMC= Area under the first moment curve; T_{max}=Time to reach maximum plasma concentration
* - The standard deviation of T_{max} is zero because not many blood samples were collected immediately after drug administration.

5.1.5 Discussion

The LC-MS method described here for measurement of articaine and its metabolite articainic acid in the plasma of red deer is simple and sensitive with acceptable precision and accuracy. The sensitivity of the LC-MS method was greater than previously reported methods with lower limit of quantification of 5 ng/mL and 10 ng/mL for articaine and articainic acid, respectively. Richter and Oertel (1999) reported a lower limit of quantification of 10 ng/mL for articaine using an HPLC-based method and Hoizey *et al.* (2009) reported a lower limit of quantification of 78.1 ng/mL for articaine using a different LC-MS method. The sample preparation method used here involved a simple protein precipitation step which yielded good recoveries of the analytes. Previously reported extraction methods used liquid–liquid extraction or a solid phase extraction step followed by drying (Richter and Oertel 1999; Hoizey *et al.* 2009), which are more time consuming than the simple protein precipitation

step used in our method. Though the recoveries from deer plasma are good, internal standards should have been used to improve the precision of the method.

This is the first study to investigate the pharmacokinetics of articaine hydrochloride and its metabolite articainic acid in red deer. Following S/C injection, articaine hydrochloride was rapidly absorbed with a mean C_{max} of 1013.9 ng/mL and T_{max} of 0.17 hours. The time when maximum concentrations were detected was when the first sample was collected after administration of articaine, therefore in future studies, blood samples should be collected earlier than 10 minutes after administration to more accurately determine the C_{max} and T_{max}. Rapid absorption of the articaine hydrochloride can be attributed to its vasodilatory effect, similar to most other local anaesthetics (Snoeck 2012). The rapid absorption of articaine hydrochloride has also been reported in humans following oral nerve blocks and epidural administration (Muller *et al.* 1991; Vree *et al.* 1997).

The mean terminal half-life of 1.12 hours indicates that articaine was rapidly eliminated following S/C administration in red deer. Articaine was rapidly hydrolysed to its metabolite articainic acid, with maximum concentrations of articainic acid being detected 30 minutes after administration. Rapid hydrolysis has been reported to be due to the presence on the articaine molecule of an ester group, which makes it a suitable substrate for plasma esterases (Oertel *et al.* 1997). Articainic acid was also eliminated rapidly with a mean terminal half-life of 0.90 hours. Pharmacokinetic studies in humans found that articaine was excreted primarily as articainic acid (64%), and the glucuronide conjugate of articainic acid (13%). Only 1.5% was excreted as the parent drug, articaine hydrochloride (Vree *et al.* 1997).

No signs of toxicity associated with over-dose of local anaesthetic, such as muscle tremor, sedation or convulsion, were observed following administration of articaine hydrochloride in the current study. Rapid hydrolysis of articaine hydrochloride by plasma esterases to an

inactive metabolite results in short half-life of the drug thereby reducing the risk of systemic toxicity (Oertel *et al.* 1997). In contrast, lidocaine hydrochloride is metabolised to active metabolites which require hepatic clearance. In addition, in vitro and in vivo preclinical toxicity studies in rats, rabbits and dogs indicated that articaine hydrochloride did not demonstrate any pathomorphological or mutagenic effects at doses up to 70 mg/kg (Leuschner and Leblanc 1999).

In conclusion, a method for the detection and quantification of articaine and articainic acid using LC-MS was developed and validated, and found to be simple, accurate and sensitive. Articaine hydrochloride was shown to have favourable pharmacokinetic properties in red deer including rapid absorption, hydrolysis and elimination. Moreover, we observed no adverse effects following S/C administration of articaine hydrochloride as a complete ring block around the antler pedicle in red deer. Articaine hydrochloride may therefore be a good alternative to the commonly used local anaesthetic lidocaine for velvetting in red deer. Use of articaine hydrochloride in animals has not been authorised in any animal species and further studies are required to evaluate the safety and efficacy of the drug at different concentrations, and to establish maximum residue limits, before it can be recommended for use as a local anaesthetic in red deer.

5.1.6 Acknowledgements

We acknowledge the technical support provided by Geoff Purchas, Trevor Loo, and David Lun. This work was undertaken with financial support from the Institute of Veterinary Animal and Biomedical Sciences, Massey University and the McGeorge Research Fund.

5.1.7 References

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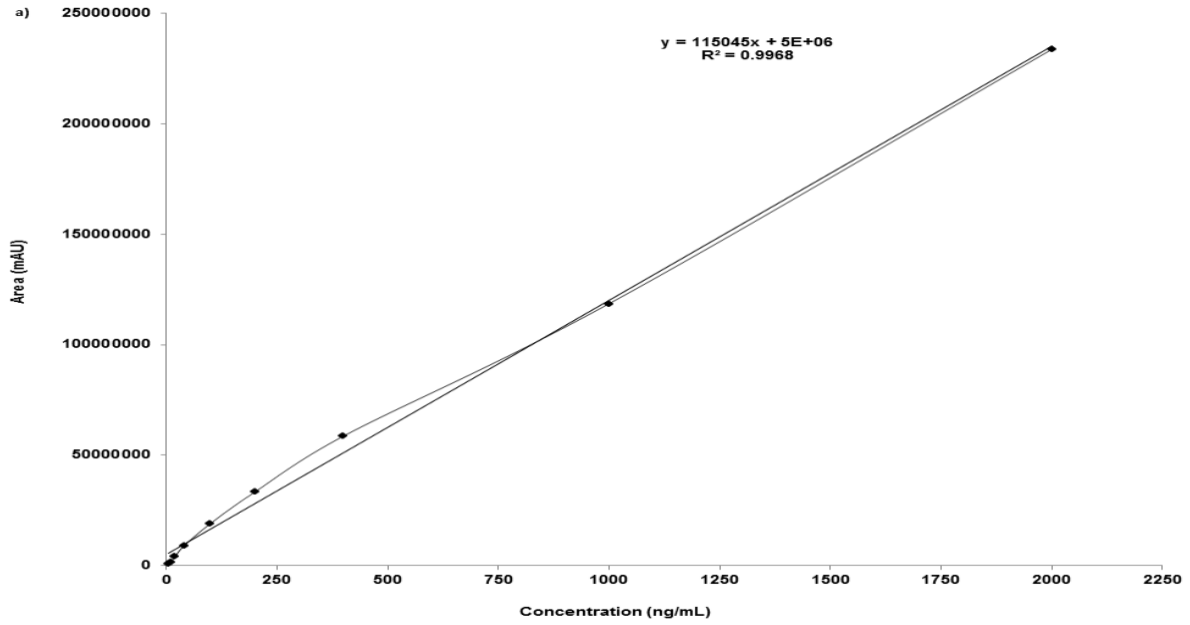
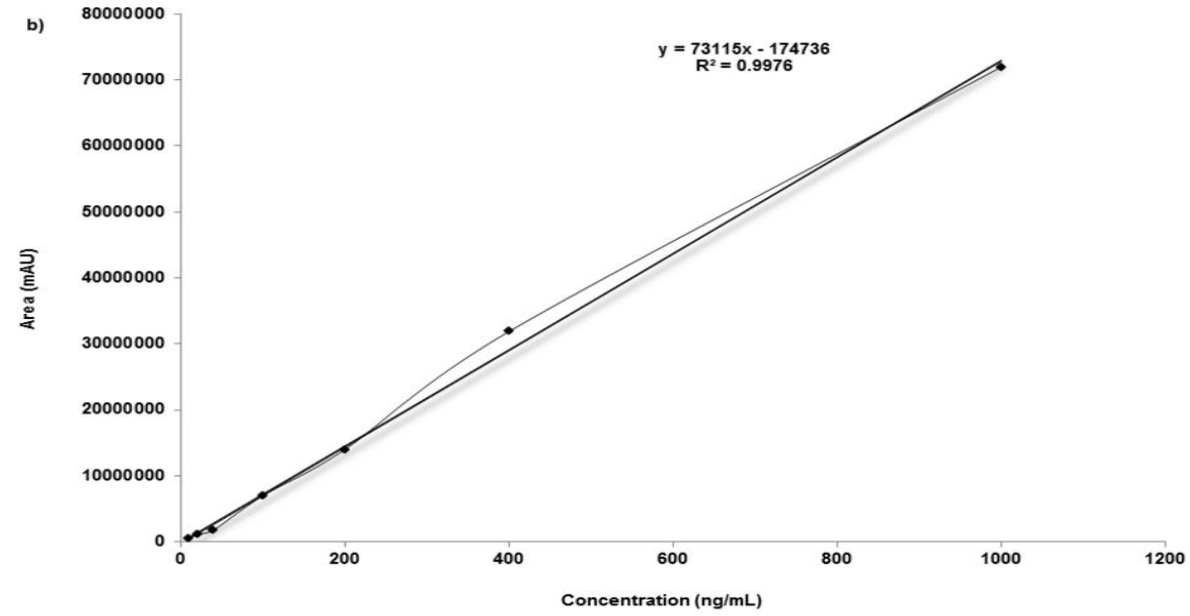
5.1.8 Supplementary materials

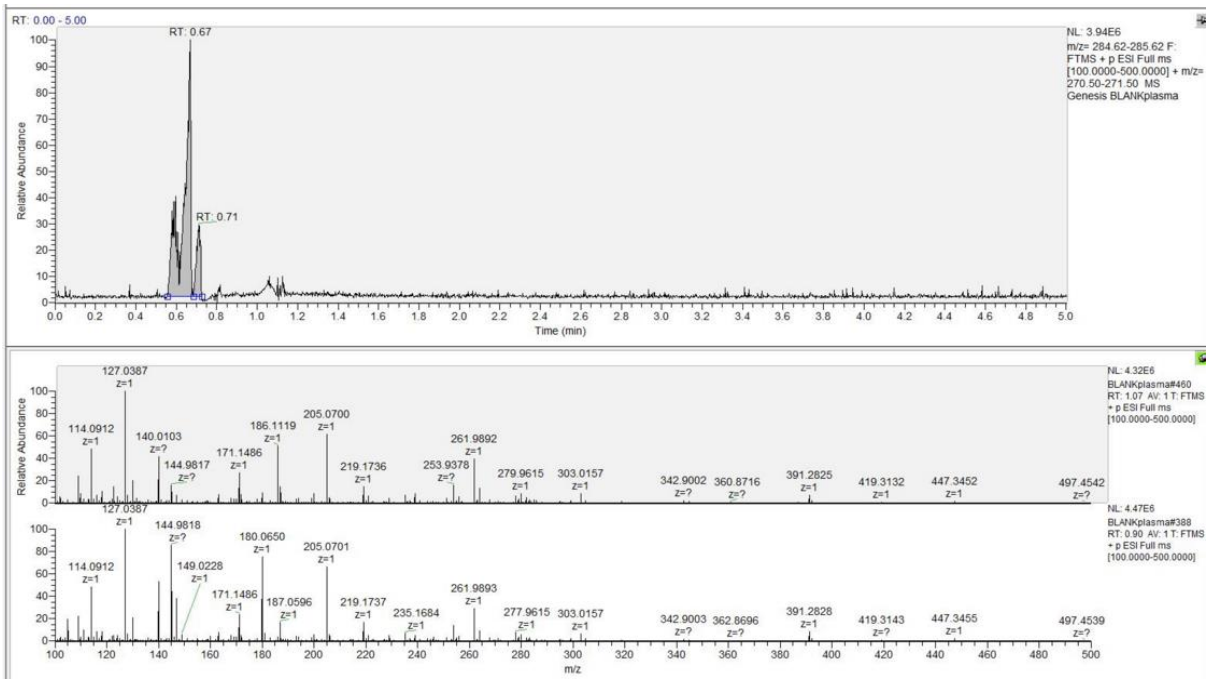
Supplementary Figure 5.1. Representative calibration curves of (a) articaine hydrochloride and (b) articainic acid constructed by spiking pooled plasma from untreated red deer (*Cervus elaphus*) with different concentrations of each analyte.



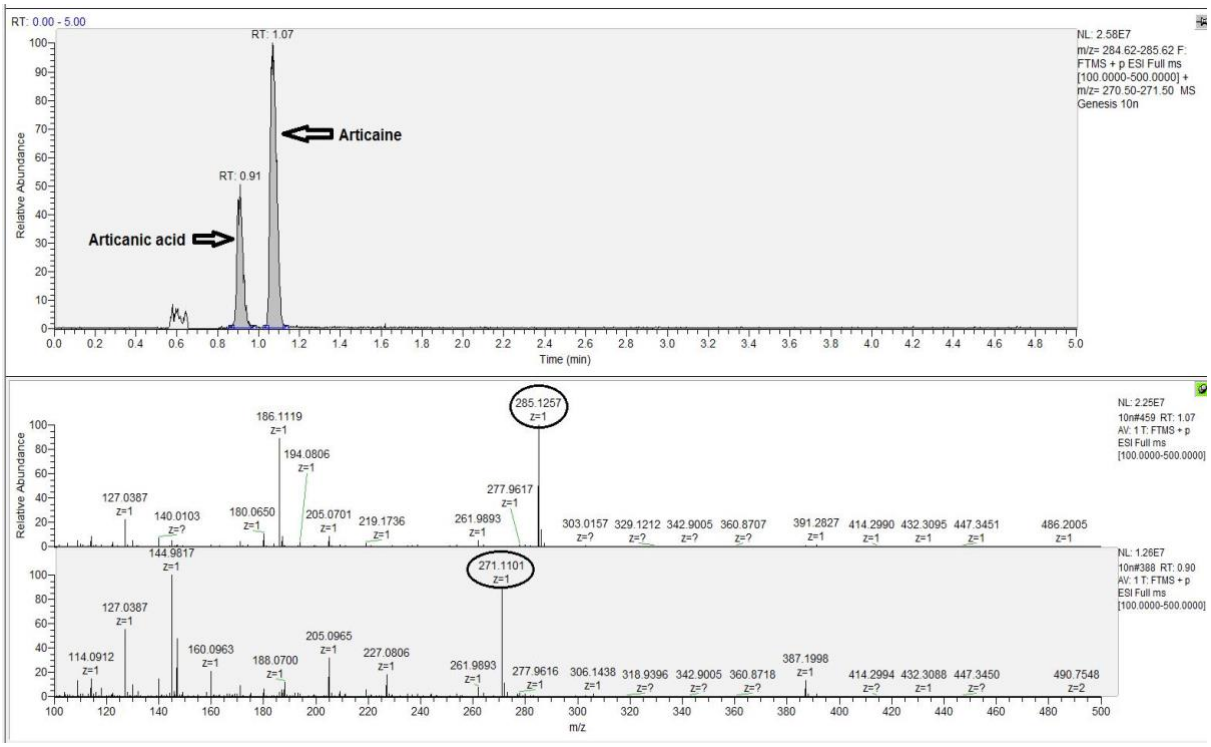


Supplementary Figure 5.2. Chromatogram and electrospray ionization full scan MS of (a) plasma from untreated red deer (*Cervus elaphus*), (b) untreated plasma with 20 ng/mL articaine and articainic acid added, and (c) plasma from a red deer after S/C administration of 40 mg/mL articaine hydrochloride (1 mL/cm pedicle circumference) as a complete ring block around the antlers.

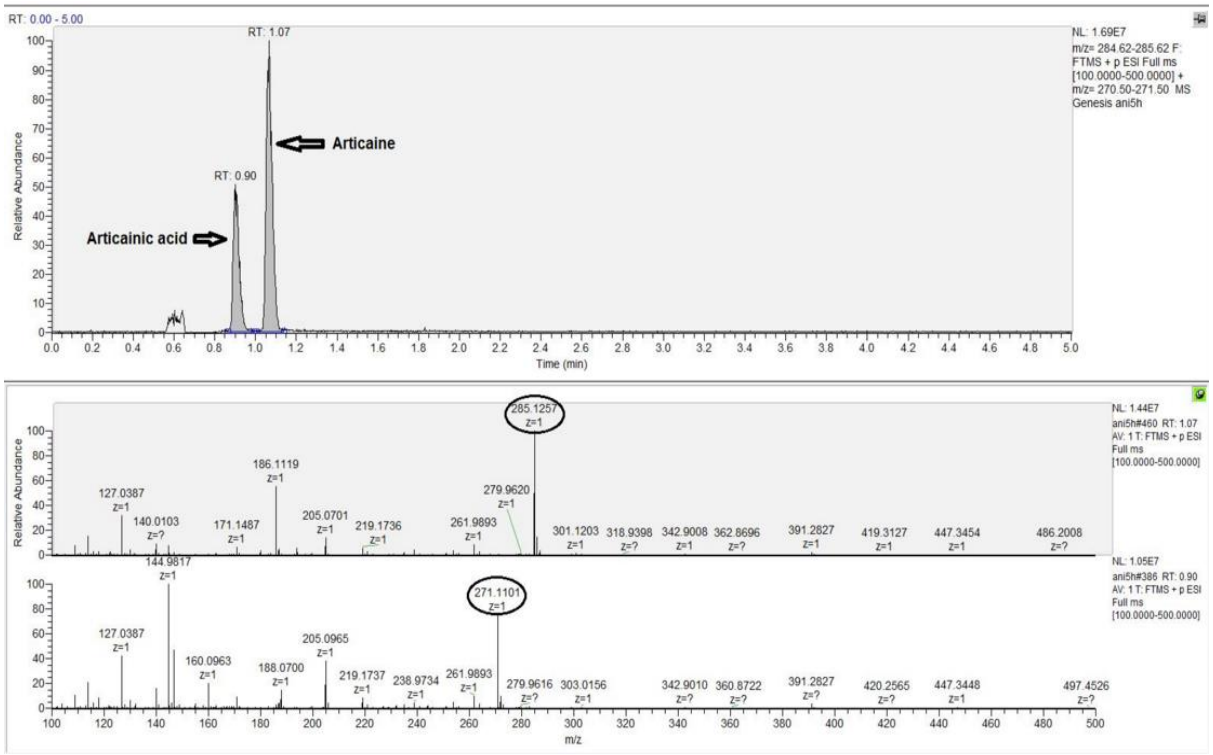
a)



b)



c)


RT: 0.00 - 5.00
RT: 1.07
RT: 0.90
Articaine
Articainic acid
Relative Abundance
Time (min)
NL: 1.69E7
m/z= 284.62-285.62 F: FTMS + p ESI Full ms [100.0000-500.0000] + m/z= 270.50-271.50 MS Genesis ani5h
285.1257 z=1
186.1119 z=1
127.0387 z=1
279.9620 z=1
140.0103 z=?
171.1487 z=1
205.0701 z=1
219.1736 z=1
261.9893 z=1
301.1203 z=1
318.9398 z=?
342.9008 z=1
362.8696 z=?
391.2827 z=1
419.3127 z=1
447.3454 z=1
486.2008 z=?
NL: 1.44E7 ani5h#460 RT: 1.07 AV: 1 T: FTMS + p ESI Full ms [100.0000-500.0000]
144.9817 z=1
271.1101 z=1
127.0387 z=1
205.0965 z=1
160.0963 z=1
188.0700 z=1
219.1737 z=1
238.9734 z=1
261.9893 z=1
279.9616 z=?
303.0156 z=1
342.9010 z=?
360.8722 z=?
391.2827 z=?
420.2565 z=?
447.3448 z=1
497.4526 z=?
NL: 1.05E7 ani5h#386 RT: 0.90 AV: 1 T: FTMS + p ESI Full ms [100.0000-500.0000]
m/z

5.1.9 Appendix

Table 5.4. Intra-day and inter-day accuracy and precision of articaine in deer plasma

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-day	20	20.8±0.7	3.4	3.8
	200	194.2±5.0	2.6	-2.9
	1000	1048.5±43.5	4.1	4.9
Inter-day	20	18.3±1.8	10.0	-8.7
	200	205.0±22.4	10.9	2.5
	1000	1046.9±46.3	4.4	4.7

Table 5.5. Intra-day and inter-day accuracy and precision of articainic acid in deer plasma

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-day	20	19.3±1.0	5.3	-3.6
	200	204.4±6.2	3.0	2.2
	1000	996.5±4.9	0.5	-0.3
Inter-day	20	17.5±1.4	8.3	-12.6
	200	186.1±19.9	10.7	-6.9
	1000	1050.3±82.7	7.9	5.0

Table 5.6. Individual animal plasma concentrations (ng mL^{-1}) of articaine following S/C administration of 40 mg/ml articaine hydrochloride (1 mL/cm pedicle circumference) as a complete ring block around the antler pedicle of red deer (n=6).

Time points	1	2	3	4	5	6	Mean ± SD
10	844.76	882.74	430.93	658.95	1526.36	1739.75	1013.92±510.12
20	632.04	637.98	340.99	596.92	714.80	664.85	597.93±131.85
30	584.92	595.01	261.73	473.49	529.13	453.32	482.93±122.47
40	495.83	493.65	229.00	366.15	408.93	420.13	402.28±98.79
60	301.43	317.42	146.33	266.71	333.70	281.83	274.57±67.25
120	100.82	103.80	113.21	167.38	121.94	91.01	116.36±27.15
240	54.32	42.71	46.01	51.11	34.86	54.96	47.33±7.74
360	12.11	9.13	15.10	17.19	8.18	17.00	13.11±3.93

Table 5.7. Pharmacokinetic parameters of articaine following S/C administration of 40 mg/ml articaine hydrochloride (1 mL/cm pedicle circumference) as a complete ring block around the antler pedicle of red deer (n=6).

Parameter	1	2	3	4	5	6	Mean ± SD
C_{max} (ng mL^{-1})	844.76	882.74	430.93	658.95	1526.36	1739.75	1013.9±510.1
T_{max} (hour)	0.17	0.17	0.17	0.17	0.17	0.17	0.17±0.00*
$t_{½\lambda z}$ (hour)	0.99	0.94	1.38	1.22	1.03	1.20	1.12±0.17
AUC (hour ng mL^{-1})	957.84	950.16	634.00	958.26	1059.33	1061.99	936.9±157.1
AUMC (hour ng mL^{-1})	1329.45	1201.47	1226.19	1578.30	1201.19	1422.22	1326.5±151.0
MRT (hour)	1.39	1.26	1.93	1.65	1.13	1.34	1.45±0.29

Table 5.8. Individual animal plasma concentrations (ng mL^{-1}) of articainic acid following S/C administration of 40 mg/ml articaine hydrochloride (1 mL/cm pedicle circumference) as a complete ring block around the antler pedicle of red deer (n=6).

Time points	1	2	3	4	5	6	Mean ± SD
10	252.7	334.9	203.6	394.3	420.1	295.3	316.8±82.9
20	437.3	661.3	436.7	453.3	602.5	621.9	535.5±103.9
30	741.1	890.7	820.1	811.8	646.3	665.7	736.4±139.0
40	693.1	777.4	667.4	690.6	590.3	533.7	680.8±60.3
60	569.3	601.6	587.1	493.4	556.2	508.3	556.9±39.1
120	197.5	197.8	190.8	185.4	198.6	117.2	181.2±31.8
240	44.4	44.2	53.5	57.1	58.3	59.9	52.9±7.0

Table 5.9. Pharmacokinetic parameters of articainic acid following S/C administration of 40 mg/ml articaine hydrochloride (1 mL/cm pedicle circumference) as a complete ring block around the antler pedicle of red deer (n=6).

Parameter	1	2	3	4	5	6	Mean ± SD
C_{max} (ng mL^{-1})	741.1	890.7	820.1	811.8	646.3	665.7	762.6±95.4
T_{max} (minute)	0.5	0.5	0.5	0.5	0.5	0.5	0.50±0.00
$t_{½\lambda z}$ (hour)	0.8	0.8	0.9	0.9	1.0	1.0	0.90±0.07
AUC (hour ng mL^{-1})	1185.8	1301.6	1209.2	1188.5	1234.9	1056.0	1196.0±80.7
AUMC (hour ng mL^{-1})	1638.1	1682.2	1744.9	1731.6	1840.9	1572.3	1701.7±93.1
MRT (hour)	1.4	1.3	1.4	1.5	1.5	1.5	1.43±0.08

DRC 16

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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:	DINAKARAN VENKATACHALAM
Name/title of Primary Supervisor:	Dr. PREET SINGH
Name of Research Output and full reference:	
[redacted]	
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GRS Version 4– January 2019

Name of research output and full reference: Venkatachalam D, Chambers JP, Kongara K, Singh P (2018). Pharmacokinetics of articaine hydrochloride and its metabolite articainic acid after subcutaneous administration in red deer (*Cervus elaphus*). *New Zealand Veterinary Journal*, 66(1):16-20.

5.2 Analgesic efficacy of articaine hydrochloride for velvet antler removal in red deer (*Cervus elaphus*) and analysis of drug residues in the harvested velvet antlers

5.2.1 Abstract

AIMS: To investigate the analgesic efficacy of articaine hydrochloride for antler removal in red deer (*Cervus elaphus*) following S/C administration as a ring block, and to quantify the residue concentrations of articaine compared to lignocaine in the harvested antlers.

METHODS: Articaine hydrochloride (40 mg/mL) was administered to 10 male red deer as a ring block around the base of each antler at 1 mL/cm of pedicle circumference. Analgesia was evaluated by determining the response to a saw cut test every 1-minute, until no response was observed. Behaviour during and following removal of antlers was also recorded. Twenty commercially harvested velvet antlers were also collected following S/C administration of 2% lignocaine hydrochloride. A liquid chromatography-mass spectrometry (LC-MS) method for quantification of residues of articaine and lignocaine in velvet antlers was developed and validated.

RESULTS: In administered 4% articaine hydrochloride as a ring block, the median interval to analgesia was 4 (min 3, max 5) minutes and no deer showed withdrawal responses during antler removal. There were no signs of toxicity or adverse effects up to 2 hours after administration. The sample preparation method developed was simple and had acceptable extraction recoveries of articaine and lignocaine from the velvet antlers. The lower limits of quantification of lignocaine and articaine were 5 ng/g and 50 ng/g, respectively. Mean concentrations of articaine in antlers following ring block with 4% articaine hydrochloride were 1.50 (SD 1.09) mg/kg, and of lignocaine following ring block with 2% lignocaine hydrochloride were 0.66 (SD 0.71) mg/kg.

CONCLUSIONS AND CLINICAL RELEVANCE: A ring block with 4% articaine hydrochloride at a dose of 1 mL/cm of pedicle circumference provided effective analgesia for velvet antler removal in red deer. The LC-MS method developed and validated to quantify articaine and lignocaine was simple and sensitive. Based on these results, articaine hydrochloride appears to be an effective alternative to lignocaine hydrochloride for velvet antler removal. However, further studies to evaluate the safety and residue concentrations of articaine and articainic acid are required before it can be recommended for use in deer.

KEY WORDS: *Articaine hydrochloride, antler removal, red deer, analgesia, liquid chromatography-mass spectrometry*

%CV	Percent co-efficient of variation
DMA	2,6-dimethylaniline
LC-MS	Liquid chromatography-mass spectrometry
MEGX	Monoethylglycinexylidide
MRL	Maximum Residue Levels
pK_a	Dissociation constant

5.2.2 Introduction

Deer are commercially farmed in New Zealand and other countries for the production of venison, velvet antlers and by-products. Velvet antler is highly innervated and vascularised growing tissue and its removal (velvetting) without appropriate analgesia is extremely painful and stressful (Matthews and Cook 1991; Wilson and Stafford 2002). In New Zealand, antler removal must be performed only by a veterinarian or a farmer certified by the National Velvetting Standards Body under veterinary authorisation, using the approved methods of analgesia which include a ring block around the base of antler using local anaesthetic or

compression analgesia using a rubber ring in yearlings (Anonymous 1992; Walsh and Wilson 2002; Nicol *et al.* 2009). Other analgesic methods studied for antler removal are regional nerve block of the zygomaticotemporal nerve or the infratrochlear nerve using local anaesthetic (Innervation of antler pedicle is shown in Figure 5.2), general anaesthesia, compression analgesia and electroanaesthesia (Wilson *et al.* 2000; Woodbury *et al.* 2002; Flint 2012).

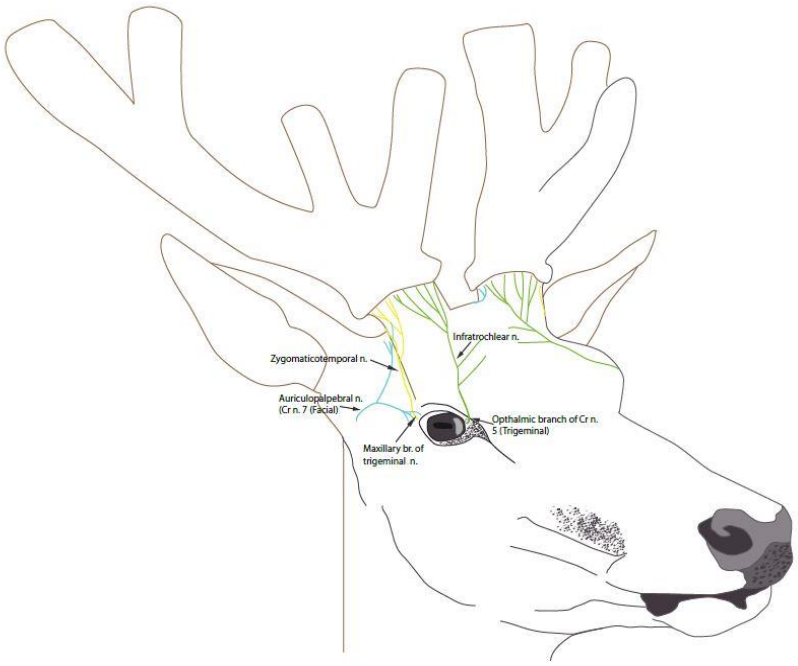

Figure 5.2. Innervation of the antler pedicle showing the course of the infratrochlear (green), auriculopalpebral (blue) and zygomaticotemporal (yellow) nerves (Flint 2012).

Among these methods, a ring block around the base of the antler using the local anaesthetic lignocaine hydrochloride was reported as being the most reliable and commonly used technique (Woodbury *et al.* 2002; Johnson *et al.* 2005). However, concerns were raised about the presence of lignocaine residue in harvested antlers (Woodbury *et al.* 2002). Velvet antlers are consumed as human health supplements (Gilbey and Perezgonzalez 2012; Tang *et al.* 2015), and ingestion of lignocaine residue present in the velvet antlers or their products can lead to the absorption and metabolism of lignocaine to several metabolites, including 2,6-

dimethylaniline (DMA) (Nelson *et al.* 1977). DMA has been reported to be toxic and carcinogenic in humans and animals. In a carcinogenicity study following oral administration of DMA in rats, several types of cancers including adenoma, carcinoma and sarcoma were observed in dosed rats (Anonymous 1990). In humans, several epidemiological studies suggest that DMA is associated with increased risk of bladder cancer (Duan *et al.* 2008; Tao *et al.* 2013). Therefore, the presence of lignocaine in harvested velvet antlers intended for human consumption has been a concern to the deer industry, as the usual method of avoiding residues, applying a withholding time, cannot apply to antler removal (Woodbury *et al.* 2002).

We previously reported the pharmacokinetics of articaine hydrochloride in red deer (*Cervus elaphus*) following use as a ring block (Venkatachalam *et al.* 2018). Articaine hydrochloride (4-methyl-3-(2-propylaminopropionamido) thiophene-2-carboxylic acid methyl ester hydrochloride) is a unique amide-type local anaesthetic with an ester group which allows it to be metabolised by plasma esterases to an inactive metabolite, articainic acid (Snoeck 2012). Although it is classified as an amide-type local anaesthetic, it is not metabolised to DMA, unlike other amide-type local anaesthetics (Nai *et al.* 2015).

Articaine hydrochloride has been widely used in humans for local and regional anaesthesia, particularly for dental procedures, and is considered safe and effective (Oertel *et al.* 1997; Vree and Gielen 2005; Su *et al.* 2016). Pharmacokinetic studies in humans and deer reported that articaine was rapidly eliminated with elimination half-lives of 20 minutes and 67.2 minutes, respectively (Oertel *et al.* 1997; Venkatachalam *et al.* 2018). In addition, the clearance of articaine (8.9 L/minute) in humans was reported as being several times greater than that of lignocaine (0.9 L/minute) (Simon *et al.* 1998). Preclinical toxicity studies in rats and dogs following repeated administration of articaine hydrochloride showed no pathomorphological systemic changes, and *in vitro* and *in vivo* studies found no mutagenic

properties associated with articaine, even at cytotoxic concentrations (Leuschner and Leblanc 1999).

There appear to be no previous publications on the use of articaine hydrochloride for nerve blocks in veterinary medicine. Considering the concerns on the carcinogenicity of DMA and the reported favourable pharmacokinetic properties of articaine hydrochloride in red deer (Venkatachalam *et al.* 2018), articaine hydrochloride may be a useful alternative to the commonly used lignocaine hydrochloride for antler removal. However, it must be noted that although articaine hydrochloride is commonly used in humans around the world, including New Zealand, it has not yet been approved for use in veterinary medicine and no studies have been conducted in target animals to evaluate the efficacy and safety of articaine or its metabolites.

The objectives of this study were to investigate the analgesic efficacy of articaine hydrochloride for antler removal in red deer following S/C administration as a ring block, and to develop and validate a liquid chromatography-mass spectrometry (LC-MS) method to quantify the concentrations of articaine and lignocaine in harvested velvet antlers.

5.2.3 Materials and methods

Reagents and drug preparation

Articaine hydrochloride and articainic acid were purchased from SCI Pharmtech (Taoyuan, Taiwan) and Toronto Research Chemicals (Toronto, Canada), respectively. Lignocaine hydrochloride, monoethylglycinexylidide (MEGX) and DMA were purchased from Sigma Aldrich (Auckland, NZ). LC-MS-grade acetonitrile, water and formic acid were purchased from Fisher Scientific (Auckland, NZ). Perchloric acid and sodium tetraborate decahydrate were reagent-grade and were purchased from BDH (Auckland, NZ) and Merck (Auckland, NZ), respectively. Articaine hydrochloride solution (40 mg/mL) for injection was prepared

fresh each day by dissolving an appropriate amount of the drug in sterile water and making up the volume with normal saline. The pH of the drug solution was measured using a pH meter (Sevencompact, Mettler Toledo; Columbus, OH, USA). The solution was then filtered through a syringe filter prior to injection (0.45 μm; Phenomenex Inc, Auckland, NZ).

Analysis of drug residues

A simple LC-MS method for the quantification of lignocaine and articaine in harvested velvet antlers was developed and validated. The instrumentation and conditions used were the same as those described by Venkatachalam *et al.* (2018) for measurement of articaine and articainic acid in plasma, except that the column (100 mm × 2.1 mm; Accucore Auckland, New Zealand) was maintained at a temperature of 25°C. Quantitation was performed in the full scan MS mode using peak-area ratios of the target ions of articaine (mass to charge ratio 285.12), and lignocaine (mass to charge ratio 235.17), and the metabolites, articainic acid (mass to charge ratio 271.11), MEGX (mass to charge ratio 207.14) and DMA (mass to charge ratio 120.09).

Preparation of calibration standards and quality controls

Standard stock solutions (1 mg/mL) of articaine and lignocaine were prepared by dissolving in LC-MS-grade water and working standards were prepared by serially diluting these in LC-MS grade water. The standard stock solutions and working standards were stored at -20°C until use. Calibration standards of articaine (50–2,500 ng/g) and lignocaine (5–2,500 ng/g) were prepared fresh each day by fortifying homogenised drug-free antlers with appropriate amounts of working standards (as described below). Quality control samples for articaine (50, 250 and 2500 ng/g) and lignocaine (5, 250 and 2500 ng/g) were prepared similarly using homogenised drug-free antlers.

Sample preparation

Drug-free velvet antlers (n=4) harvested from yearling red deer following the application of NaturO ring (NaturO; AgResearch, NZ), and 20 velvet antlers harvested following administration of 2% lignocaine hydrochloride as a ring block (1 mL/cm circumference) around the base of the antlers, were obtained from Massey University Deer Research Unit (Palmerston North, NZ). These antlers were stored at −80°C until use.

Drug-free velvet antlers were sliced and homogenised at 3000 rpm using a homogeniser (Ultra-Turrax T25; IKA, Rawang, Selangor, Malaysia). Two grams of the homogenised velvet antler (semi-solid) was mixed in a 15 mL conical centrifuge tubes with 100 µL of working standards containing articaine or lignocaine by vortexing for 2 minutes to prepare calibration standards. For the detection of metabolites, standard solutions of articainic acid, MEGX and DMA were individually prepared in methanol and added to homogenised antlers to obtain 100 ng/g of each metabolite.

After mixing, samples were left to stand for15 minutes, then 200 µL perchloric acid (70%) was added, mixed and left for 10 minutes, before 2 mL borate buffer (0.1 M) and 2 mL of mobile phase (0.1% formic acid and acetonitrile (70:30, V/V)) were added and centrifuged to extract the analytes. The resulting supernatant was filtered (0.45 µm, Phenomenex Inc, Auckland, NZ) and 10 µL was injected on to the column. This sample preparation procedure was also used for the analysis of velvet antlers (base piece) obtained from animals following administration of a local anaesthetic ring block, except that no drug was added to the samples.

LC-MS validation

The specificity of the method was determined by comparing the chromatograms from drug-free antlers and drug-free antlers spiked with the analytes, to ensure that there were no interfering peaks at the retention times of the analytes. The linearity of the calibration

standards was determined by linear regression analysis. The lower limit of quantification of the compounds was determined as a signal to noise ratio >10:1. Extraction recoveries of the analytes were calculated by comparing the peak areas of drug-free antlers spiked with analytes prior to extraction with the peak areas of the standards added after extraction. Intra- and inter-day precision and accuracy of the method were determined by processing quality control samples (50, 250 and 2500 ng/g) three times on the same day and on three different days, respectively. Carry-over from the system was assessed by injecting drug-free antlers after an injection of spiked samples containing 2,500 ng/g of the analytes.

Animals and experimental procedure

Ten healthy 2-year-old male red deer (weighing 102 – 111 kg) with velvet antlers were brought in to a holding area and individually restrained in a padded hydraulic crush. The pedicle circumference of each antler was measured using a tape measure and the standard procedure for antler removal in New Zealand, using articaine instead of lignocaine, was followed. A compression tourniquet was applied just above the site of injection as described by Flint (2012) to control haemorrhage and reduce distribution of the local anaesthetic into the antler. Using a 20 gauge needle attached to a 20 mL syringe, 5–6 injections of 2–2.5 mL articaine hydrochloride (4%) was injected S/C as a ring block around the base of the antlers. The maximal total volume injected was 28 mL (approximately 10 mg/kg).

The effect of the nerve block was tested using a nick or saw-cut test (Wilson *et al.* 2000). This test involved making a superficial cut with a meat saw (Wells, Auckland, NZ) on the lateral aspect of the velvet antlers about 10 mm above the pedicle/antler junction. The response was determined using the behavioural signs summarised in Table 5.10. If any response was observed, the saw was removed and the test was repeated (just above the previous location) at 1-minute intervals until no response was observed.

The antlers were then removed using the meat saw and animals were observed for behavioural signs during removal. Following drug administration, animals were visually observed for signs of local anaesthetic toxicity such as muscle tremor, sedation or convulsion at 15, 30, 45 minutes and 1, 1.5 and 2 hours while unrestrained in a pen. The harvested velvet antlers were individually labelled and stored at −80°C. The experimental protocol was approved by the Massey University Animal Ethics Committee (Palmerston North, NZ).

Samples from antlers collected following administration of 4% articaine hydrochloride were individually analysed for concentrations of articaine and articainic acid, whereas samples from antlers obtained following administration of 2% lignocaine were analysed for lignocaine, MEGX and DMA, using the LC-MS assay.

Table 5.10. Behavioural signs used to assess the reaction to the saw cut test.

No.	Description
1	No response
2	Flinch
3	Slight movement of head
4	Movement of antlers away from the saw
5	Head down or shaking
6	Flight response
7	Whole body struggle

5.2.4 Results

LC-MS validation

Representative chromatograms and the mass spectra of drug-free antler, drug-free antler spiked with lignocaine (5 ng/g), articaine (50 ng/g), articainic acid (100 ng/g), MEGX (100 ng/g) and DMA (100 ng/g), and antler samples obtained following ring block using 4 % articaine hydrochloride and (h) 2% lignocaine hydrochloride are shown in Supplementary

Figure 5.3. The absence of interfering peaks at the retention times of articaine and lignocaine in the drug-free antler confirms the specificity of the method.

The calibration curves (Supplementary Figure 5.4) were linear over the concentration range of 50 – 2,500 ng/g for articaine and 5 – 2,500 ng/g for lignocaine with correlation coefficient (r^2) of 0.994 and 0.999, respectively. The extraction recoveries for articaine ranged from 52–76% and the recoveries of lignocaine ranged from 58–84%. The intra-day CV for articaine and lignocaine were <6.8 and 7.8%, respectively, and inter-day CV for articaine and lignocaine were <15.7 and 11.0%, respectively. No carry-over effect was observed following injection of 2,500 ng/g of the analytes. The lower limit of quantification for articaine was 50 ng/g and for lignocaine 5 ng/g.

Analgesic efficacy of articaine hydrochloride

The pH of the articaine hydrochloride solution used for ring block around the antlers was 6.0. The circumference of the pedicle ranged from 10.5–14.0 cm and the corresponding volume of articaine hydrochloride injected ranged from 10.5–14.0 mL per velvet antler. The interval to onset of analgesia, based on response to the saw-cut test, for individual deer is presented in Table 5.11. The median interval was 4 (min 3, max 5) minutes. None of the deer showed withdrawal response during removal of velvet antlers. Systemic toxicity signs associated with local anaesthetics such as muscle tremor, sedation or convulsion were not observed in any of the deer.

The mean residue concentration of articaine measured in harvested antlers following ring block with 4% articaine hydrochloride was 1.50 (SD 1.09) mg/kg, and of lignocaine following ring block with 2% lignocaine hydrochloride was 0.66 (SD 0.71) mg/kg. Concentrations in individual antlers are shown in Table 5.12. The main metabolite of articaine, articainic acid, was detected in 6/20 antlers harvested following administration of

4% articaine hydrochloride, but the metabolites of lignocaine, MEGX and DMA were not detected in the antlers following administration of 2% lignocaine hydrochloride.

Table 5.11. Time to onset of analgesia following S/C administration of 4% articaine hydrochloride (1 mL/cm pedicle circumference) as a ring block around the antlers in red deer (*Cervus elaphus*).

Animal no.	Onset of analgesia (minutes)	
	Left antler	Right antler
1	3	5
2	4	3
3	5	4
4	5	5
5	4	3
6	3	4
7	4	3
8	5	5
9	4	3
10	3	4
Median (range)	4 (3-5)	

Table 5.12. Concentrations (mg/kg) of articaine and lignocaine in the harvested velvet antlers following S/C administration of 4% articaine hydrochloride and 2% lignocaine hydrochloride, respectively.

Animal no.	Articaine (mg/kg)		Lignocaine (mg/kg)	
	Left antler	Right antler	Left antler	Right antler
1	1.19	0.51	0.18	0.10
2	2.69	3.39	0.77	1.02
3	3.15	0.48	0.10	1.77
4	1.48	1.51	0.13	0.39
5	3.00	0.44	0.13	0.63
6	1.38	1.20	0.59	0.13
7	2.76	1.02	0.98	2.76
8	0.23	0.12	0.49	0.40
9	0.71	0.13	1.76	0.45
10	2.66	1.99	0.21	0.11

5.2.5 Discussion

Administration of 4% articaine hydrochloride as a ring block (1 mL/cm) around the base of the antlers of red deer produced good analgesia, with no response to velvet antler removal and no signs of toxicity or adverse effects. Similarly, no local anaesthetic-associated toxicity was previously reported following S/C administration of the same dose in red deer (Venkatachalam *et al.* 2018). The results of the saw-cut test suggest that articaine hydrochloride desensitised the velvet antlers between 3–5 minutes following S/C administration as a ring block around the base of the antlers. This is longer than the 1–2 minute interval to desensitisation of antlers reported following ring block with lignocaine hydrochloride in deer (Wilson *et al.* 2000). In contrast, in humans, articaine hydrochloride (4%) had shorter onset of action compared to 2% lignocaine hydrochloride (Rebolledo *et al.* 2007; Tortamano *et al.* 2013). A possible reason for the slight delay in the onset of action of articaine hydrochloride in this study could be the pH of the drug solution used, which was 6.0. The dissociation constant of articaine is 7.8 (Snoeck 2012), and the penetration and thus onset of action of local anaesthetics depends on the degree of ionisation of the local anaesthetic (Becker and Reed 2006). Adjusting the pH of the drug solution to close to its dissociation constant value should increase the proportion of the lipid-soluble form of the drug and thereby shorten the onset of action (Covino 1986).

This is the first study that evaluated the analgesic efficacy of articaine hydrochloride for antler removal in deer. Lack of response to the cutting of velvet antlers using a saw suggests that administration of 4% articaine hydrochloride as a ring block was able to provide effective analgesia for antler removal. However, post-operative analgesia was not evaluated.

The LC-MS method used for the quantification of the drug residues in the harvested velvet antlers is simple, sensitive and minimally time consuming. The lower limit of quantification for lignocaine (5 ng/g) was slightly greater than the previously reported 10 ng/g (Bagonluri *et*

al. 2005). The sample preparation method used was simple although did not remove all the contaminants, and cleaning of the sweep cone of the ion source area was required after running many samples. However, this did not affect the analysis. The concentrations of lignocaine in the velvet antlers harvested following administration of 2% lignocaine hydrochloride were below the current maximum residue level (MRL) of 5 mg/kg of velvet antler (Anonymous 2018). None of the metabolites of lignocaine were detected in the samples whereas the metabolite of articaine, articainic acid, was detected in a few velvet antlers. This could be because articaine was rapidly hydrolysed in plasma to articainic acid whereas lignocaine requires hepatic metabolism and hence more time is required for detectable limits in the antlers than articainic acid. Considerable variations in the residue levels of articaine and lidocaine were observed in the harvested velvet antlers between animals (Table 5.12). Differences in the tourniquet pressure, wait time (3-5 minutes), injection volume (10.5–14.0 mL) and size of the antlers would have contributed to the variations in the residue levels of the drugs in the velvet antlers. Post hoc power analysis using the data obtained from this study suggest that the sample size used in this study was not sufficient and at least 13 animals would be required to show statistical difference in the residue levels.

Articaine hydrochloride has not yet been approved for veterinary use, hence no MRL has been set. If articaine hydrochloride is approved in future for velvet antler removal, this study provides preliminary information on the residue concentrations of articaine. Our results indicate that a similar MRL to lignocaine, 5 mg/kg, should be practical. Several studies in humans showed that even 2% articaine hydrochloride was effective in providing analgesia and so the use of 2% solution may be effective and may reduce the residue concentrations of articaine in the harvested antlers (Hintze and Paessler 2006).

Non-chemical alternatives for analgesia have been investigated, however they were not as reliable and effective as lignocaine ring block (Woodbury *et al.* 2002; Johnson *et al.* 2005; Woodbury *et al.* 2005). However, alternatives to lignocaine are required as a metabolite of lignocaine, DMA, is classified as a possibly carcinogenic compound (Anonymous 1990; Tao *et al.* 2013). DMA is not only a metabolite of lignocaine but also of most other amide-type local anaesthetics with the exception of articaine. Individual local site blocks of the zygomaticotemporal and infratrochlear nerves may reduce the residue levels in the velvet antlers but previous studies have shown that individual nerve blocks are not as effective as ring blocks (Wilson *et al.* 1999; Wilson *et al.* 2000). Articaine is metabolised mainly in plasma to an inactive metabolite, articainic acid which is further metabolised and excreted in urine (Oertel *et al.* 1997; Yapp *et al.* 2011). Our previous study in red deer demonstrated that articaine was rapidly hydrolysed to articainic acid and rapidly eliminated with an elimination half-life of 1.12 hours (Venkatachalam *et al.* 2018). Rapid hydrolysis to an inactive metabolite and short elimination half-life are favourable properties of articaine that reduce the risk of systemic toxicity (Oertel *et al.* 1997; Snoeck 2012).

The results of the present study and the reported favourable pharmacokinetic parameters suggest that articaine hydrochloride is a safe and effective local anaesthetic for antler removal in red deer and may therefore be a suitable alternative to the commonly used lignocaine. Although articaine appears to be effective and should be safer for consumers, target animal safety studies and more efficacy studies with different concentrations are required before recommending the drug for velvet antler removal.

5.2.6 Acknowledgements

This study was supported by McGeorge research funds and IVABS post grad funds from Massey University. We acknowledge the technical support provided by Geoff Purchas.

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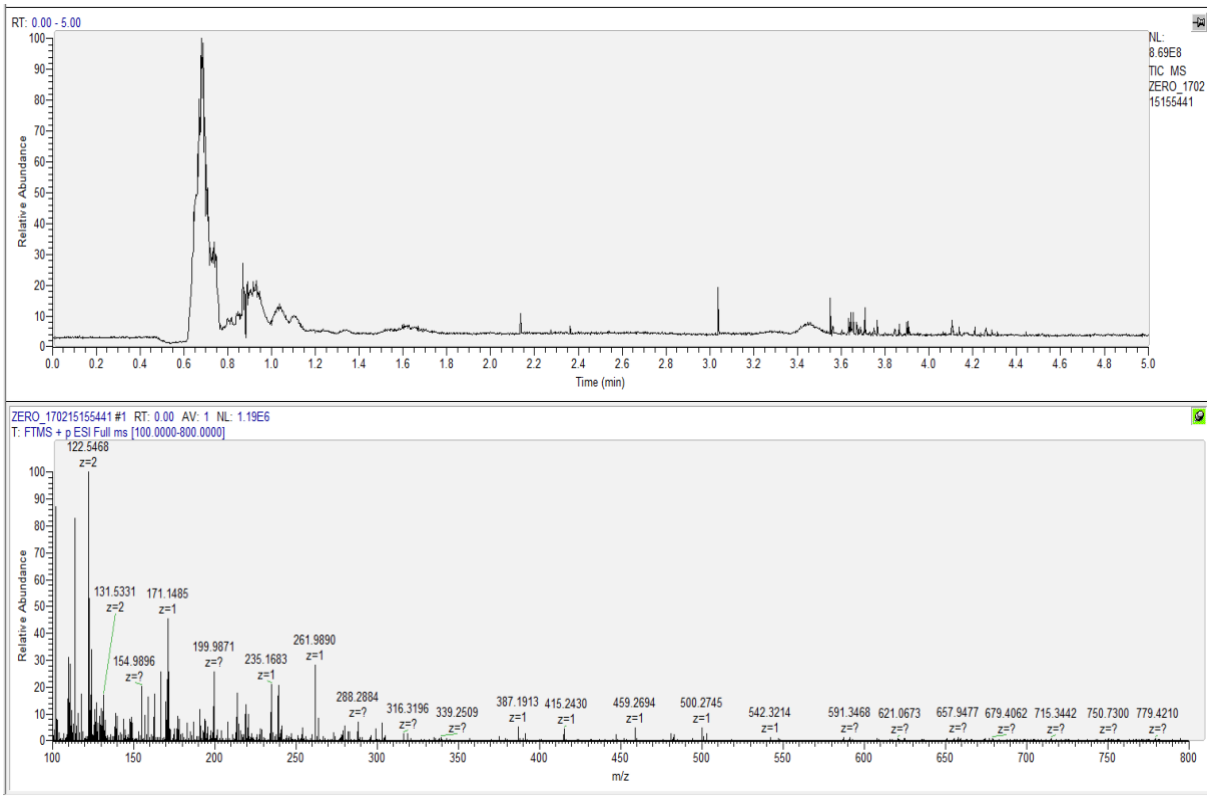
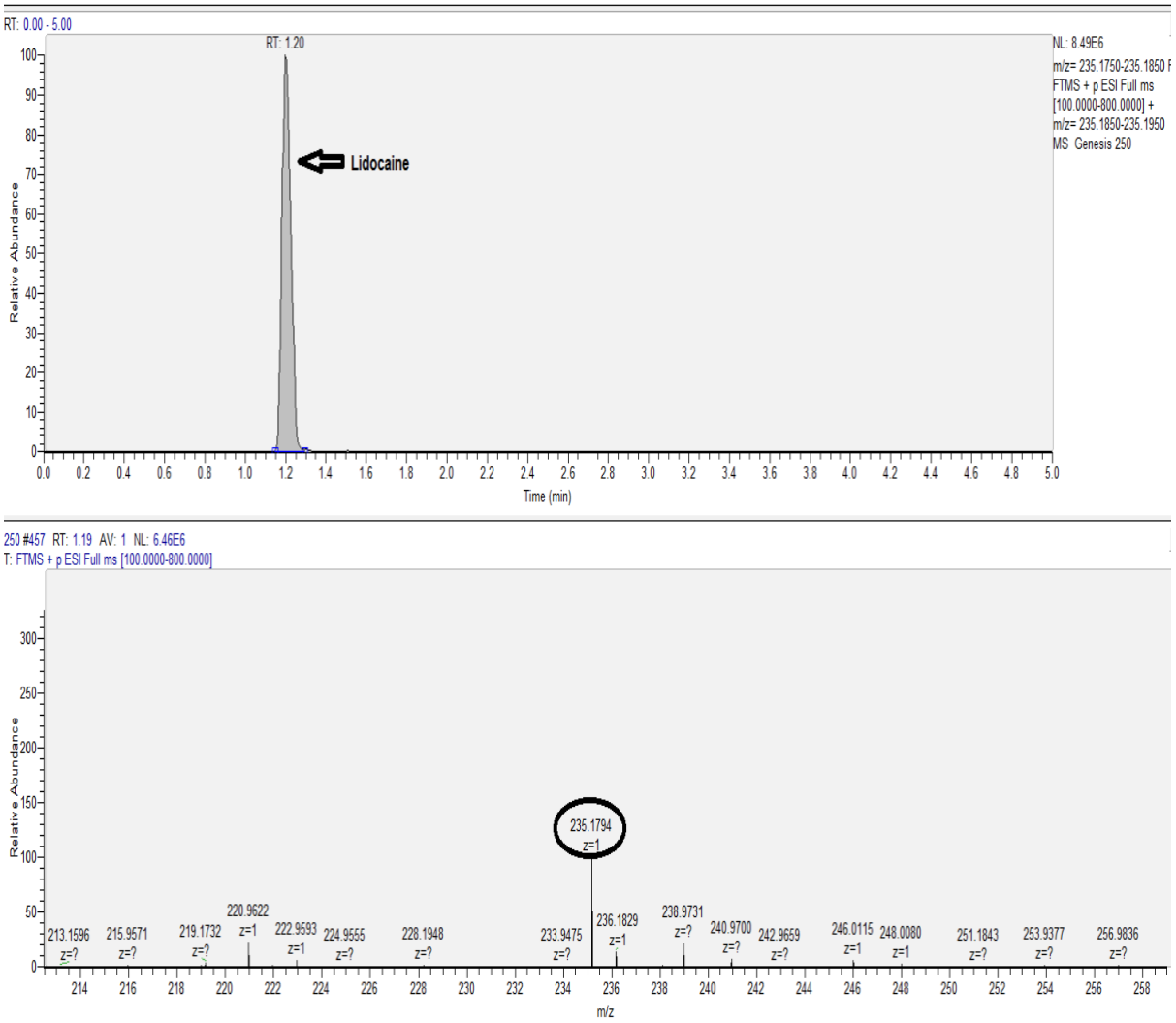
5.2.8 Supplementary materials

Supplementary Figure 5.3: Representative chromatograms and the mass spectra of (a) drug-free antler, drug-free antler spiked with (b) lignocaine (5 ng/g), (c) articaine (50 ng/g), (d) articainic acid (100 ng/g), (e) monoethylglycinexylidide (MEGX) (100 ng/g), (f) 2,6-dimethylaniline (DMA) (100 ng/g), and antler samples obtained following ring block of (g) 4 % articaine hydrochloride and (h) 2% lignocaine hydrochloride in red deer (*Cervus elaphus*).

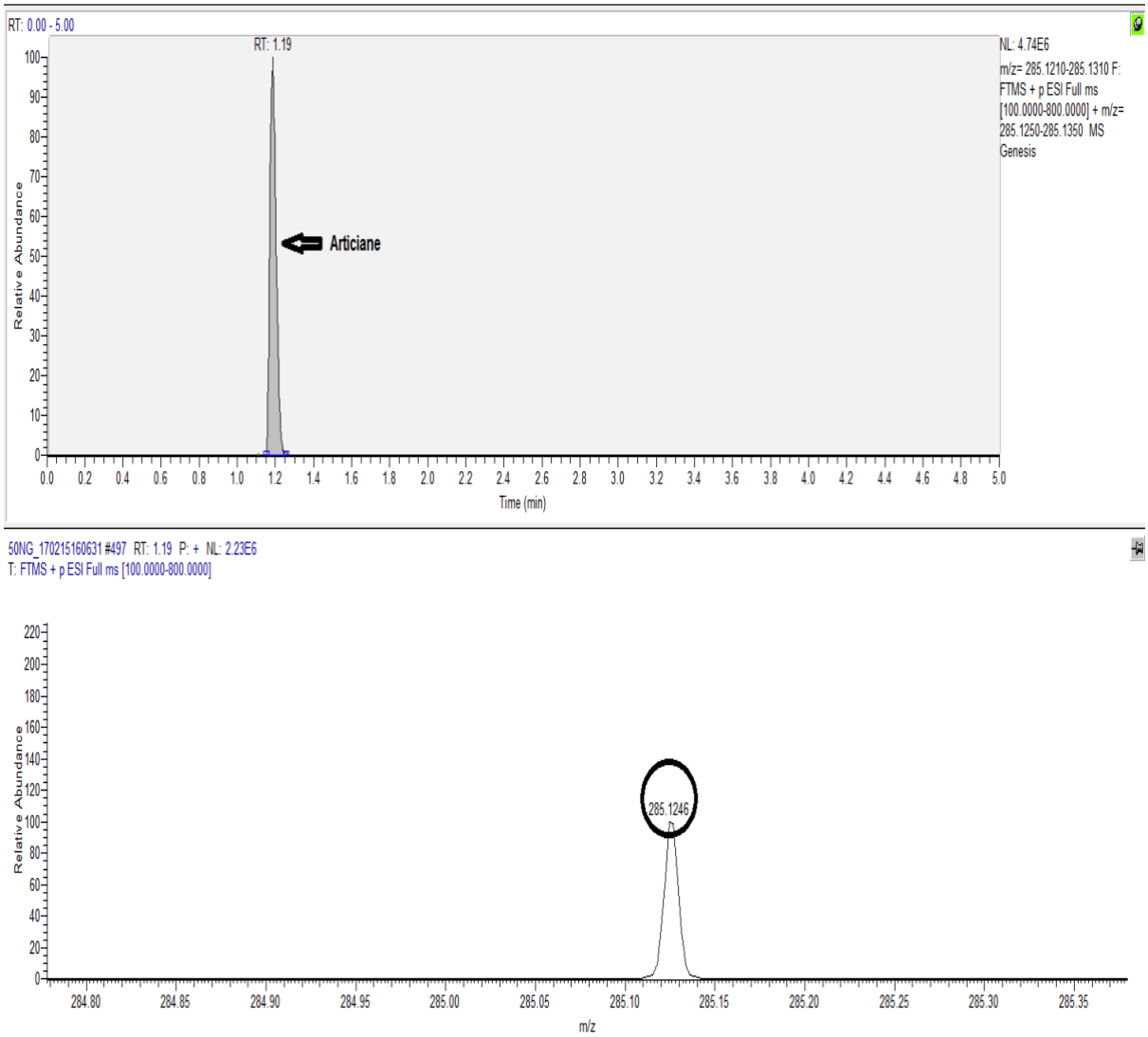
a) Drug-free antler



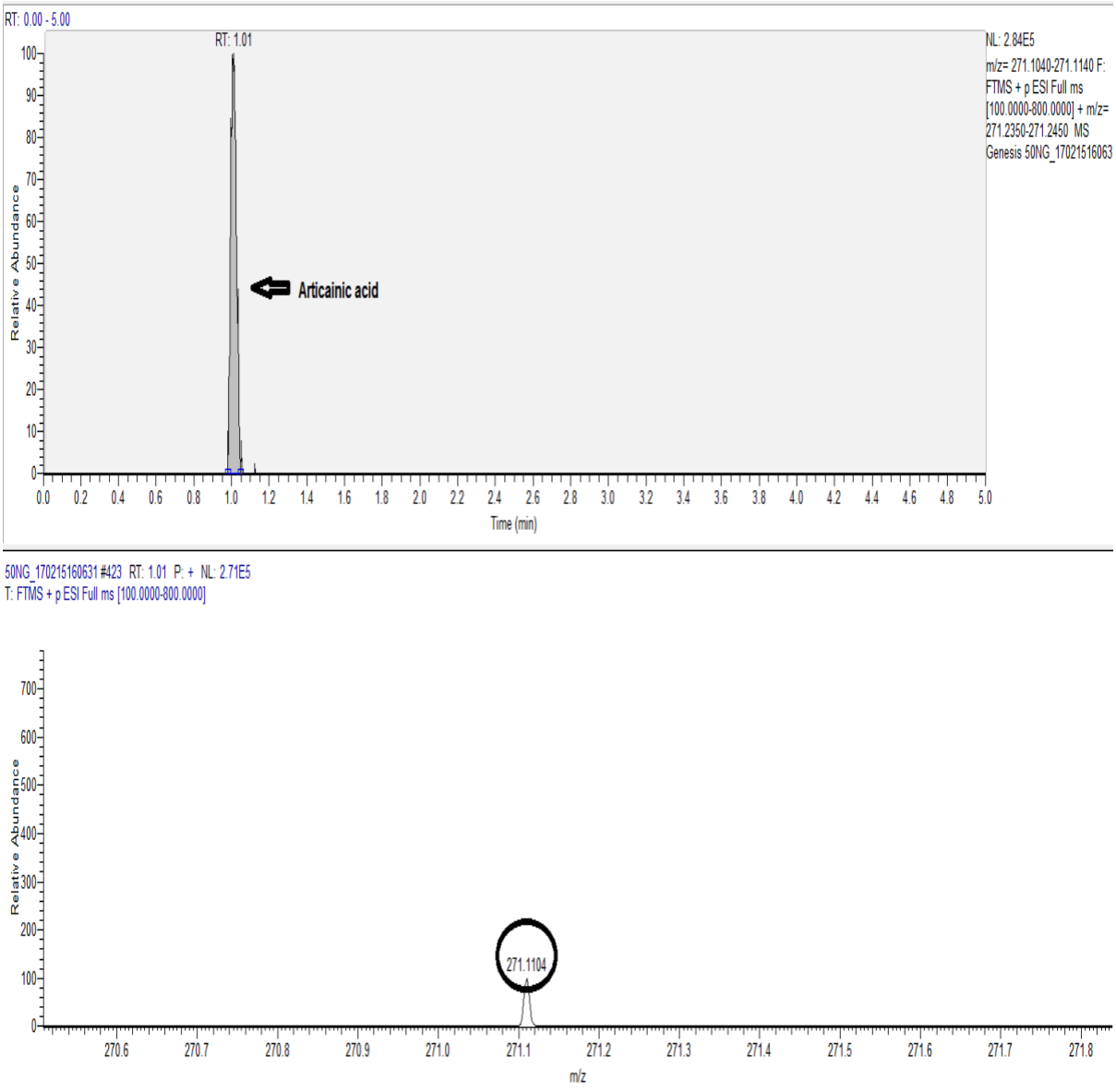
b) Drug-free antler spiked with lignocaine (5 ng/g)



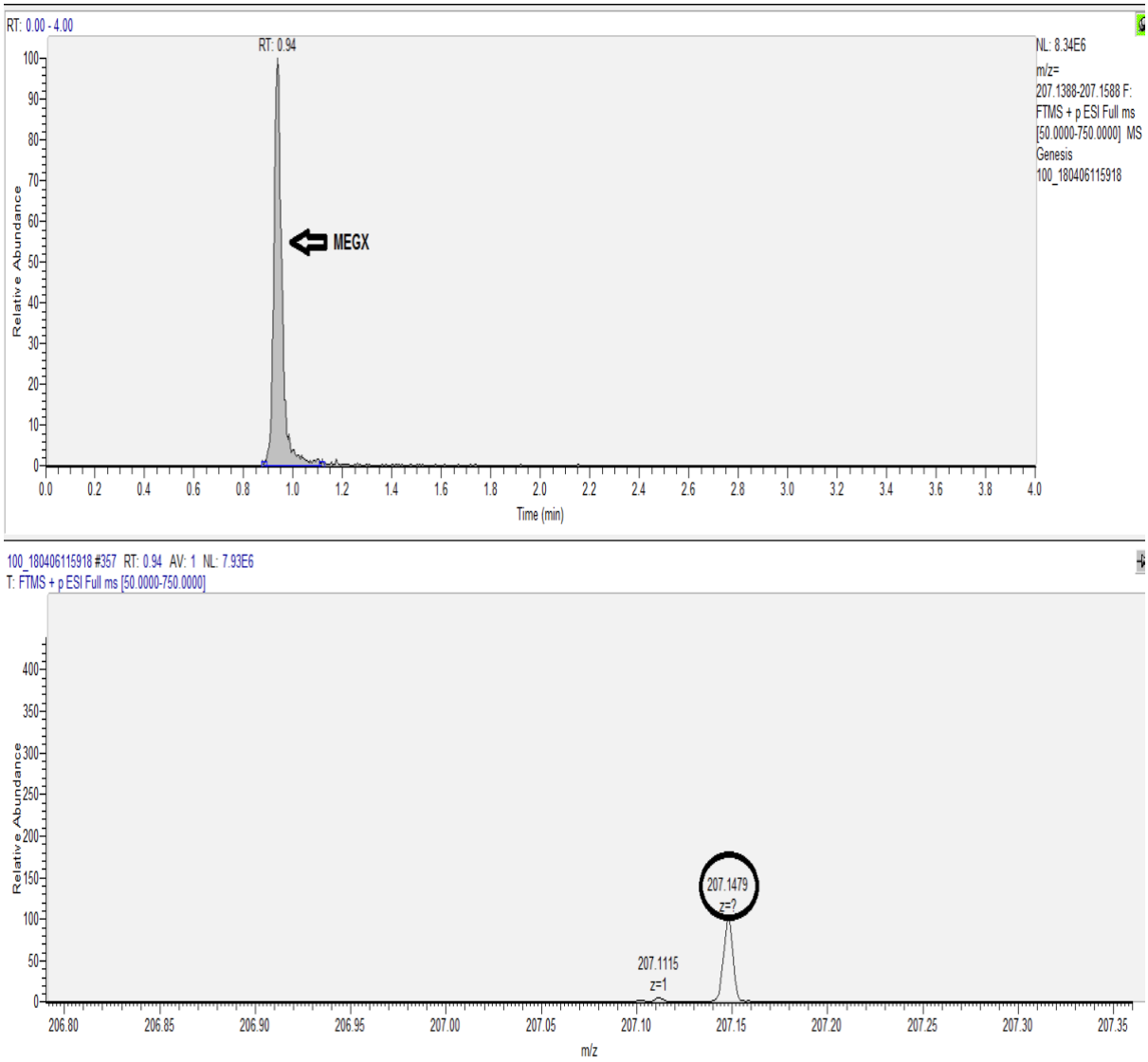
c) Drug-free antler spiked with articaine (50 ng/g)



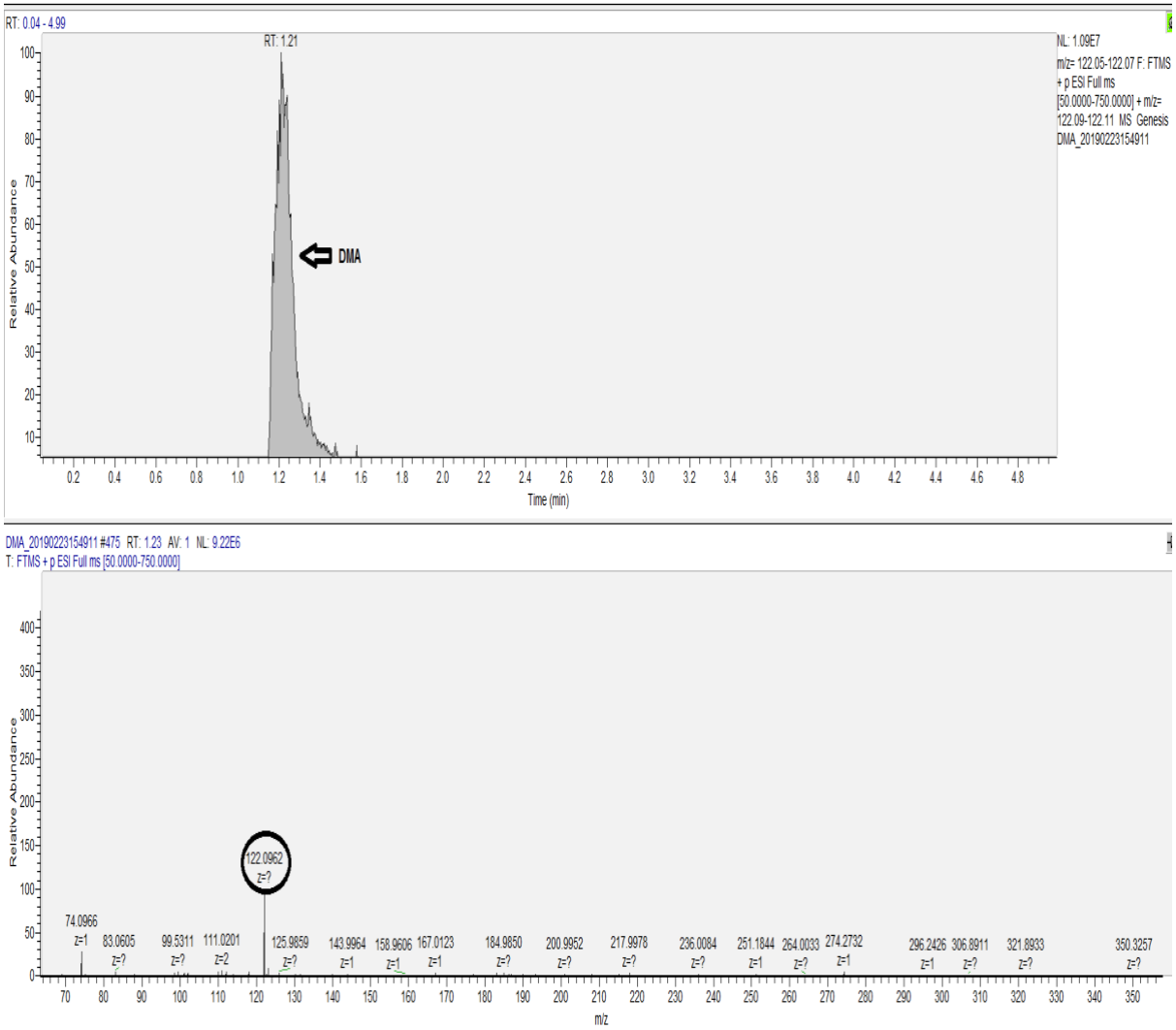
d) Drug-free antler spiked with articainic acid (100 ng/g)



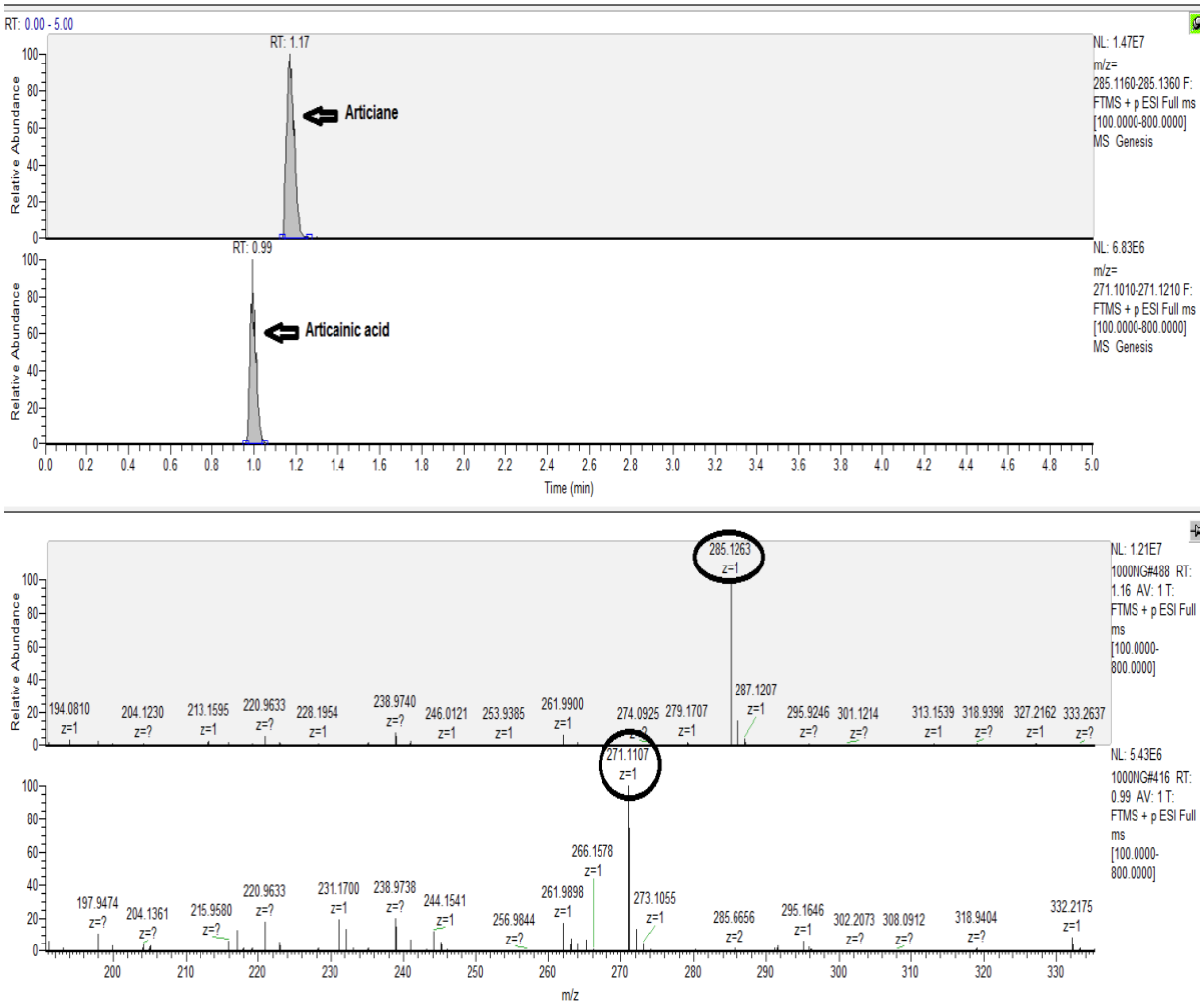
e) Drug-free antler spiked with monoethylglycinexylidide (MEGX) (100 ng/g)



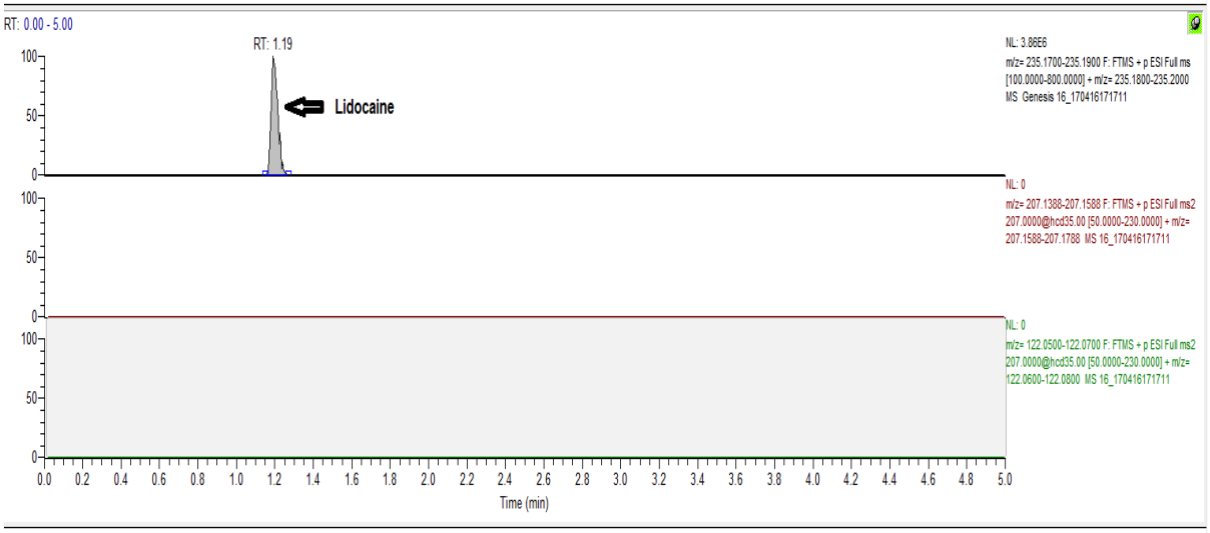
f) Drug-free antler spiked with 2,6-dimethylaniline (DMA) (100 ng/g)

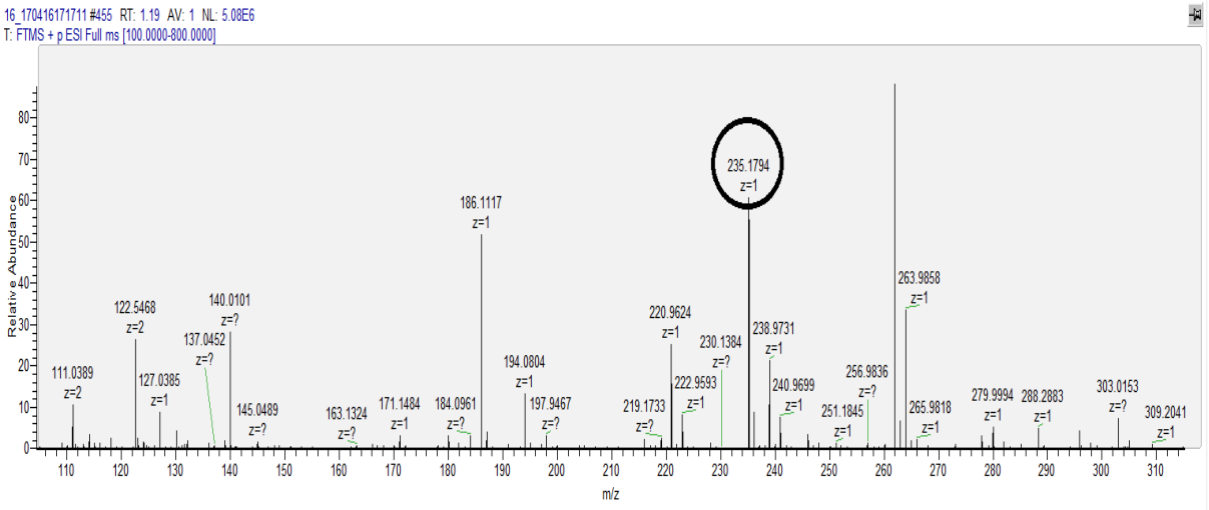


g) Antler samples obtained following ring block of 4 % articaine hydrochloride in red deer



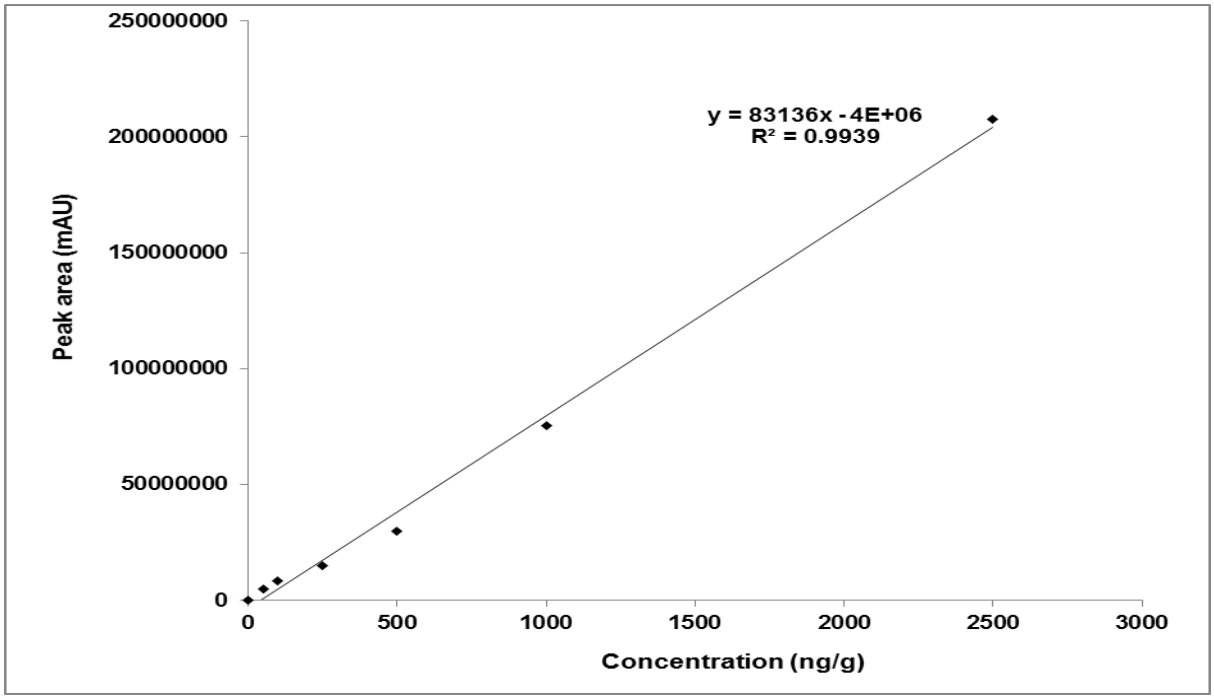
h) Antler samples obtained following ring block of 2% lignocaine hydrochloride in red deer



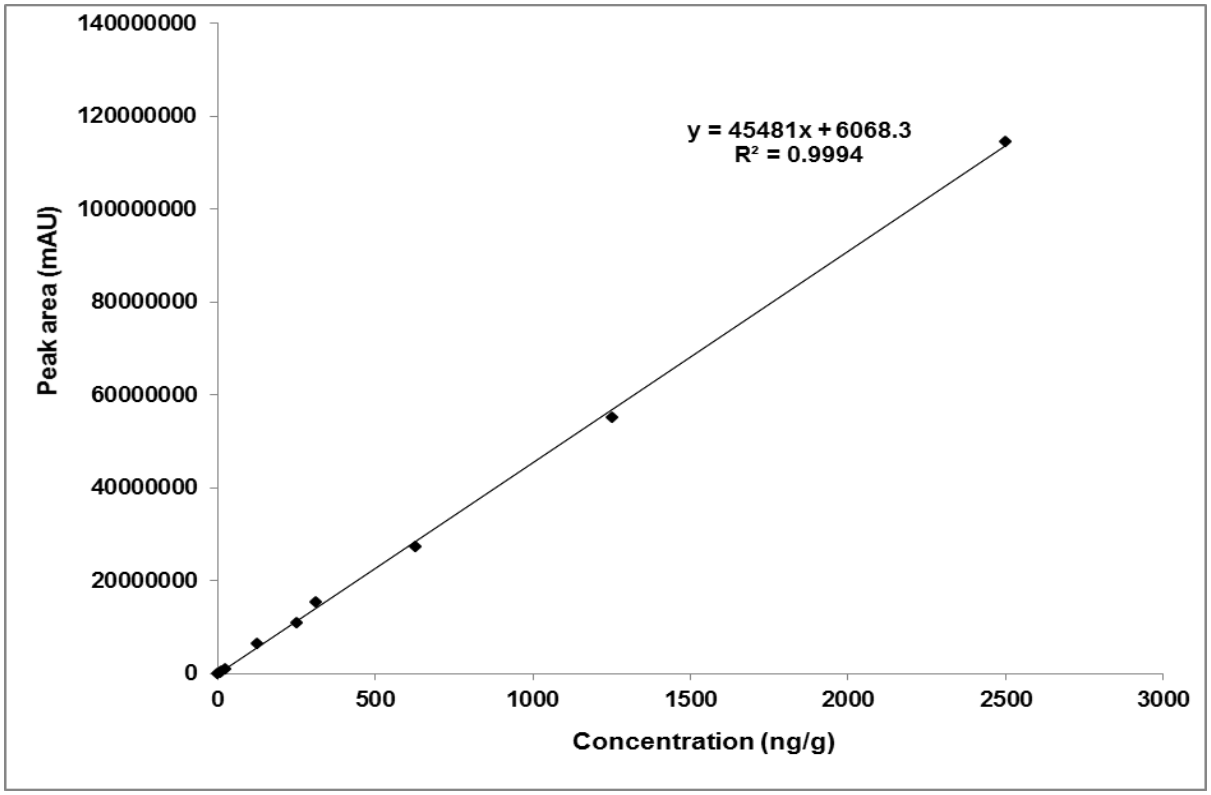


Supplementary Figure 5.4: Calibration curves of (a) articaine and (b) lignocaine constructed by spiking different concentrations of articaine and lignocaine, respectively, in drug-free velvet antlers.

a) Articaine



b) Lignocaine



5.2.9 Appendix

Table 5.13. Intra-day and inter-day accuracy and precision of articaine in deer velvet antler.

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-day	50	52.9±8.3	2.4	5.8
	250	235.4±18.0	6.8	-5.8
	2500	2534.1±168.1	6.6	1.4
Inter-day	50	48.8±10.5	15.6	-2.5
	250	272.3±86.1	11.5	8.9
	2500	2733.8±440.3	15.7	9.4

Table 5.14. Intra-day and inter-day accuracy and precision of lignocaine in deer velvet antler.

	Nominal concentration (ng/mL)	Observed concentration ± SD (ng/mL)	Precision (CV%)	Accuracy (RE%)
Intra-day	5	5.9±0.5	7.8	-3.4
	250	247.6±13.2	5.3	1.3
	2500	2302.0±172.5	7.5	-6.3
Inter-day	5	6.0±0.3	5.5	-5.8
	250	278.3±30.7	11.0	12.4
	2500	2176.6±231.1	10.6	-10.2

Table 5.15. Extraction recoveries of articaine and lignocaine from deer velvet antlers.

Concentration (ng/mL)	Articaine (%)	Lignocaine (%)
5	-	84.9
50	52.4	-
250	76.8	73.0
2500	60.4	58.6

DRC 16

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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:	DINAKARAN VENKATACHALAM
Name/title of Primary Supervisor:	Dr. PREET SINGH
Name of Research Output and full reference:	
[redacted]	
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:	
Dinakaran had a primary role in study design, data collection, analysis, interpretation, writing of the manuscript and addressing reviewers comments, with guidance from supervisors.	
For manuscripts intended for publication please indicate target journal:	
The New Zealand Veterinary Journal	
Candidate's Signature:	V. Dinakaran
Date:	26-04-2019
Primary Supervisor's Signature:	
Date:	26/April/2019

(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)

GRS Version 4– January 2019

Name of research output and full reference: Venkatachalam D, Chambers JP, Kongara K, Singh P (2019). Efficacy of articaine hydrochloride for velvet antler removal in red deer and analysis of drug residues in the harvested velvet antlers. This manuscript has been accepted for publication in the *New Zealand Veterinary Journal*.

CHAPTER 6

GENERAL DISCUSSION

Pain is one of the important indicators of poor animal welfare (Wikman *et al.* 2013). It not only has negative impact on the wellbeing of the animals but also affects the productivity and economics of farming (Mclennan 2018). Public concern on the painful procedures on farm animals has been increasing for years (Bennett and Blaney 2003; Anonymous 2015). These animal welfare concerns may decrease the consumers demand for animal products and affect the market of the products. In response to public concerns and consumers' expectations animal welfare legislation on the painful procedures have been changing around the world to protect the animals from pain and suffering, e.g., In New Zealand, it will be illegal from October 2019 to disbud a calf unless it is "under the influence of an appropriately placed and effective local anaesthetic" (Anonymous 2018). The animal welfare legislations vary between countries. For example, disbudding in goat kids is prohibited in Austria, while in the UK and Germany it must be carried out only by veterinarians under anaesthesia, whereas in other countries like Australia and New Zealand it is recommended but not mandatory to use pain relief. Lack of simple, cost-effective, on-farm methods could be the reason why most of the painful husbandry procedures are not performed humanely. Many studies have been carried out to find methods that are safe, cost-effective, less painful and practical in commercial farming conditions.

This thesis was undertaken to investigate various novel, and safer, methods of producing analgesia for surgical disbudding in goat kids and velvet removal in red deer, and to assess if simple and novel techniques can prevent horn bud growth in goat kids. The studies in the thesis (**chapters 2-5)** were written as manuscripts and therefore the results of the experiments were discussed in the respective chapters. This chapter includes an overall discussion of the

principal findings of all experiments, limitations of the research, future studies, and conclusions. There may be some repetition of the points to provide comprehensive results of the thesis however, wherever possible, every effort has been made to avoid unnecessary repetition of the points.

6.1 Principal findings and limitations

6.1.1 Articaine hydrochloride and lignocaine hydrochloride for disbudding in goats

Chapter 2 and 3 reported the findings of the dose-ranging study, pharmacokinetics, toxicity, and efficacy of articaine hydrochloride and lignocaine hydrochloride, respectively, in goat kids. The dose-ranging study suggested that doses up to 8 and 7 mg/kg of articaine hydrochloride and lignocaine hydrochloride, respectively, were well tolerated and can be used safely for perineural injections in goat kids. The safety of these doses was determined based on the absence of signs of toxicity observed for over 60 seconds following intravenous administration of the drugs. The risk of accidental intravenous injection of local anaesthetics is difficult to predict and so it was assumed that in the worst-case scenario it would require at least 60 seconds for the total dose to reach systemic circulation during cornual nerve block (four injection sites). Limitations of the dose-ranging study were that no cardiovascular parameters were monitored and only one animal per dose was used. Future studies should monitor cardiovascular parameters and increase sample size to determine the maximum tolerated dose of articaine and lignocaine in goat kids.

The pharmacokinetic data of articaine hydrochloride in goat kids suggested that articaine was rapidly hydrolysed and cleared following systemic absorption. In comparison, the metabolism and elimination of lignocaine were slower than articaine in goat kids. Similar to other species,

articaine was hydrolysed to an inactive metabolite, articainic acid in goat kids. Rapid hydrolysis to inactive metabolite and short elimination half-life are favourable pharmacokinetic parameters of articaine that could reduce the risk of systemic toxicity during overdose which can occur during cornual nerve block in goat kids.

Both lignocaine and articaine demonstrated similar signs of systemic toxicity such as sedation, ataxia, and tonic-clonic convulsions when toxic plasma concentrations were achieved. However, the total dose of articaine required to produce convulsions in goat kids was higher than that of lignocaine. The pharmacokinetic and toxicity data suggest that articaine appears to be a safe local anaesthetic in goat kids. One of the limitations of the toxicity study was that the cardiovascular and respiratory parameters such as heart rate, cardiac output, and oxygen saturation were not monitored. Future studies should include the cardiovascular and respiratory parameters to confirm the safety of the drugs in a large population.

Both articaine hydrochloride (1.5%) and lignocaine hydrochloride (1%) provided effective anaesthesia of horn buds following cornual nerve block (0.5 mL/site) in goat kids. The peak plasma concentrations of articaine and lignocaine following cornual nerve block remained well below the convulsive plasma concentrations of articaine and lignocaine, respectively. Articaine hydrochloride (1.5%) and lignocaine hydrochloride (1%) appear to be safe and effective for alleviating the acute pain during disbudding in goat kids. Even though articaine hydrochloride is safe and widely used in people it has not been approved for veterinary use. More target animal safety and efficacy studies are required before recommending articaine for disbudding. Lignocaine hydrochloride has been approved and widely used in veterinary medicine, future studies to determine the safety and efficacy of 1% lignocaine for cornual nerve block in large population of goat kids may be advantageous.

Low concentrations of articaine (1.5%) and lignocaine (1%) provided analgesia only for 20 to 40 minutes following disbudding. Behavioural and cortisol responses following cautery disbudding in goat kids indicate that pain persists for at least 2 hours following disbudding in goat kids (Alvarz *et al.* 2015, Hempstead *et al.* 2018). Therefore, in addition to local anaesthetic nerve blockade, it is recommended to provide systemic analgesics such as non-steroidal anti-inflammatory drugs (NSAIDS) to minimize the post-operative pain. Meloxicam has been reported to alleviate the post-disbudding pain in goat kids hence the administration of meloxicam along with local anaesthetic may provide better analgesia during and after disbudding. Long acting local anaesthetics such as bupivacaine may not be a good choice for goat kids as they possess a greater risk of toxicity than articaine and lignocaine.

Even though cornual nerve block is the currently available cheap and effective analgesic technique for disbudding, the injection of local anaesthetics at four sites in goat kids was stressful and painful. Administration of sedatives such as xylazine may reduce the stress and make it easier to administer local anaesthetics. Therefore, the ideal pain relief protocol for disbudding goat kids should include sedatives, local anaesthetics and NSAIDs. However, the cost and availability of drugs to the farmers are obstacles in most the countries. Also, the administration of these drugs requires skill and experience. In addition, there is also a risk of skull and brain damage during thermal cautery disbudding in goat kids. Therefore, there is a need for a simple, safe, cost-effective technique to prevent the growth of horn buds in goat kids.

6.1.2 Novel analgesic and disbudding methods in goat kids

Chapter 4 evaluated the different analgesic and disbudding methods in goat kids. Methoxyflurane administered using the hand-held inhaler (Penthrox inhaler) produced cutaneous analgesia but failed to provide sufficient analgesia for disbudding in goat kids. Methoxyflurane has been proven to be an effective analgesic even at sub-anaesthetic doses for various painful conditions (Grindlay and Babl 2009; Dyan 2016). Self-administered methoxyflurane using the Penthrox inhaler has been widely used by Australia and New Zealand army forces and ambulatory services for emergency relief of moderate to severe pain, and has been recently approved for use in the UK and Europe. In humans, inhalation of low-dose methoxyflurane via Penthrox inhaler produces rapid and effective analgesia for trauma associated pain and for pain caused by various procedures such as wound dressing, colonoscopy, and incision-drainage of abscess. Methoxyflurane did not provide analgesia for disbudding in goat kids possibly because the minimum alveolar concentration (MAC) required to produce effective analgesia could not be achieved using the Penthrox inhaler in goat kids. Penthrox inhaler was not suitable for the administration of methoxyflurane in goat kids as it was specially designed for self-administration in humans. Design of a device that can effectively administer methoxyflurane in goat kids may improve the efficacy of this technique. Even though low-dose methoxyflurane is a safe and effective analgesic, administration of methoxyflurane in farm animals may result in exposure to farm workers and produce toxicity. The Penthrox inhaler is attached with an activated carbon chamber to reduce environmental contamination. Similar chamber can be considered in the design of the new device for animals. Further investigation of this novel technique could be valuable because unlike local anaesthetic techniques, inhalant analgesics can produce rapid analgesia and may eliminate the waiting period. Also, administration of inhalant analgesics may be relatively easier and less painful than local anaesthetic injections.

The novel topical formulation and EMLA cream produced cutaneous anaesthesia but failed to provide effective anaesthesia of horn buds. Investigation of depth of skin analgesia of the novel formulation around the horn bud region may provide more information on the efficacy of the formulation. The use of topical formulation may not be practical in farming conditions because of the requirement of long waiting time and double handling of animals. However, the novel formulation may be useful for procedures that require a longer duration of dermal or wound analgesia as the formulation provided cutaneous anaesthesia for several hours.

Subcutaneous injection (0.2 mL) of both eugenol and mepacrine under the horn buds produced necrosis of the horn buds in most of the goat kids but failed to completely arrest the growth of horns in all the animals. Several studies have reported that injection of clove oil and its active ingredient, eugenol were effective in preventing the growth of horns in goat kids and calves (Molaei *et al.* 2015; Sutherland *et al.* 2019). Increasing the injection volume may increase the efficacy and completely arrest the horn growth. The use of non-invasive or minimally invasive techniques such as needless jet injector or needle assisted jet injector may reduce the injection pain. The absence of pain-related behaviours following the injection of the formulations indicate that post-operative pain relief may not be required if these formulations are used. Unlike thermal cautery disbudding, this novel technique (injection of mepacrine) may not cause skull or brain damage as the compounds are injected subcutaneously under the horn buds. Since this novel technique appears to be simple, safe and fast, future research should be carried out to improve the efficacy of this novel technique.

6.1.3 Articaine hydrochloride for velvet antler removal in Deer

Chapter 5 described the pharmacokinetics and efficacy of articaine hydrochloride for velvet antler removal in red deer as well as compared the residue levels of articaine and lignocaine in the harvested velvet antlers. Like goat kids, articaine was rapidly hydrolysed to articainic

acid and eliminated in red deer following subcutaneous administration. Administration of 4% articaine hydrochloride (1 mL/cm) as a ring block around the base of the antlers in red deer provided safe and effective analgesia for velvet antler removal in deer. The residue levels of articaine in the harvested velvet antlers following ring block were similar to lignocaine. Use of 2% articaine solution may be effective and reduce the residue levels in the harvested velvet antlers. Future studies should evaluate the efficacy of 2% articaine for velvet antler removal. Though articaine appears to be a safe and effective local anaesthetic, more safety and efficacy studies with different concentrations in a large population are required before recommending the drug for velvet antler removal.

One of the limitations of this study was that the analgesic duration of articaine was not evaluated following the administration of the drug in red deer. However, it must be noted that the identification of pain-related behaviours is challenging in deer. Deer being a prey species that does not overtly express pain or weakness which makes it difficult to recognize and assess pain in deer (Mclennan 2018). There are no established ethograms to identify pain in deer. Only a limited number of studies have evaluated the post-operative pain following velvet antler removal in deer. Pollard *et al.* (1992) reported that some behavioural changes such as ear flicking, head shaking and grooming were more common in velveted deer than the control group. Mathew *et al.* (1990) reported that behavioural and physiological responses following velvetting were not significantly different between velveted stags and control stags.

Walsh and Wilson (2002) reported that ring block using a combination of lignocaine and other long acting local anaesthetics such as bupivacaine and mepivacaine increased the duration of analgesia. The use of long acting local anaesthetics may increase the duration of analgesia but at the same time, they may introduce the residues in the harvested antlers. Like lignocaine, most of the amide-type local anaesthetics including bupivacaine and mepivacaine are also metabolised to DMA (Walsh and Wilson 2002). To provide post-operative analgesia

and to avoid local anesthetic residues in the harvested velvet antlers, the novel topical formulation (Chapter 4) may be evaluated in deer. The novel formulation may provide analgesia for several hours because of the high concentration of long acting local anaesthetics.

Despite widely used in humans, the carcinogenicity studies of articaine or its metabolites have not been conducted in animals yet. Unlike lignocaine, the metabolic pathway of articaine is different and the metabolites of articaine may not possess carcinogenicity. However, future studies to evaluate the safety of articaine and its metabolites are required before recommending the drug for velvet antler removal in deer.

6.1.4 Analytical method

The LC-MS methods used for the analysis of the local anaesthetics and their metabolites were simple, specific, sensitive and accurate. Short-run times (5 minutes) and easy sample preparation procedures were useful for the analysis of large quantities of samples from pharmacokinetic and *in vitro* skin permeation studies. Simple protein precipitation was used for the preparation of plasma samples in some of the experiments. Even though protein precipitation was an easy method, it did not remove all the contaminants from plasma as the contaminants were precipitated on the sweep cone of the ion source area which required frequent cleaning. The use of phospholipid removal tubes along with protein precipitation reduced the contaminants in the plasma and allowed analysis of many samples without the need for cleaning the ion-source area of the mass spectrometer. The extraction recoveries of the analytes from plasma using the sample preparation methods used in the analytical methods were low and variable. One of the major limitations of the analytical methods was that no internal standards were used during the analysis. Internal standards are normally used in chromatographic analysis to increase the precision of the analysis. An ideal internal

standard should have similar structure to the analyte to be quantified. Isotopically labeled analogues of the analytes are the most appropriate compounds to be used as internal standards. In the absence of isotopically labeled analogues, compounds that have retention times close to the anlytes can be used as internal standards. An internal standard is used to correct for variations in the instrument response, normalization of recovery differences and to correct for the loss of analyte during sample preparation, injection and ionization. Future studies should use internal standards to increase the precision and accuracy of the method.

The sensitivity of the LC-MS method for the quantification of articaine and articainic acid was greater than the previously reported sensitivity (Hoizey *et al*. 2009). High sensitivity was possible because of the Orbitrap technology in the mass spectrometer. Orbitrap is the newest addition to the class of high-resolution mass spectrometers that provides high-resolution, high-mass accuracy, and sensitivity (Hu *et al.* 2005; Zubarev and Makarov 2013). The use of LC-MS offered greater advantages over HPLC such as greater sensitivity, smaller sample volume and faster analysis.

6.2 Conclusion

The following conclusions can be made based on the results of the studies presented in this thesis:

1) Articaine hydrochloride and lignocaine hydrochloride appear to be safe up to 8 mg/kg and 7 mg/kg, respectively, following intravenous administration over 60 seconds in goat kids. These doses should be safe for perineural injections in goat kids.
2) Articaine demonstrated favourable pharmacokinetic properties such as rapid hydrolysis and clearance in goat kids. The pharmacokinetic and toxicity data in goat kids suggest that articaine is a safe local anaesthetic.
3) Cornual nerve block using 1.5% articaine and 1% lignocaine appears to be safe and effective for alleviating the acute pain during thermal cautery disbudding in goat kids.
4) The novel topical formulation and methoxyflurane did not provide sufficient analgesia for disbudding.
5) Injection of 0.2 mL of mepacrine and eugenol under the horn buds of goat kids produced necrosis of horn buds but did not completely prevent the growth of horn buds.
6) A high dose ring block using 4% articaine hydrochloride produced safe and effective analgesia for velvet antler removal in red deer.

6.3 Future Directions

6.3.1 Articaine hydrochloride and lignocaine hydrochloride for disbudding in goat kids

Articaine hydrochloride appears to be a safe and effective local anaesthetic for cornual nerve block in goat kids. Future studies with different doses and concentrations of articaine hydrochloride in a large population are required to establish the safety and efficacy of articaine hydrochloride for disbudding in goat kids. Both articaine hydrochloride (1.5%) and lidocaine hydrochloride (1%) were effective in alleviating the acute pain during thermal cautery disbudding. More studies to determine the behavioural, physiological and neurohormonal responses to disbudding following cornual nerve block using these drugs are required before recommending these drugs for clinical use in goat kids. In addition to local anaesthetic nerve blockade, it is also necessary to sedate the goat kids to reduce the stress and pain during local anaesthetic injections, and provide NSAIDs for post-operative pain management. Future studies should evaluate the safety and efficacy of this protocol (sedatives + local anaesthetics + NSAIDs) for disbudding in goat kids. Oral administration of sedatives and NSAIDs may be easy and preferred in farm settings. Therefore, future studies should consider evaluating the safety and efficacy of oral formulations of sedatives and NSAIDS. Future studies should also evaluate the feasibility of this analgesic protocol in commercial farming conditions.

Post-disbudding pain could be minimized by using sustained/controlled release local anaesthetic formulations. Several sustained release formulations of local anaesthetics have been reported to produce local anesthesia for hours to days after a single application. Cornual nerve bock using such sustained release local anesthetic formulations could provide pain relief during and after disbudding. These formulations will not only increase the duration of

analgesia but also decrease the toxicity of the local anesthetics as local anaesthetics will be slowly absorbed into the systemic circulation. Development of a prolonged acting local anesthetic formulation that can provide analgesia for atleast 24 hours can address both procedural and post-procedural pain associated with routine disbudding. Prolonged acting local anaesthetic formulations could be a good choice for other painful procedures including castration, tail docking and velvet antler removal as a single injection of the formulation can provide pain-relief during and following the procedure. Future studies should be conducted to develop a cost-effective formulation that can be used in a commercial farming setting.

6.3.2 Articaine hydrochloride for velvet antler removal

Articaine hydrochloride appears to be a safe and effective local anaesthetic for velvet antler removal. More studies with different concentrations of articaine hydrochloride are required in a large population to further establish the safety and efficacy of articaine for velvet antler removal in deer. As velvet antlers are intended for human consumption, future research to determine the maximum residue levels (MRL) for articaine and its metabolites are required before recommending articaine for velvet antler removal. Additionally, studies should also evaluate the duration of analgesia produced following the administration of articaine hydrochloride for velvet antler removal in deer.

Since the metabolic profiles of drugs can vary between species, it is important to identify the different metabolites of articaine in deer. Future studies to evaluate the toxicity (especially carcinogencity and mutagenicity) of articaine metabolites should be conducted before recommending articaine for velvet antler removal. There is paucity of data on the behavioural responses of deer following velvet antler removal therefore future studies should study the behavioural changes following velvet antler removal in deer to understand the intensity and duration of pain. To minimize the pain following antler removal, sustained release local

anesthetic formulations, long acting topical local anaesthetic formulations and NSAIDs can be investigated.

The residue levels of local anaesthetics in the harvested velvet antlers can be reduced by decreasing the absorption of the local anaesthetics from the site of injection. This can be achieved by preparing liposomal local anaesthetic formulations, formulating local anesthetics in thermosensitive gels (poloxamer gels) or by encapsulating local anesthetics in biodegreadable carriers. All these formulations have been shown to slow down the systemic absorption of the local anaesthetics from the site of administration and also increased the duration of local anesthesia. These formulations should be evaluated for velvet antler removal as they can reduce the residue levels in the harvested velvet antlers and increase the duration of analgesia. It is also necessary to do a cost-benefit analysis to use such formulations in commercial farms.

6.3.3 Novel analgesic techniques for disbudding

As methoxyflurane provided some degree of analgesia in goat kids, it may be useful to further evaluate its analgesic efficacy for disbudding in goat kids. A cost-effective device that can effectively deliver methoxyflurane to produce sufficient analgesia for disbudding may be useful in a commercial farm setting as inhalation analgesics produce rapid analgesia and are relatively easy to administer than local anaesthetics. In addition to disbudding, methoxyflurane can be evaluated for other painful husbandry procedures such as castration, tail docking and mulesing in farm animals.

Even though the novel topical formulation failed to provide sufficient analgesia for disbudding in goat kids, the formulation may be useful for procedures that require cutaneous or wound analgesia. The novel formulation can be evaluated for post-operative analgesia

following various painful procedures such as disbudding/dehorning, castration, velvet antler removal, hot branding, tail docking and mulesing in farm animals.

6.3.4 Novel disbudding techniques

Injection of mepacrine or eugenol under the horn buds could be an effective technique to stop horn bud growth in goat kids if some refinements are made. Future studies should investigate the safety and efficacy of different injection volumes and concentrations of these compounds. Non-invasive drug administration techniques such as needleless jet injector or microneedle arrays can be evaluated to reduce the injection pain. As both the compounds appear to produce necrosis without pain, it is worth to study their anaesthetic potency using *in vitro* and *in vivo* models. Future studies should be carried out to compare the safety and efficacy of this novel technique and traditional disbudding methods.

In addition to mepacrine, several other compounds including calcium chloride, polidocanol, sodium tetradecyl sulphate, proflavine and tetracycline may destroy the horn buds. Injection of alcoholic solutions of calcium chloride into the horn buds has been reported to prevent the growth of horns in calves. However, no information on the adverse effects or pain were reported by the authors. Injection of alcoholic solution can be painful therefore future studies should consider using different solvents to reduce pain. Polidocanol has been reported to posses local anesthetic and sclerotic properties so injection of polidocanol into the horn buds may effectively destroy the horn buds with minimal pain.

In order to effectively disperse the compounds around the horn buds, spreading agents such as hyaluronidase can be investigated. Injection pain can be reduced by mixing the cytotoxic drugs with local anesthetics. Future studies should be carried out to identify compounds that are cheap, effective, painless and non-toxic following systemic absorption. This novel

technique might provide a simple, cost-effective, safe and on-farm method to prevent the growth of horn buds in goat kids.

6.4 References

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