LOCAL ANESTHESIA PRODUCED BY QUATERNARY LIDOCAINE DERIVATIVES: THE COMPARATIVE EFFICACY AND SAFETY OF QX-314, QX-572, AND QX-222 IN MICE

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LOCAL ANESTHESIA PRODUCED BY QUATERNARY LIDOCAINE DERIVATIVES: THE COMPARATIVE EFFICACY AND SAFETY OF QX-314, QX-572, AND QX-222 IN MICE

submitted by Desmond H. Fung in partial fulfillment of the requirements for

the degree of Master of Science

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Abstract

Poorly managed acute pain, particularly postoperative pain, reduces quality of life, delays recovery, and increases financial burden. Current therapeutic regimens focus on nonsteroidal anti-inflammatory drugs and systemic opioids. Unfortunately, these analgesics are associated with notable and potentially life-threatening adverse events. Thus, there is a great need for analgesic alternatives.

Local anesthetics are among the most effective non-opioid analgesic options for acute pain. However, conventional agents are limited by their short duration as well as local and systemic toxicity. Previous research at the UBC Hugill Anesthesia Research Centre, showed that the quaternary lidocaine derivative, QX-314, produces long-lasting local anesthesia in animals. However, QX-314 was found to be more toxic compared to its parent compound. The quaternary lidocaine derivatives, QX-572 and QX-222, have not been studied systematically to compare their duration and toxicity.

In this thesis, I tested the primary hypothesis that perineural injection of QX-572 and QX-222, similar to QX-314, will produce long-lasting local anesthesia. I assessed nerve blockade in mice and found that QX-572 concentration-dependently produced long-lasting sensory and motor blockade. In contrast, QX-222 produced long-lasting sensory blockade but did not produce extended motor blockade. I determined the order of potency among quaternary lidocaine derivatives to be: QX-572 > QX-314 > QX-222. Additionally, I tested the secondary hypothesis that QX-572 and QX-222 have more favorable local and systemic toxicity profiles relative to QX-314. I found that QX-314 and QX-222 induced myofibre degeneration whereas QX-572 induced ischemic necrosis of the mouse tail. Adverse reactions were observed that manifested as death. To investigate the possibility of systemic toxicity, I developed a novel liquid chromatography tandem

mass spectrometry method to measure plasma concentrations of quaternary derivatives. I found that QX-314 and QX-222 were absorbed more readily than QX-572 or lidocaine.

The results from this thesis suggest that differences in therapeutic and toxic local anesthetic effects are mediated by structural differences. Overall, the present findings do not unreservedly support the transition of QX-314, QX-572, or QX-222 into human studies for the treatment of acute postoperative pain, however, they illustrate a potentially promising pharmacological avenue for further research and provide insight into quaternary anesthetic pharmacology.

Lay Summary

Inadequately controlled acute postoperative pain continues to be a widespread health care problem. Current pain treatments are associated with significant adverse events. Therefore, there is a great need for analgesic alternatives. Local anesthetics may be an important part of the solution, however, current agents are limited by duration and toxicity. It has been shown that one chemical derivative of lidocaine, QX-314, produces long-lasting local anesthesia. However, associated toxicity restricted its clinical potential. The goal of the present study was to investigate local anesthetic effects of two other quaternary compounds, QX-572 and QX-222. I conducted behavioral assessments in mice, performed microscopic analyses of tissues, and developed a new analytical method to quantify these agents in mice plasma. I demonstrated that QX-572 and QX-222 possess long-lasting local anesthetic effects but found that they also produce toxicity. This work provides insight into the pharmacology of quaternary anesthetics, and direction for future drug development.

Preface

The contents of this dissertation are based on experimental studies conducted under the supervision of Dr. Stephan Schwarz. The author, Desmond H. Fung, helped to design all the experiments in collaboration with Drs. Stephan Schwarz, Ernest Puil, and Bernard MacLeod. The research presented in this dissertation has been prepared in separate draft manuscripts that will be submitted for publication. Content found in Chapters 2, 3, and 4 of this dissertation have been modified from draft manuscripts written by the author. The contributions of the author and collaborators for each chapter are summarized below:

Chapter 1: This chapter was written by Desmond H. Fung. Drs. Stephan Schwarz, Ernest Puil, Bernard MacLeod, and Brian Cairns provided suggestions and comments to this section.

Chapter 2: The materials and methods used in all the experiments are described by Desmond H. Fung. Sections from this chapter will be included in manuscripts to be published. Drs. Stephan Schwarz, Ernest Puil, Bernard MacLeod, and Brian Cairns were involved in revisions of this work. Drs. Aaron Shapiro and Sergei Likhodi, from the BC Provincial Toxicology Centre, were involved in developing the liquid-chromatography tandem mass spectrometry (LC-MS/MS) method used in pharmacokinetic experiments investigating toxicity. Drs. Ian Welch and Catherine Schuppli, from the Centre for Comparative Medicine at UBC, were involved in designing experiments for histopathological analyses of biological samples.

Chapter 3: Desmond H. Fung conducted the experiments, analyzed the data, and produced the figures presented in this dissertation. Sections from this chapter will be included in manuscripts to

be published and may be modified prior to publication. Dr. Hye-Young Kim helped collect data for sensory blockade experiments. Ms. Helia Shariati and Mr. Michael Smith helped collect data for motor blockade experiments. Drs. Stephan Schwarz, Ernest Puil, Bernard MacLeod, and Brian Cairns were involved in revisions to figures prepared in draft manuscripts. Drs. Ian Welch and Catherine Schuppli were involved in the histological analyses of tissue samples. Drs. Aaron Shapiro and Sergei Likhodi were involved in the analyses of data generated from validation procedures for LC-MS/MS.

Chapter 4: This chapter was written by Desmond H. Fung. Sections from this chapter will be included in manuscripts to be published. Drs. Stephan Schwarz, Ernest Puil, Bernard MacLeod, and Brian Cairns were involved in revisions of this work.

Chapter 5: This chapter was written by Desmond H. Fung. Drs. Stephan Schwarz, Ernest Puil, Bernard MacLeod, and Brian Cairns provided suggestions and comments to this section.

All studies were approved by the Animal Care and Biosafety Committees at The University of British Columbia:

Evaluation of Local Anesthesia in Mice produced by Quaternary Lidocaine Derivatives:

Certificate #A17-0283

Analysis of quaternary lidocaine derivatives in murine blood samples:

Certificate #A19-0005

Analysis of QX derivatives in murine blood:

Certificate #B19-0058

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List of Abbreviations

ANOVA	Analysis of variance
ARRIVE	Animal Research: Reporting In Vivo Experiments
ASRA	American Society of Regional Anesthesia and Pain Medicine
CI	Confidence interval
CNS	Central nervous system
COX-1/COX-2	Cyclooxygenase 1/2
CVS	Cardiovascular system
EMLA	Eutectic mixture of local anesthetics
GX	Glycinexylidide
HARC	Hugill Anesthesia Research Centre
IASP	International Association for the Study of Pain
IFNγ	Interferon gamma
IL-17	Interleukin 17
IS	Internal standard
IVRA	Intravenous regional anesthesia
LA	Local anesthetic
LC-MS/MS	Liquid chromatography tandem mass spectrometry
logP	1-octanol/water partition coefficient
MEGX	Monoethylglycinexylidide
NSAIDs	Nonsteroidal anti-inflammatory drugs
OIH	Opioid-induced hyperalgesia

pK_a	Dissociation constant
PNS	Peripheral nervous system
SD	Standard deviation
TFL	Tail flick latency
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1
VGSC	Voltage-gated sodium channel

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First and foremost, I would like to thank my supervisor, Dr. Stephan Schwarz, for his mentorship, wisdom, kindness, and perpetual support. I would not have been able to complete this journey without his guidance. Apart from helping me become a better learner, his teachings have also made me a better person. For all of this and more, I remain grateful.

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Dedication

To Adrian and Ophelia

Chapter 1: Introduction

1.1 Pain Classification and Function

According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP, 1979). This definition was first described by Harold Meskey in 1964, and later published with revisions by the IASP. There is consensus among researchers and clinicians that pain is more than just a simple sensory response to noxious stimuli. This definition implies that individuals feel pain physiologically, emotionally, and psychologically with or without actual tissue damaging events. Moreover, it implies that pain is subjective among individuals. By having a clear definition of pain, clinicians will be better equipped to understand their patients in order to treat them effectively. Similarly, one unified definition will foster better communication between pain research and clinical practice.

The role of pain perception in biological systems is to help prevent further damage to an organism. This type of pain has been characterized as "physiological" because it serves a purpose (Woolf, 1989). With physiological pain, sensory impulses that arise from noxious stimuli undergo several levels of central nervous system (CNS) processing before pain perception occurs. Pain perception leads to a change in behavioral responses including avoidance of injured tissue from damaging stimuli, and reduced mobility to promote wound healing. Physiological pain is often associated with acute pain which is defined as pain with a duration of less than three months. However, pain can sometimes persist longer than expected for an injury and may eventually interfere with recovery. When pain persists for longer than three months, this pain state is classified as chronic. Physiological changes may occur in the body resulting in changes to the way in which

one responds to stimuli. These changes may occur in the peripheral nervous system (PNS) and CNS, and ultimately lead to altered connections. The ability for both the PNS and CNS to make altered connections is known as plasticity (Besson & Chaouch, 1987). When this occurs, conventional pain treatment is often ineffective and the chronic state is termed "pathological" pain (Woolf, 1995). For the reason that one's nervous system inputs can change, beliefs about pain have since shifted to appreciate that pain is a complex process.

1.1.1 Postoperative Pain

Surgery is an invasive procedure that can be associated with potential harm to tissues. In 2015, nearly 266 million surgical procedures were performed around the world (Holmer et al., 2019). Pain experienced by an individual after surgery is known as acute postoperative pain. According to the US Institute of Medicine (2011), roughly 80% of people that undergo surgery experience postoperative pain, and among those, 88% report moderate to severe pain. Inadequately controlled acute postoperative pain continues to be a widespread health care problem (Gan, 2017; Warfield & Kahn, 1995). The consequences of poorly managed acute postoperative pain are delayed recovery, prolonged opioid use during and after hospitalization, impaired physical function (Joshi & Ogunnaike, 2005), reduced quality of life (Peters et al., 2007), increased morbidity and overall cost of care (Chan et al., 2013; Coley et al., 2002). More importantly, studies have shown that the presence and intensity of acute postoperative pain are significant risk factors for the development of chronic postoperative pain (Gan, 2017).

Chronic postoperative pain was first defined by Macrae and Davies in 1999 as persistent pain which exists for a minimum of two months following surgical intervention (Tawfic et al., 2017). Since then, the definition has been revised to match the definition of chronic pain by IASP.

Several notable mechanisms associated with the development of chronic pain involve inflammatory processes, tissue and nerve damage, and central sensitization (Rashiq & Dick, 2014). Following surgical intervention, damage to peripheral nerves may lead to a number of physiological changes. Damaged nerves may alter their rate of firing and begin to produce ectopic activity due to clustering of sodium channels around damaged axons (Devor et al., 1993; England et al., 1996). As well, mechanoreceptors may become highly sensitized to natural stimuli as a result of constant stimulation by inflammatory mediators. Modulation of sympathetic nerve fibre function may also result in the activation of sensory fibres through alpha-adrenergic receptor pathways (Tawfic et al., 2017). Together, these changes can cause spontaneous pain and sensitize the CNS to exhibit enhanced responses to normal stimuli. In fact, this is represented by hyperalgesia and allodynia which are often associated with chronic pain. Hyperalgesia is the exaggerated pain response to stimuli that are normally painful (Shaikh et al., 2010). In contrast, allodynia is the development of painful responses to stimuli that are normally innocuous (Shaikh et al., 2010). In summary, postoperative pain is a burden to individuals and the society from a welfare and financial perspective.

1.2 Multimodal Analgesia

Due to the dynamic nature of the pain pathway, it was proposed that optimal analgesia should be achieved using a combination of analgesics rather than a single drug class (Kehlet & Dahl, 1993). By attenuating multiple pain pathways, acute pain and the risk of peripheral and central sensitization may be decreased. This multimodal approach is effective at reducing the frequency and intensity of perioperative pain, and minimizing the impact after surgery in terms of progression to chronic postoperative pain (Gan, 2017). However, attempts to produce analgesia

must take patient morbidity and any physiological changes into account. The nature of the noxious stimuli and degree of tissue damage will influence the level of pain, and types of analgesic medicines required. Currently, the mainstays for acute postoperative pain management are nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and local anesthetics.

1.2.1 Nonsteroidal Anti-Inflammatory Drugs

It is common for patients with acute inflammatory pain to receive systemic NSAIDs after surgery (Shankar et al., 2004). NSAIDs exert their mild-moderate analgesic effects by inhibiting the *de novo* synthesis of prostaglandins. This process is achieved by inhibiting cyclooxygenase 1 and 2 (COX-1/COX-2) enzymes (Candido et al., 2017). However, NSAIDs also produce ulceration along the gastrointestinal tract, renal dysfunction, and cardiovascular system (CVS) complications (Scheiman & Hindley, 2010). Investigation into the mechanism causing toxicity revealed that homeostatic functions of COX-1 enzymes are important for maintaining a healthy gastrointestinal system. Thus, if enzyme function is inhibited by non-selective NSAIDs, protective functions are also abolished (Sostres et al., 2010). Once the need for specificity was apparent, selective inhibitors for the COX-2 enzyme were produced. These selective inhibitors are known as coxibs. Unfortunately, concurrent with the decreased incidence of gastrointestinal complications was the increased risk of CVS complications, such as myocardial infarctions, stroke, and heart failure (Solomon et al., 2005). It is also important to distinguish between acute or chronic use of NSAIDs as this will influence the incidence and risk of complications. Although NSAIDs reduce postoperative morphine consumption (Elia et al., 2005), associated toxicities and the lack of specificity limit their clinical use.

1.2.2 Systemic Opioids

Opioids are another class of analgesics that interact with pain receptors and suppress the synaptic transmission of nociceptive signals. These compounds exert their antinociceptive actions at supraspinal and spinal sites with evidence of peripheral action. In the mesencephalic periaqueductal gray matter, activation of μ opioid receptors inhibits tonic γ-aminobutyric acid (GABA) release (Goodman et al., 2011). This leads to the release of downstream monoamine neurotransmitters in the spinal dorsal horn which can attenuate dorsal horn excitability (Yaksh, 1997). In addition to the CNS, all three receptor subtypes have been identified in the spinal cord and PNS (Barber & Gottschlich, 1992; Stein, 1993). In the spinal cord, opioids exert their presynaptic effects by reducing the release of primary afferent peptide transmitters (Yaksh et al., 1980). This effect corresponds with the inhibitory effect of opioids on voltage-gated calcium channels which are required for transmitter release. As well, opioids exert their postsynaptic effects by activating voltage-gated potassium channels, leading to hyperpolarization and inhibition of dorsal horn neurons (Goodman et al., 2011). The effects of opioids in the periphery are less clear (Stein & Lang, 2009). Nevertheless, opioids have been used as a mainstay of postoperative pain therapy because they are efficacious for moderate-severe pain.

Systemic opioids are, on the other hand, associated with significant adverse effects. These undesirable side effects include respiratory depression, sedation, puritis, constipation, nausea, vomiting, and delayed recovery (Bowdle, 1998; Campbell, 1990; Green & Jonsson, 1993). Recently, it has been demonstrated that there is also an inherent risk of new persistent opioid use after minor or major surgery (Brummett et al., 2017). In this study, rates of prolonged opioid use were no different for those who underwent major or minor surgery, and individuals with more comorbidities were at greater risk. This becomes problematic because long-term opioid

consumption can lead to the development of analgesic tolerance, dependence, and/or opioid-induced hyperalgesia (OIH) (Colvin et al., 2019; Koppert & Schmelz, 2007; Lee & Yeomans, 2014; Mercadante et al., 2019). But in spite of these adverse effects, widespread use of opioids for many acute and chronic pain states continues (Ballantyne, 2017).

Opioid tolerance is defined as a reduction in drug potency and analgesic effect following prolonged administration (Dumas & Pollack, 2008). This clinical problem develops differently for individuals and can occur after a short or long period of opioid use. Physiological factors that are known to influence the development of tolerance include interactions between opioids and receptors, dosing, and frequency of administration. The mechanisms may involve upregulation of enzymes responsible for drug metabolism, downregulation of receptors, or desensitization of receptor signaling (Cahill et al., 2016; Lau & Vaughan, 2014). For patients who have developed tolerance, increasing the dose of opioids will normally result in restoration of analgesia. In contrast, increased dosing will not relieve pain associated with OIH. Physiological mechanisms for OIH may involve reversal of cyclic-AMP mediated actions by opioids, and uncoupling of associated G-proteins (Gintzler & Chakrabarti, 2004). Opioids can also interact with glial cells leading to the production of inflammatory mediators and subsequent sensitization of neurons (Harada et al., 2013). On top of adverse events associated with opioids, the propensity for opioid abuse and addiction in the general population has collectively led to the public health epidemic known as the opioid crisis (Ballantyne, 2017). Problems associated with adverse effects produced by opioids are not only challenging and debilitating, but also significantly increase hospitalization costs (Oderda et al., 2007). In summary, there is a great need for non-opioid alternatives for the treatment of acute postoperative pain.

1.2.3 Local and Regional Anesthesia

Local anesthetic (LA) agents are used in local and regional anesthesia for the treatment of postoperative pain. Regional anesthesia is popular because it provides superior control of postoperative pain (Albrecht & Chin, 2020; Wildsmith & Armitage, 1987). Studies have shown that the combination of an opioid with LA is more effective at producing analgesia than either alone (McQuay et al., 1988). Additionally, LA use has been associated with reduced overall opioid consumption following major surgical intervention (Jo et al., 2014). LAs can be used for topical, infiltration, minor or major nerve plexus, epidural, or spinal blocks. Technological advancement with ultrasound-guided techniques have helped make numerous regional anesthetic procedures safer, simpler, and more reliable. Regional anesthesia is also more effective at reducing the stress-induced physiological response to surgery and trauma compared to general anesthesia and systemic opioids (McClure & Wildsmith, 1991). In some cases, regional anesthesia may even obviate the need for general anesthesia. This would eliminate the risks associated with general anesthesia, and reduce the cost associated with surgery for both the patient and the hospital. In fact, regional anesthesia has been shown to reduce the cost of medical treatment, improve efficiency of surgical procedures (Head et al., 2011; Mercereau et al., 2012), and minimize risk of postoperative nausea and vomiting (Bridenbaugh, 1983). In general, regional anesthesia is associated with a reduced risk of cardiac, pulmonary, and gastrointestinal morbidity (Buggy & Smith, 1999; Liu et al., 1995). LAs are therefore excellent non-opioid alternatives for effective and safe postoperative pain control.

1.3 Local Anesthetics

1.3.1 History

The origins of local anesthesia are often interwoven with early attempts at producing neural blockade. In 1845, Francis Rynd (1801-1861) described a procedure to expose peripheral nerves to a solution of morphine with the hope to ameliorate neuralgic pain (Rynd, 1845). A few years later in 1848, the Scottish obstetrician, James Simpson (1811-1870) conducted experiments using liquids and vapors to produce numbness that he mistakenly described as local anesthesia (Simpson, 1848). Around 1853, Alexander Wood (1817-1884) thought to combine needle with hypodermic syringe to inject morphine into tissues. While his attempt to treat neuralgia using a local injection technique failed, the idea of injecting a drug locally was conceptualized. Approximately five years later, Benjamin Ward Richardson (1828-1896) attempted to produce local anesthesia using methods that involved nerve compression, electricity, temperature, and finally succeeded with the development of the ether spray (Richardson, 1858). However, by the mid 19th century, word of the systemic effects produced by chewing coca leaves (*Erythroxylon coca*) had spread into Europe. In 1860, a German chemist by the name of Albert Niemann (1834-1861) extracted and purified an alkaloid from coca leaves at the University of Göttingen. This compound was cocaine, and Niemann noted that numbness of the tongue was produced after tasting the powder for the purpose of confirming the bitterness of a plant alkaloid. Cocaine was subsequently used to treat morphine addiction, and news of this spread to an Austrian by the name of Sigmund Freud (1856-1939). This budding psychologist and researcher took the news and sought help from his friend, Carl Koller (1857-1944). Koller, being the aspiring ophthalmologist he was, came to realize that cocaine produced local anesthesia when applied topically on the eye (Arthur & Strichartz, 1987). Not long after, Koller performed the first public demonstration of local anesthesia produced by the

LA, cocaine, for eye surgery. Koller's experimental results on himself, animals, and colleagues were eventually shared with the scientific community on September 15, 1884, marking the beginning of local anesthesia (Becker, 1963; Leonard, 1998). This brief report on the history of local anesthesia simply does not capture enough of the story. Therefore, I am inclined to mention that cocaine was also used in several noteworthy regional techniques including infiltration (Schleich, 1894), spinal (Bier, 1899), and intravenous anesthesia (Bier, 1908).

1.3.2 Pharmacology

To appreciate the pharmacology of LAs, one must have a basic understanding of nerve physiology. Peripheral axons are covered by a double layer phospholipid membrane (Tetzlaff, 2000). The (polar) hydrophilic phosphate head groups face the intercellular fluid region, whereas the (non-polar) hydrophobic lipid groups are neatly packed against each other in the center of the membrane. Embedded in the membrane are large proteins which include enzymes, receptor channels, transport pumps, and ion channels. With respect to LA function, the most important proteins are the voltage-gated ion channels (Nau & Wang, 2004). Ion channels have a pore region that permits passage of ions into and out of the nerve. Some channels also have a selectivity filter which acts to ensure that the correct ion passes through (Yu & Catterall, 2003). Simply put, many channels have an activation gate that regulates entry, and a sensory mechanism that responds to changes in membrane potential (Stuhmer et al., 1989). Changes to the membrane potential induces conformational changes in voltage-gated ion channels which causes the activation gate to open and allow passage of ions.

Normally, nerve fibres have a resting membrane potential of around -70 mV (de Jong, 1994; Hodgkin & Huxley, 1939). This charge difference reflects an inside portion of a nerve fibre

that is negative relative to the outside. When nerves are stimulated, a transient depolarization followed by repolarization, and hyperpolarization of the resting potential occurs. If an activation threshold is reached from stimulatory inputs, this process occurs in milliseconds and is known as the action potential (Figure 1). Further information about the biophysical properties of the action potential and impulse propagation will not be discussed here as it goes beyond the scope of this dissertation (Hodgkin & Huxley, 1952). In short, the action potential is the most important functional unit of the nervous system and is responsible for neuronal transmission along nerves.

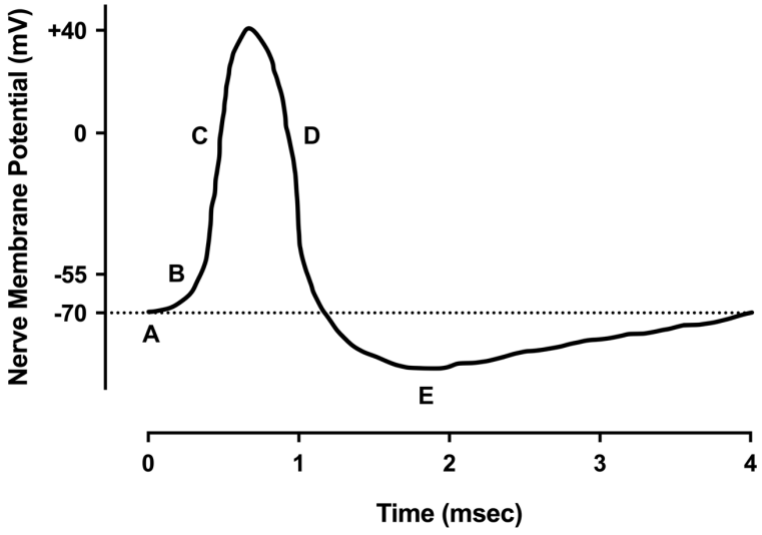


Figure 1 The neuronal action potential. At rest, (A) the nerve membrane potential is at -70 mV. (B) Nerve stimulation gradually increases the membrane towards a threshold potential. When the threshold is reached, (C) depolarization occurs. Inactivation of sodium channels and activation of potassium channels result in (D) repolarization followed by (E) hyperpolarization.

1.3.2.1 Pharmacodynamics

LA drugs block the excitability of nerves principally by blocking voltage-gated sodium channels (VGSC) (Taylor, 1959). If enough channels are blocked, this results in a complete, reversible block of neuronal transmission (Wildsmith & Armitage, 1987). Indeed, *in vitro* experiments on isolated squid giant axons using quaternary, neutral, and tertiary forms of LAs showed that these compounds exert their pharmacological effect by binding intracellularly to VGSC (Frazier et al., 1970; Narahashi et al., 1970; Narahashi et al., 1969). Although less important in terms of nerve blockade, traditional LAs have also been found to block voltage-gated calcium, voltage-gated potassium, hyperpolarization-activated cyclic nucleotide-gated channels, and other receptors involved in neurotransmission (Coyle & Sperelakis, 1987; Putrenko & Schwarz, 2011; Strichartz, 1990).

All clinically used LAs have a common molecular structure (Figure 2) which is composed of a lipophilic group attached to a hydrophilic group by an intermediate carbon linkage (Strichartz et al., 1990). Possession of both groups renders the LA amphipathic which allows them to be prepared, in most cases, as an acid solution of the hydrochloride salt in water. In fact, the hydrophilic group typically is a tertiary amine that becomes protonated (ionic). Because of small differences between the dissociation constant (pK_a) of the amine group and physiological pH, ionization increases the overall water solubility of the drug. Once injected, equilibrium with physiological buffering systems result in a ratio being formed between the deprotonated (neutral) and protonated (ionic) form of the LA. For most tertiary LAs, it is the neutral form that allows diffusion of the drug across nerve membranes (Bernards & Hill, 1992). Once inside the nerve, tertiary LAs, with the exception of benzocaine, again reach equilibrium with intracellular pH so that the protonated form can then bind to VGSCs.

For years researchers focused on identifying the specific binding site (Ragsdale et al., 1994; Strichartz, 1976), and use-dependent blocking properties of LAs (Courtney, 1980; Schwarz et al., 1977). Use-dependent block was discovered because there was an observed difference between the resting state and stimulated blocks produced by LAs. In laboratory experiments, the amplitude, duration, and rate of pre-pulses and test pulses that were given to simulate the nerve (use-dependent) resulted in different blocking effects of LAs compared to the absence of stimulation (resting state). It was later determined that this difference is the result of different LA binding affinities for activated, inactivated, and closed states of the sodium channel (Hille, 1977a). LAs have a weak binding affinity for the closed state, and strong binding affinity for the activated and inactivated states. With help from technological advancements, the specific binding interactions between LAs and voltage-gated sodium channels have finally been identified and crystalized (Catterall, 1999; Gamal El-Din et al., 2018).

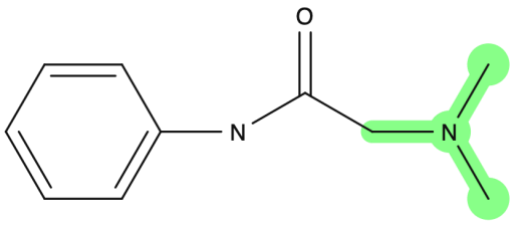


Figure 2 General molecular structure of tertiary amide-type local anesthetics. In this example, the compound has a lipophilic group (aromatic ring) attached to a hydrophilic group (tertiary amine; green) connected by an intermediate carbon linkage which contains an amide bond.

1.3.2.2 Pharmacokinetics

Pharmacokinetic properties of all LAs are determined by biochemical factors and structural features. The main biochemical factors to consider are molecular weight, pK_a, lipid solubility, and extent of plasma protein binding. Since all LAs have different biochemical factors and molecular structures, it is important to understand general pharmacokinetic principles in terms of systemic

absorption, local distribution, metabolism and elimination. Knowledge of LA pharmacokinetics will allow for efficacious and safe use of LAs.

To determine levels of systemic absorption, the blood or plasma drug concentrations are collected and measured sometime after perineural injection. Measured values are then compared to predetermined threshold values known to be associated with CNS or CVS toxicity. For example, the standard CNS threshold value for lidocaine given intravenously is around 5 μg/mL in plasma. With bupivacaine, the CNS threshold value is around 2 μg/mL which is expected since it is much more potent compared to lidocaine (Wildsmith & Armitage, 1987). Although most clinically relevant LAs have published threshold values (Tucker & Mather, 1979), anesthesiologists must be mindful about whether these recommended values are respective for plasma or blood, bound or unbound drug, racemic mixture or pure enantiomer, active compound or metabolite, and the rate of administration. The site of blood sampling, whether it be from an artery or vein is also important (Tucker, 1986). In clinical toxicology, liquid chromatography tandem mass spectrometry (LC-MS/MS) is used to quantify the amount of drug in systemic circulation (Maurer, 2004). Development of agent-specific analyses methods requires time, funding, and considerable input from biochemical and toxicological experts. Once a novel method is developed, it is put through a series of validation tests. If the novel method passes all validation testing, it can be used for high-throughput drug analyses.

Systemic absorption depends on the lipid solubility of the compound, local blood flow, and extent of LA tissue affinity. For most clinical LAs, diffusion across the cell membrane and into systemic circulation is not limited by lipid solubility since diffusion occurs with their neutral forms. Thus, the main determinants are local blood flow and extent of tissue binding. Compounds have different affinities for different tissues which means that the rate of diffusion into systemic

circulation will be influenced by the site of injection. As well, some LAs will have an increased rate of systemic absorption if they also produce vasodilatory effects in peripheral tissues (Hickey et al., 1990; Johns et al., 1985). The site of injection, dose, and presence of vasoconstrictors, formulation, and pathological features of the patient will affect the total quantity of LA absorbed systemically (Tucker & Mather, 1998). With regard to local tissue binding, sequestering of more lipid soluble LAs in fatty tissues of the body will decrease the amount of systemic absorption. In general, absorption rates depend on the injected site, and decrease in the order of intercostal > caudal > epidural > brachial plexus > sciatic and femoral nerve blocks (Tucker & Mather, 1998).

The local distribution of LAs are affected by the anesthetic procedure and biochemical properties of the agent (Wildsmith & Armitage, 1987). Factors like the bulk flow of the injection, extent of blood flow at the site of injection, and skill of the anesthesiologist will significantly influence the amount of LA deposited at the intended site. As well, the specific pK_a of the agent will determine the rate at which equilibrium occurs with local tissues around the injection site. Since most clinically relevant LAs are tertiary amines which are poorly soluble in water, they are prepared as an acid solution of the hydrochloride salt in water (section 1.3.2.1). As a salt solution, LAs dissociate between the quaternary amine cation and the tertiary amine base (neutral). The ratio between LA cation and base is determined by the pK_a or pH of the environment. Because the pK_a of a specific agent is constant, this ratio is therefore governed by the pH of surrounding tissues. However, it is important to note that it is the base form of LA that determines how much drug is transported across the biological membrane, and thus how much is available to bind to the sodium channels. Lidocaine and bupivacaine have pK_a values of approximately 7.8 and 8.1, respectively (Denson, 1991; Strichartz et al., 1990). At physiological pH (~ 7.4), lidocaine exists in base form at approximately 30%. In contrast, the percentage of bupivacaine in base from is approximately

17%. In most instances, the closer the LA's pK_a is to physiological pH, the greater the amount of base that will be formed. The extent of plasma protein binding, which is also agent specific, will determine the ratio of bound to unbound drug near the site of injection. In general, the local distribution of LA compounds can be controlled with sound knowledge of the pharmacokinetic properties of the agent being used and anesthetic procedure.

The lung is considered an important first-pass organ for LA agents. It helps modulate the initial arterial drug concentration to prevent toxic levels from reaching the brain and heart (Tucker & Boas, 1971). The amount of LA taken up by the lung is dependent on binding affinity to lung tissue, and pH gradients between plasma and the lung environment (Palazzo et al., 1991). After passage through the lungs, LAs are redistributed preferentially to organs with the highest demand for blood. Postoperatively, there can be an accumulation of LA in plasma due to the stress response of the body from surgery (Burm et al., 2000; Erichsen et al., 1996; Tucker, 1986; Tucker & Mather, 1975). The stress response results in the production of plasma proteins and thus, unbound LAs in the plasma will quickly become bound. As a general rule, the extent of binding to the plasma protein, α_1-acid glycoprotein, is greatest for lipid-soluble agents (Burm et al., 1994; Emanuelsson et al., 1995; van der Meer et al., 1999). Since plasma proteins cannot diffuse out of blood capillaries, LAs with the highest affinity for plasma proteins will stay in the blood for longer (Patrick, 2013).

The metabolism of LAs depend on the class of the agent, whereas elimination depends on patient factors. For ester-type LAs, plasma cholinesterases metabolize these compounds rapidly. This is why it is difficult to measure ester-type LAs in the blood after regional blockade. In contrast, amide-type LAs are unaffected by plasma cholinesterases. Instead, biotransformation occurs in the liver and metabolites can be found in the urine. Biotransformation is necessary since

renal excretion of unchanged drug is less than 5% (de Jong, 1994). Lidocaine belongs with the amide family of LAs. Since the amide linkage is stable in the blood, clearance is mostly controlled by rate of metabolism in the liver. With regard to drug elimination, individuals who are diseased may have more difficulty eliminating LAs as a result of renal complications from diabetes (Peeyush et al., 1992). Ageing can also result in decreased clearance and disposition volumes (Cusack et al., 1980; Fukuda et al., 2000; Nation et al., 1977). Equally important, gender differences may influence volume of distribution kinetics (Wing et al., 1984). Overall, LA metabolism is primarily influenced by enzymes, whereas elimination is mostly influenced by patient factors.

1.3.3 Notable Aminoesters

Cocaine is significant because it was the first agent used in clinical practice (Koller, 1884). Cocaine produces local anesthetic effects when applied on mucous membranes found in the cornea, gastrointestinal tract, and airway. It is manufactured as a liquid solution in the 1-10% range and used at 4-5% for topical anesthesia. Cocaine is no longer used for infiltration, spinal, epidural, or peripheral nerve blocks. Aside from local anesthesia, cocaine is known for producing robust vasoconstriction which is attributable to norepinephrine uptake inhibition (cf. below) and increased endothelin-1 (Sáez et al., 2011). Investigation into the effect of vasoconstriction on the CVS has confirmed that it causes coronary vasospasm which can lead to myocardial infarction, extreme hypertension, and death (Lange et al., 1989; Lustik et al., 1997; Riezzo et al., 2012). Additionally, cocaine is well known for being a drug of abuse due to its profound pharmacological effects on monoamine transport systems in the CNS and PNS (Riezzo et al., 2012). Specifically, cocaine blocks the dopamine, serotonin, and norepinephrine reuptake transporters found on

presynaptic terminals which result in potentiation of monoamine neurotransmission. The toxicity produced by cocaine and potential for abuse have somewhat limited its applicability to modern medicine, though, it is still used for topical anesthesia (Wildsmith & Armitage, 1987). With regard to anesthetic practice, cocaine was limited by its short duration of action. It was later determined that the benzoic acid ester component of cocaine was the active molecule responsible for producing local anesthesia (Tetzlaff, 2000). Ultimately, due to the limited duration, concern for toxicity, and abuse potential of cocaine, the development of novel ester-based LAs quickly ensued.

Procaine is significant because it was the first successful LA alternative to cocaine that produced less toxicity and irritation to tissues (Dunsky, 1997). This ester-based LA was discovered in 1904 by Alfred Einhorn (1856-1917). Procaine is prepared as a hydrochloride salt in solution and manufactured in different concentration ranges depending on the anesthetic procedure. For example, 1-2% is used for peripheral nerve blocks while 10% is used in combination with dextrose for spinal anesthesia. Procaine is a safer alternative to cocaine because there are no addictive properties, however, due to poor lipid solubility [pK_a, 8.9; (Butterworth & Strichartz, 1990)] there is almost no topical effect. As well, procaine is unstable as it is readily hydrolyzed in an alkaline environment or by direct exposure to sunlight. Procaine was commonly used for infiltration anesthesia of short duration and spinal anesthesia (Aminev, 1973), but now it is limited to infiltration anesthesia and diagnostic purposes (Goodman et al., 2011). The issue with short duration anesthesia produced by ester-based compounds, e.g. procaine, started to become apparent at this juncture in research and clinical practice. Moreover, the downfalls of procaine included a lack of heat stability, short shelf life, and incidences of metabolite-induced toxicity. Although toxicity with procaine is uncommon, it was later discovered that toxicity could be produced with high doses due to an intrinsic ability to inhibit plasma cholinesterases (Tetzlaff, 2000). Overall,

procaine allowed for further enhancement and practice of other techniques that were once limited by cocaine. More importantly, the advancement of LA drug research and subsequent discovery of other ester and amide based LAs would not have occurred without these two notable aminoesters.

1.3.4 The First Aminoamide

The most significant event in the history of LA medicine arguably occurred in 1943 when Nils Löfgren (1913-1967) discovered the aminoamide lidocaine (Arthur & Strichartz, 1987). Lidocaine (Figure 3) is favored among other LA agents because of its predictable nature when used in clinical practice. This tertiary amide is prepared as a hydrochloride salt solution and commonly mixed with epinephrine. Addition of epinephrine allows for an extended duration of action as systemic absorption is decreased; however, this results in a more acidic solution which can be irritating for some patients. Compared to all other amide-type LAs, only lidocaine is achiral (Tetzlaff, 2000). Lidocaine is considered to have a moderate level of lipid solubility making it suitable for intermediate duration anesthesia. Lidocaine is also considered to have an intermediate level of plasma protein binding affinity (~ 60% plasma-bound LA; (de Jong, 1994)). As with all other aminoamides, lidocaine is metabolized primarily in the liver with very little excreted intact in urine. The process of metabolism begins as a series of N-dealkylation steps, followed by hydrolysis of the amide bond, and finally aromatic hydroxylation (Tetzlaff, 2000). Lidocaine is broken down into monoethylglycinexylidide (MEGX), glycinexylidide (GX), and 4-hydroxy-2,6-xylidine. Despite metabolic breakdown, MEGX retains the CVS and CNS actions of lidocaine which has been associated with toxicity (Fukuda et al., 1980). The breakdown of lidocaine to MEGX is mediated by CYP1A2 hepatic enzymes at low concentrations, and CYP3A4 at high concentrations.

Lidocaine is recognized for its clinical efficacy, versatility, and relatively safe toxicity profile. For topical anesthesia, lidocaine is mixed with prilocaine and available as the eutectic mixture of local anesthetics (EMLA) cream. This preparation allows for deep anesthesia of the skin, albeit, onset is rather slow. This is particularly useful during surgical procedures because topical application of lidocaine can reduce the incidence of autonomic responses to intubation of the airway (Prengel et al., 1993). Injection of 0.2-0.5% lidocaine for infiltration anesthesia, achieves rapid anesthesia in cutaneous and subcutaneous tissues that lasts around 60-90 minutes (Langham & Harrison, 1992). Lidocaine can also be used for peripheral and major plexus blocks in the 1-1.5% range. At this concentration, complete sensory and motor anesthesia occurs with rapid onset, and lasts for around 90-120 minutes (McCoy & Wilson, 1991). For epidural anesthesia, lidocaine is capable of producing analgesia alone at 0.5% while at 1-2%, sensory block is complete with some motor blocking effects. Lidocaine is used for spinal anesthesia as well, however, incidences of nerve toxicity have diminished its popularity in clinical practice (cf. below). Another application for lidocaine is intravenous regional anesthesia (IVRA). When given systemically, lidocaine is a potent suppressant of the cough reflex (Poulton & James, 1979). Intravenous lidocaine can also be effective for the management of chronic and acute postoperative pain. However, in most adults, toxicity ensues if the dose of lidocaine, with co-injected epinephrine, exceeds 7-8 mg/kg (Lee et al., 2019). In the presence of acidosis, plasma proteins are decreased and the maximum tolerated dose becomes even smaller (Burney et al., 1978). Studies show that lidocaine increases nociceptive thresholds and suppresses pain transmission in the dorsal horn of the spinal cord, thus making it effective for the treatment of chronic pain (Dohi et al., 1979). Lidocaine is versatile because like many other LAs, with the exception of cocaine, it also produces peripheral vasodilation due to the relaxation of arteriolar smooth muscle (Johns et al.,

1985; Newton et al., 2007). Additionally, lidocaine has inhibitory effects on the repolarization phase of the cardiac action potential. It decreases the incidence of ectopic beats making it an effective anti-arrhythmic agent (Tetzlaff, 2000). To summarize, lidocaine is the most popular local anesthetic in the world and current gold-standard agent to which all clinically useful LAs are compared against.

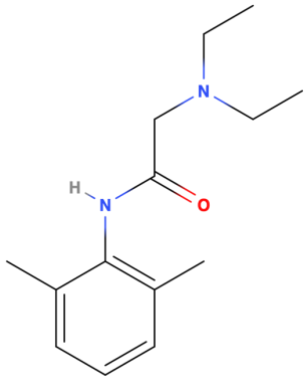


Figure 3 Chemical structure of lidocaine.

1.3.5 Limitations of Clinical Local Anesthetics

1.3.5.1 Duration

Despite its rapid onset, relatively safe toxicity profile, and versatility, lidocaine is not without limitations. Lidocaine is limited by its short duration of effect, potential for toxicity, and relative inability to block pain transmission while leaving motor function unimpeded. For procedures that require intermediate duration anesthesia (60-90 minutes), lidocaine is second to none (Langham & Harrison, 1992; McCoy & Wilson, 1991). The reality of regional anesthesia is that each administration technique has its own rate of onset, duration, and risk of producing systemic toxicity (Winnie et al., 1977). Anatomical features like the thickness of peripheral nerve coverings or extent of blood supply to the area of injection undeniably play a role in affecting the

onset, duration, and toxicity of LAs (Cohen, 1968). Initial efforts to extend duration were to mix LAs with vasoconstrictors. The pharmacological effect of vasoconstrictors limit the extent of systemic absorption leaving higher concentrations of LA available for diffusion across nerve membranes. However, this approach is contraindicated for distal nerve blocks, e.g. in the distal extremities, or for IVRA because it can lead to tissue ischemia (Myers & Heckman, 1989; Wildsmith & Armitage, 1987). Another issue with the use of epinephrine is that it has systemic adrenergic effects that can lead to tachycardia, hypertension, myocardial ischemia and infarction (Pearson et al., 1987). For these reasons, the use of epinephrine to prolong LA duration is associated with limitations.

Another approach for extending duration is the use of high concentrations. Reports of concentrations as high as 5% were initially used for spinal anesthesia. This concentration is no longer used in modern medicine due to toxicity as previously mentioned (Lambert & Hurley, 1991; Rigler et al., 1991; Schell et al., 1991). Investigations revealed that the concentration of lidocaine was responsible for producing neurologic injury known as “transient neurologic symptoms”, or in its most extreme manifestation, cauda equina syndrome, confirming the neurotoxic potential of lidocaine (Hampl et al., 1996; Hashimoto et al., 1998).

A third technique to extend the duration of anesthetic block involves the use of indwelling catheters. Indwelling catheters can maintain analgesia for as long as necessary, however, issues with infection, malposition, or discomfort limit the clinical effectiveness of this approach (Darbyshire et al., 1985). There are also technical issues that relate to human resources, time, and cost associated with these techniques.

From a different perspective, advancements in drug delivery systems using liposomes have been effective at extending the duration of LA agents (Prabhakar et al., 2019). In fact, the use of

liposomes as drug carriers started in the 1950s (Byers & Friedman, 1960). Liposomes are small lipid vesicles that be manufactured to carry many types of drugs (Lian & Ho, 2001; Yamaguchi & Mizushima, 1994). Pharmaceutical formulations using encapsulated liposomes containing LAs allow for a slow release, prolonging the duration of analgesic action and lowering the risk of systemic toxicity. Liposomal bupivacaine was approved for infiltration blocks by the US FDA in 2011 as EXPAREL® (Pacira Pharmaceuticals, Inc., San Diego, California). Since then, the effectiveness of liposomal bupivacaine for prolonged analgesia has been demonstrated in several studies (Gabriel & Ilfeld, 2019; Hutchins et al., 2016; Vandepitte et al., 2017). However, many investigators still question its true effectiveness for the treatment of postoperative pain (Hamilton et al., 2016). Other challenges associated with drug production, high cost, off-target effects, and stability of the liposomal agents highlight the need for LA alternatives. In summary, there exists a compelling and urgent need to identify LA molecules that inherently produce long-lasting blockade (and associated pain relief) after a single injection.

1.3.5.2 Toxicity

All LAs have the potential to cause harm through local and systemic toxicity. Local toxicity refers to damage to tissues that are exposed to LA compounds upon injection (Zink & Graf, 2008). Two tissue types that are most affected by the administration of LAs are muscles and nerves. Sometimes, local tissue toxicity may be the result of poor patient positioning during injection or trauma from the needle, catheter, or procedure (Aitkenhead, 1994). However, all LAs are known myo- and neurotoxic agents (Cox et al., 2003; Radwan et al., 2002; Zink & Graf, 2004). As previously mentioned, spinal injection of lidocaine has been found to produce transient radicular irritation (see previous page), and cauda equina syndrome (Hutter, 1990; Kennedy et al., 1950).

Damage to muscle by all LAs is dose-dependent, with histopathological changes following a distinct pattern of hypercontracted myofibrils, lytic degeneration of the sarcoplasmic reticulum, myocyte edema, and calcified necrosis (Zink & Graf, 2004). In fact, disruption of intracellular calcium is the most damaging to muscle fibres (Zink & Graf, 2008).

LAs can also produce systemic toxicity which is often the result of accidental intravascular injection or systemic absorption following an overdose (Tetzlaff, 2000). If concentrations in the systemic circulation reach toxic thresholds, LAs will inhibit ion channels in the cells of the heart and brain. Importantly, anatomical barriers like the blood-brain barrier help limit absorption into the CNS. The clinical signs of LA-induced systemic toxicity progress as numbness of the tongue, dizziness, tinnitus, acute excitation, slurred speech, followed by drowsiness, loss of consciousness, seizures, cardiorespiratory collapse, and death (Cousins & Bridenbaugh, 1998). In the case of a rapid bolus intravenous injection, the onset of signs can occur within minutes (Di Gregorio et al., 2010). Systemic toxicity is less common for aminoesters due to their rapid metabolism in the periphery (Foldes et al., 1965). That said, most clinically relevant LAs are aminoamides which are metabolized much more slowly. As such, the best way to prevent occurrence of systemic toxicity induced by aminoamides will be through repetitive needle aspiration upon administration, careful incremental administration of the intended dose, ongoing patient monitoring (Mulroy, 2002), and/or development of LA compounds that are not easily absorbed into systemic circulation.

1.3.5.3 Motor Blockade

LAs are used clinically to provide analgesia. This is achieved by interfering with the conduction of action potentials through the blockade of VGSC on peripheral nerves. However, spinal nerve bundles contain not just sensory, but motor and autonomic nerve fibres as well (Figure

4). The anatomical arrangement also varies depending on the particular region on the body. In general, the pattern of onset for nerve block is affected by the arrangement of fibres within the mixed peripheral nerve. An assumption is that motor fibres of the peripheral nerve exist at the core, innervating distal regions, while sensory fibres are at the surface innervating proximal regions (Arthur & Strichartz, 1987; de Jong, 1994). As LAs diffuse into a nerve bundle, they are expected to block core motor fibres last (de Jong, 1994; Winnie et al., 1977). Differential nerve block is the clinical observation that different modalities of nerve function are not blocked at the same rate (Goodman, 1996). This phenomenon depends on many factors like the site of injection, specific agent, and concentration used. Regardless of differential blockade, most LAs, including lidocaine, produce motor as well as sensory block. In some instances, prolonged impairment of motor function may be desirable. However, for postoperative pain management, prolonged motor block is associated with increased recovery time, risk of infection, and other complications related to immobility (De Negri et al., 2004; Stundner et al., 2014).

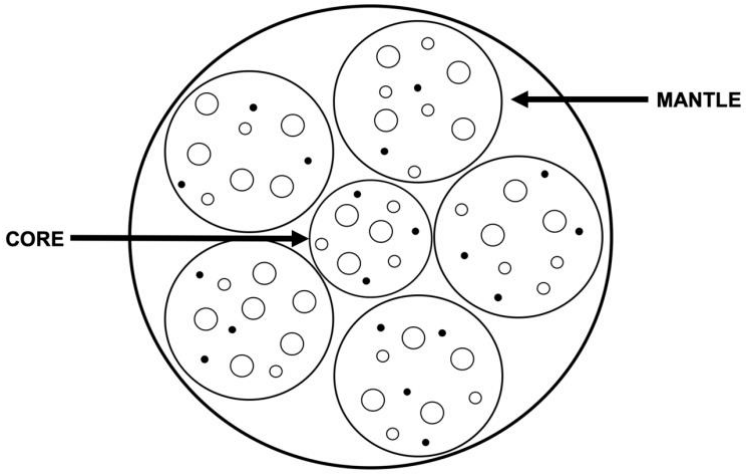


Figure 4 Cross-section of a peripheral nerve. LAs must diffuse across several membranes to reach individual nerve fibres. Nerve fibres which innervate proximal regions are located in the periphery (mantle), whereas fibres which innervate distal regions are situated in the center (core) of peripheral nerves. Individual nerve bundles contain a mixture of sensory, motor, and autonomic nerve fibres depicted above as different circular entities.

1.4 Quaternary Lidocaine Derivatives

1.4.1 Early Applications in Electrophysiology

Besides revolutionizing the practice of local anesthesia, the discovery of lidocaine also expanded the field of electrophysiological research. This field helped to shape our knowledge about the mechanism of action regarding nerve conduction (Hodgkin & Huxley, 1952), and especially, interactions with LA drugs at the molecular level. Notably, two fundamental issues regarding the mechanism of action needed answering. The first was the location of the binding site. After a series of *in vitro* experiments using lidocaine and two tertiary lidocaine derivatives, Narahashi and colleagues (1970) postulated that the binding site was intracellular. To test the proposed hypothesis, Frazier and colleagues (1970) conducted voltage clamp experiments using lidocaine and two quaternary lidocaine derivatives, QX-314 and QX-572. Quaternary compounds are unique because they exist as permanently charged cations, and because of this charge, they are not expected to readily cross biological membranes. Indeed, Frazier and colleagues (1970) found that the quaternary derivatives could only produce sodium channel blockade when applied on the inside of nerve fibres. Investigators were troubled though, by discrepancies in the degree of block produced by tertiary, quaternary, and neutral LAs between voltage clamp experiments. In an effort to resolve these discrepancies, Strichartz (1973) conducted voltage-clamp studies using QX-314 and QX-222 to investigate binding affinities of quaternary agents. The main conclusions from this study were that quaternary agents bind to and dissociate from open, voltage-activated channels and cannot bind to or dissociate from closed channels. Regarding mechanism of action, the second issue that remained unresolved was the way in which LA compounds reached their binding site. To investigate this, a series of voltage-clamp experiments were conducted using tertiary, neutral, and quaternary LA compounds (Hille, 1977b). Results from these experiments led to the

development of the “modulated receptor hypothesis” (Hille, 1977a). This model explains that LAs must first cross the neuronal membrane barrier in order to reach the intracellular binding site. From these historical studies, it is evident that quaternary lidocaine derivatives (Figure 5) were used only as laboratory tools to investigate molecular properties of LAs.

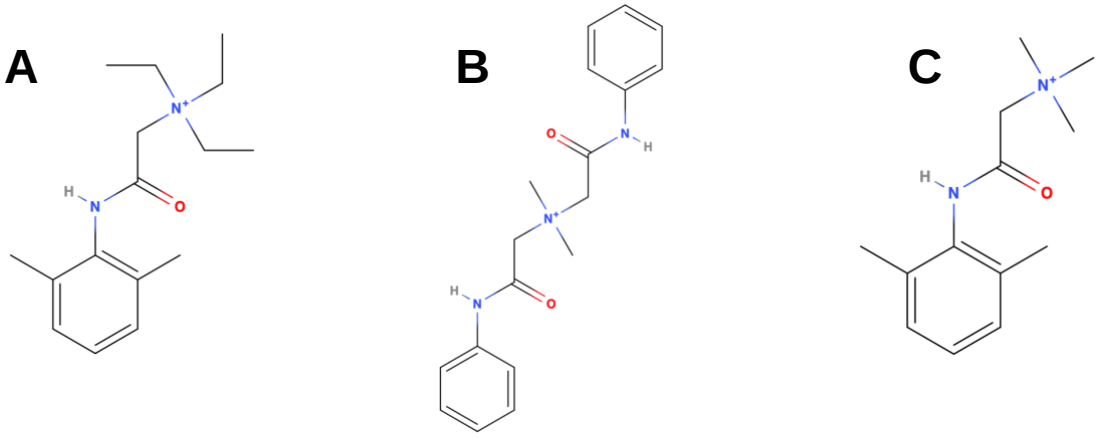


Figure 5 Chemical structures of quaternary lidocaine derivatives.
(A) QX-314, (B) QX-572, and (C) QX-222.

1.4.2 The Emergence of QX-314

The search for the “Holy Grail” in LA medicine, namely, to provide long-lasting analgesia after a single-shot with minimal toxicity, has since led investigators back to the quaternary lidocaine derivative, QX-314. From early electrophysiology studies with quaternary compounds, it was confirmed that the degree of sodium channel blockade produced by QX-314 was greater compared to its tertiary relative (Frazier et al., 1970). However, due to the traditional pharmacological dogma that permanently charged compounds do not readily cross biological membranes, QX-314 was not considered further for clinical use in the peer-reviewed literature.

Not so long ago, the Hugill Anesthesia Research Centre (HARC) demonstrated that local injection of QX-314 alone, contrary to pharmacological dogma, produces robust nociceptive,

sensory, and motor blockade of long duration compared to lidocaine *in vivo* (Lim et al., 2007). Around the same time, an *in vitro* study showed that QX-314 can be selectively introduced into nociceptive neurons to produce long-lasting, nociceptive-specific blockade without loss of motor function (Binshtok et al., 2007). However, application of a painful substance, capsaicin, to induce analgesia made this ill-suited for clinical use. Despite these findings, subsequent research at HARC involving intrathecal (Schwarz et al., 2010) and systemic (Cheung et al., 2011) administration of QX-314 indicated, somewhat surprisingly, that this compound possesses a worse toxicity profile than lidocaine.

1.5 Research Question and Hypotheses

Poorly controlled postoperative pain continues to be a global healthcare problem. The solution may lie with LAs since they are among the best non-opioid alternatives for pain control. However, the issues of short duration, local or systemic toxicity, and undesirable motor blockade remain problematic for current LA agents. Discovery of novel LA compounds that produce long-lasting, nociceptive-specific blockade with minimal local and systemic toxicity would improve the overall practice of regional anesthesia, and hence, postoperative pain management. One topic in LA research that has generated enthusiasm, yet remains relatively unexplored, is the pharmacology of quaternary lidocaine derivatives. QX-314 is one possible solution to achieve longer lasting local anesthesia, but findings associated with systemic toxicity curtail its clinical potential. However, the local anesthetic effects and toxicity profile produced by other quaternary lidocaine derivatives such as QX-572 and QX-222 remain unknown.

This dissertation challenges traditional pharmacologic dogma and considers the possibility that these quaternary compounds also possess local anesthetic effects when applied externally. To

investigate this, a series of *in vivo* laboratory experiments were conducted in mice. The goal of these experiments was to test the primary hypothesis that QX-572 and QX-222, similar to QX-314, will produce long-lasting local anesthesia. To examine the primary supposition, I compared sensory and motor nerve blocking effects of QX-314, QX-572, and QX-222 to lidocaine using the mouse tail flick and sciatic nerve blockade assays, respectively. My specific objectives were to determine the duration and concentration-dependence of sensory and motor blockade produced by quaternary agents.

In efforts to understand the toxicity of quaternary lidocaine derivatives, a series of toxicological studies were also conducted in mice. The goal of these studies was to test the secondary hypothesis that QX-572 and QX-222 have more favorable toxicity profiles relative to QX-314. To examine the secondary supposition, I functionally, macroscopically, and histopathologically assessed concentration-dependent local tissue toxicity produced by quaternary agents and lidocaine. My specific objective was to compare histological changes after acute exposure to QX-314, QX-572, and QX-222 with those of lidocaine. Concurrent with the *in vivo* studies, I assessed general animal behaviors for signs of systemic toxicity. Preliminary findings suggested the possibility of quaternary agent induced systemic toxicity. To investigate this further, development and validation of a novel LC-MS/MS method ensued. Using this LC-MS/MS method, I sought to quantify the plasma concentrations of QX-314, QX-572, QX-222, and lidocaine after peripheral injection in mice.

The results in this dissertation provide new insights into the pharmacological and toxicological actions of quaternary lidocaine derivatives, and further highlight structure-activity relationships of LAs. The hope is that with this knowledge, candidate LA compounds can be created that may in the future be used for improved postoperative pain relief.

Chapter 2: Materials and Methods

2.1 Study Design

All animal experiments conducted in this study were approved by the University of British Columbia Animal Care Committee (Vancouver, BC, Canada) and are reported in accordance with the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines (Kilkenny et al., 2010). I conducted a series of randomized, blinded, and controlled *in vivo* studies using female CD-1 mice. Naïve mice (25-30 g; 6-8 weeks old) were acquired from Charles River Laboratories (Montréal, QC, Canada) and housed in groups of 3-5 at the Animal Resource Unit (University of British Columbia, Vancouver, BC) prior to testing. Mice were housed in 25 ºC at 45-55% relative humidity, under a 12-hour light-dark cycle, and provided access to food and water *ad libitum*. Mice were not administered general anesthesia or analgesic medication before or after experiments, and surgical procedures were not performed in these studies. Animals were used once for an experiment and euthanized at the experimental endpoint. I euthanized the animals using the University of British Columbia Animal Care Committee Standard Operating Procedure: euthanasia of adult rodents using inhalant anesthetic (5% isoflurane) followed by carbon dioxide.

Target group sizes of n = 8 were selected for vehicle control, lidocaine, and each concentration of quaternary lidocaine derivative tested. This group size was selected based on a previous study (Lim et al., 2007) with the ability to detect an effect size difference of 25% with no loss of animals during experimentation, a power of 80% and type I error of 0.05. The online sample size calculator, powerandsamplesize.com/calculators (accessed October 1, 2017) was used. Mice were randomized into experimental groups using an online randomization program, www.random.org (accessed October 1, 2017). Animal matching during group allocation was not

applicable to these studies. Blinding was conducted by laboratory assistants and the investigator was blinded to all test concentrations. Unblinding occurred at the end of all experimental testing and study endpoints. All experiments were conducted with both positive (70 mM [~2%] lidocaine) and negative (0.9% NaCl; normal saline) controls.

2.1.1 Mouse Tail Flick Assay

To assess sensory nerve blockade, I used a modified version of the tail flick assay (Grant et al., 1993). Briefly, mice were placed in a restraining tube (perforated Falcon tube) and given time to acclimatize to the environment. A small segment of the tail (~2 cm) was submerged in hot water (held at 50 ºC) and the time until the animal removed their tail from the noxious stimulus was recorded as the baseline tail flick latency (TFL). The experimental unit was the TFL response of one animal. Only mice with a baseline TFL < 3 s were selected for further testing, all others were excluded from the study. To ensure minimal animal suffering and tissue damage, a maximum hot water exposure time of 10 s was implemented. Experiments were conducted at 07:30 AM in a room separate from animal storage. The total number of mice used for the mouse tail flick assay was 168. In addition to the total number, 8 mice were made available to replace animals that did not fit the inclusion criteria. Experimental groups included: normal saline, 70 mM lidocaine, QX-314 (10, 30, 70, 140, and 280 mM), QX-572 (10, 30, 70, 140, and 280 mM), and QX-222 (10, 30, 70, 140, 280, and 560 mM).

Mice selected for further testing were subjected to a subcutaneous injection of 20 µL of test compound using a 29-gauge hypodermic needle attached to a tuberculin syringe on the left and right side ~0.5 cm from the base of the tail (Figure 6). To ensure accuracy of the administered compound around spinal nerve roots, needle tips were inserted until contact with the vertebrae and

then withdrawn by 1 mm prior to injection. After injection, mice were placed in a restraint and tested once per time point. TFL responses were measured at 0:01, 0:05, 0:15, 0:30, 1, 2, 4, 8, 16, and 32 h or until return to baseline response (max. observation interval, 136 h). The primary outcome (sensory block) was defined as a TFL > 4 s. This cut-off was implemented based on the assumption that 99% of baseline TFL responses of naïve mice would be below 4 s and thus, responses above this cut off would likely represent sensory nerve conduction blockade. This cut-off was calculated by determining TFLs in control mice observed at 3 standard deviations (SD) above the mean baseline response. The secondary outcome was the latency of sensory block onset and offset. Onset of sensory block was defined as the first time point of measurement with consecutive sensory block responses. Offset of sensory block was defined as the first time point with consecutive absence of sensory block responses. General animal behaviours were assessed for adverse drug reactions.

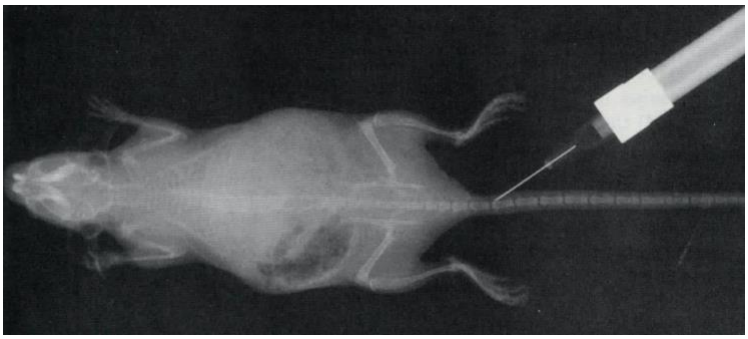


Figure 6 Mouse tail flick assay injection site. Image taken from (Grant et al., 1993).

2.1.2 Mouse Sciatic Nerve Blockade Assay

In this thesis, "motor blockade" refers to "motor function block", and to assess motor blockade, I used the sciatic nerve blockade assay (Leszczynska & Kau, 1992). Prior to testing, mice were given time to habituate to their placement on a steel wire mesh (20 x 25 cm) with

openings (5 x 5 mm). Each animal was centered on the wire mesh and baseline motor function was assessed by briefly inverting the mesh and observing mouse behaviour. Motor function is represented as the ability to grasp onto wire openings with all four limbs and climb to the top surface. Animals that failed to perform this motor task were excluded. Experiments were conducted at 07:30 AM in a room separate from animal storage. The total number of animals used for the sciatic nerve blockade assay was 120. In the event of toxicity or if animals did not meet inclusion criteria, 8 additional mice were made available for replacement. Based on unpublished local and systemic toxicity observations from the previous sensory blockade study, I restricted the range of testable concentrations to reduce the risk of toxicity and animal suffering. Experimental test groups included: normal saline, 70 mM lidocaine, QX-314 (3, 10, 30, and 70 mM), QX-572 (3, 10, and 30 mM), and QX-222 (3, 10, 30, and 70 mM).

Mice selected for further testing were placed in a restraining tube and given time to habituate. The device used for restraint was a perforated Falcon tube to ensure adequate airflow. Using forceps, the experimenter held the left hindlimb. A 30-guage hypodermic needle was inserted into the shallow depression (popliteal space) located at the back of the stifle joint of the hind limb. 50 µL of test compound were injected subcutaneously into the area of the sciatic nerve, posterior of the knee. The above procedure was performed rapidly to minimize animal suffering and tissue damage from needle trauma. After injection, mice were placed back onto the wire mesh and motor function was repeatedly assessed at 0:05, 0:10, 0:15, 0:20, 0:30, 1, 2, 4, 6, 8, 16, and 32 h post-injection or until return to baseline response (max. observation interval, 80 h).

The primary outcome (motor blockade) was defined as the inability to hang on and walk with the injected hind limb (Figure 7). The primary outcome was based on the assumption that local anesthetic activity produces loss of motor function. The secondary outcome was the latency

of motor block onset and offset. Onset of motor block was defined as the first time point of assessment with consecutive motor block responses. Offset of motor block was defined as the first time point with consecutive absence of motor block responses. General animal behaviours were assessed for adverse drug reactions throughout the experiment.

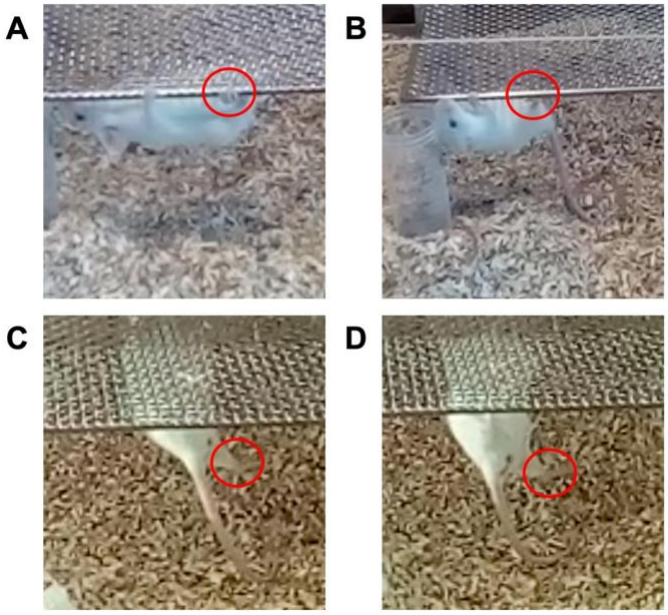


Figure 7 Assessment of motor function in the sciatic nerve blockade assay. (A and B) Naïve mice use all four limbs, including the hindlimb (red circle), to climb to the top surface. (C and D) Motor blockade reflects an inability to hang on and walk with the injected hind limb.

2.2 Assessment of Local and Systemic Toxicity

2.2.1 Histological Analysis of Tail Tissue

Mouse tails were collected from animals immediately after euthanasia. Tails were fixed in 10% formalin solution and decalcified for 48 h in formic acid (TBD-2; acquired from Thermo Fisher Scientific, Calgary, AB, Canada). Three cross-sections of the tail including the site of injection and ~0.5 cm sections both caudal and proximal to the injection site were embedded in paraffin wax for 48 h. Histology slides were stained with industry standard hematoxylin and eosin

and tissue damage was assessed by a pathologist and clinical veterinarian. Light microscopy was used for histological comparison against lidocaine treated tails. Both the pathologist and clinical veterinarian were blinded to all treatments. The tails from mice (n = 2/group) treated with 70 mM QX-222, QX-572, and QX-314 were collected 6 and 24 h following injection. Positive and negative controls included mouse tails treated with 70 mM lidocaine and normal saline, respectively. The time points of tissue collection and tested concentrations were selected to investigate the possibility of histological changes (e.g. myotoxicity and neurotoxicity) following acute compound administration (< 24 h). Histological images were selected to best reflect results of each concentration group at each time point.

2.2.2 Quantitation of Quaternary Lidocaine Derivatives using LC-MS/MS

Based on preliminary toxicity results, and subsequent discussion with toxicologists and veterinarians, I conducted pharmacokinetic studies using LC-MS/MS. With institutional approval, I was given permission to investigate the absorption of quaternary compounds in a small group of animals. This collaborative project involved Drs. Aaron Shapiro and Sergei Likhodi as well as technicians from the Provincial Toxicology Centre at the BC Centre for Disease Control. At the conclusion of this project, the novel LC-MS/MS method "Lidocaine analogues" was developed.

LC-MS/MS quantitation of quaternary compounds was performed using the Agilent 6470A triple quadrupole liquid chromatography mass spectrometer (Agilent Technologies, CA, USA). An Agilent Poroshell 120: EC-C18 column (3 mm x 100 mm, 1.9 μm) was used for chromatographic separation with isocratic elution using 0.1% formic acid (water/acetonitrile; 50:50 v/v) at a flow rate of 0.5 mL/min. The total run time for one injection was 5 min, and the temperature of the column was set at 25 ºC. The injection volume used for analysis was 1.0 μL for

plasma samples. Mass spectrometry conditions were optimized for maximal sensitivity in a positive ionization mode, which was set up with: sheath gas flow rate of 11.0 L/min; sheath gas heater temperature of 375 ºC; nebulizer pressure at 30.0 psi; and, capillary voltage at 3500 V. The multiple-reaction monitoring transitions or precursor ions for each target analyte were detected (mass-to-charge ratio; *m/z*): *m/z* 235.2 → 86 → 58.1 for lidocaine; *m/z* 222.2 → 135 → 58.1 for QX-222; *m/z* 264.2 → 86.1 → 58.1 for QX-314; *m/z* 313.3 → 58.1 → 106 for QX-572; and *m/z* 245.2 → 96.1 → 64.1 for the internal standard (lidocaine-D10, IS). Data were analyzed using the MassHunter computer program (B.09.00 Build 9.0.647.0, Agilent Technologies, CA, USA).

Stock solutions of lidocaine, QX-222, QX-572, and QX-314 (1 mg/mL) and IS (lidocaine D-10, 0.1 mg/mL) were prepared in methanol and refrigerated at 4 ºC. Fresh working solutions were prepared by diluting the stock solutions with acetonitrile in the range of 0.1-0.001 mg/mL prior to each experiment. All procedures including sample preparation and LC-MS/MS analyses were conducted at room temperature (23 ± 1 ºC). Calibration curves were prepared by spiking drug free mice plasma with working solution to produce a set of standards: 30, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0 mg/L. The resulting calibration curve was generated in the range of 0.1-30 mg/L. A quality control sample (5 mg/L) was prepared by technicians at the BC Centre for Disease Control and included in each LC-MS/MS analysis.

Female mice were placed in a restraining tube and given a subcutaneous injection of 20 µL on the left and right side ~0.5 cm from the base of the tail using 29-gauge hypodermic needles as in the tail flick assay (see section 2.1.1). Animals were euthanized 10 min post-injection as previously described (section 2.1). Based on preliminary data from sensory block studies, adverse events developed approximately 15 min post-injection. Thus, the experimental endpoint (10 min) was selected in an attempt to reduce animal suffering. The concentrations selected for quantitative

analysis were (70 mM) lidocaine, (70, and 280 mM) QX-314, (70, and 280 mM) QX-572, and (70, 280, and 560 mM) QX-222. Three additional animals were included in the study due to inconclusive results from excessive sample hemolysis, for a total of 19 mice in these experiments (n = 2/concentration). Animals were randomized into each group, blood samples were collected via intracardiac puncture (~0.8 mL/animal), then transferred to microcentrifuge tubes and centrifugated at 3500 rpm for 10 min. Plasma (~0.4 mL) was collected immediately, labelled and frozen at -15 ºC for subsequent analysis. Mice plasma (50 µL) was pipetted into a microcentrifuge tube for quantitation. Next, 250 µL of IS working solution was added to all calibration standards including the quality control, and to all study samples. Acetonitrile (1 mL) was added to all tubes to deproteinize samples. The mixture was vortexed for 1 min and centrifuged at 25,000 rpm for 5 min. The supernatant formed was transferred into labelled vials for LC-MS/MS analysis.

2.3 Validation of the LC-MS/MS Method: Lidocaine Analogues

2.3.1 Accuracy and Linearity

To determine the accuracy and linearity of the LC-MS/MS method, ten-point calibration curves were prepared for each analyte (Lidocaine, QX-314, QX-572, and QX-222). The linearity ranges for lidocaine, QX-314, QX-572, and QX-222 were (mg/L): 0.00006 to 29.78341, 0.00000 to 30.87915, 0.00000 to 32.03648, and 0.00000 to 30.49245, respectively. The purpose of this validation procedure was to determine if the selected calibration range follows a linear mathematical model within a reasonable level of accuracy. The prespecified allowable systematic error was 0.05 mg/L or 10.0% which is less conservative relative to the industry standard of 15% defined by the US FDA (2018). All data for accuracy and linearity validation were analyzed using EP Evaluator (12.0.0.11, Burlington, VT, USA).

2.3.2 Sensitivity

To determine the sensitivity of the method, replicates of samples containing no analyte (0 mg/L) and the next lowest spiked standard (0.1 mg/L) were analyzed. This data represents the limit of blank. The sensitivity is estimated by calculating the mean and standard deviation of the blank sample. An assumption is made that there is a Gaussian distribution of the analytical signals. The sensitivity of the present method for each analyte is represented as the 95% confidence interval value in an expected blank sample (Armbruster & Pry, 2008). All data for sensitivity validation were analyzed using EP Evaluator (12.0.0.11, Burlington, VT, USA).

2.3.3 Matrix Effects

To quantify quaternary analytes in LC-MS/MS, the mass spectrometer must first ionize the molecule into ions. Suppression or enhancement of ionization results in analyte signals that are not proportional to the set calibrations, further causing errors in quantitation (George et al., 2018). Ionization enhancement arises from matrix-dependent effects which alters the percentage of ions generated from the analyte in the ion source (King et al., 2000). For the present LC-MS/MS method, an electrospray ionization source was used to introduce, evaporate, and ionize the eluent. Factors that affect ionization source efficiency include competition between the analyte, and matrix components. The presence of non-volatile solutes such as salts, endogenous substances, proteins, phospholipids, e.g. in mouse plasma, could cause ionization enhancement or suppression (Annesley, 2003). To validate the present method, I performed matrix effect studies to determine whether mice plasma, the matrix, interferes with detection of analyte signals and generates ionization enhancement or suppression.

The signal responses of spiked samples (0, 5, 10, 15, 20, and 30 mg/L) in drug-free plasma were plotted against concentration and compared to those spiked in water. Linear regression analysis was performed to determine the slopes obtained from best-fit lines in signal responses from plasma and water. A slope ratio greater than one indicates ionization enhancement, and a slope ratio less than one indicates ionization suppression (personal correspondence with Drs. Aaron Shapiro and Sergei Likhodi). The prespecified matrix effect allowance was ± 0.15. To reduce the number of animals, drug-free CD-1 mice blood was collected from spare animals at the UBC Centre of Comparative Medicine. All samples were analyzed in duplicates. Matrix effects data was analyzed using Prism version 8 (GraphPad, San Diego, CA).

2.3.4 Autosampler Stability

Blank samples were prepared from drug-free plasma and kept at room temperature (23 ± 1 ºC) for five days. LC-MS/MS analysis was carried out over three trails to determine autosampler intra-stability at room temperature. Autosampler inter-stability was determined from calculated concentrations of blank samples kept at room temperature after five days. The effect of freezing was determined by comparing calculated concentrations between samples at room temperature and after one freeze-thaw cycle. Autosampler stability data was analyzed using Prism version 8 (GraphPad, San Diego, CA).

2.4 Drugs and Chemicals

Normal saline, prepared as 0.9% NaCl wt./vol. NaCl, and lidocaine HCl were purchased from Sigma-Aldrich/MilliporeSigma (Oakville, ON, Canada). Lidocaine-D10 IS for LC-MS/MS analysis was purchased from Sigma-Aldrich/MilliporeSigma (St. Louis, MO, USA). N-(2,6-

dimethylphenylcarbamoylmethyl) triethylammonium chloride (QX-314) and 2-[(2,6-dimethylphenyl)amino]-N,N,N-trimethyl-2-oxoethaniminium chloride (QX-222) were purchased from Alomone Labs (Jerusalem, Israel). N,N-dimethyl-2-oxo-N-[2-oxo-2-(phenylamino)ethyl]-2-(phenylamino)-ethanaminium chloride (QX-572) was purchased from Toronto Research Chemicals (Toronto, ON, Canada).

Lidocaine, QX-314, QX-572, and QX-222 were dissolved in normal saline at room temperature. Stock solutions of quaternary agents (1 mL) were adjusted to pH 7 ± 0.3 using 1 M $NaHCO_3$. All stock solutions were protected from light degradation, and frozen at -15 ºC. Test solutions were withdrawn 24 h prior to use and then refrigerated at 4 ºC. Before administration, test solutions were equilibrated to room temperature (23 ± 1 ºC). The concentration ranges in the mouse tail flick assay were selected to illustrate an accurate dose-response relationship for each compound. The concentration ranges in the sciatic nerve blockade assay were lowered due to toxicity observed in tail flick experiments, but they were selected to best illustrate the concentration-response relationship for each compound, nonetheless.

2.5 Statistical Analyses

Behavioural responses recorded during the mouse tail flick assay are presented in this dissertation as mean log[TFL] ± 95% confidence interval (CI) for each concentration. To determine normality of baseline TFL data, the D'Agostino & Pearson test was used. A $\log_{10}$ transformation was applied to continuous time-to-event data and the primary outcome (tail flick latency) based on the assumption that sensory perception follows the Weber-Fechner Law (Reichl et al., 2010).

To determine differences in the baseline log[TFL] responses of mice, analysis was conducted using one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. To determine concentration-dependent effects on the duration of sensory block, analysis of log[TFL] responses was conducted using two-way ANOVA with Geisser-Greenhouse correction for repeated-measures followed by Dunnett's *post-hoc* multiple comparisons test (compared to 70 mM lidocaine). To determine differences in the onset and offset times of sensory blockade, continuous log[TFL] responses are treated as quantal responses (presence or absence of sensory block) and presented as "survival curves". Statistical analyses of the onset and offset times were performed with Kaplan-Meier survival analyses using the log-rank test (compared to 70 mM lidocaine). Potency values (ED50) were determined from dose-response relationships fitted using non-linear regression analysis (equation for log[agonist] vs normalized response: y = 100/(1+10^(logED50-x)) where ED50 is the dose of agonist that gives a 50% response in the population of animals).

The dichotomous behavioural response, presence or absence of motor blockade, observed during the sciatic nerve blockade assay is presented in this dissertation as the percentage of mice with motor block for each concentration. To determine differences in quaternary compound efficacy, categorical data were analyzed using Fisher's exact test (compared to 70 mM lidocaine) and Chi-square test for trend. To determine differences in the latency of motor block onset and offset, quantal responses (presence or absence of motor block) are presented in this dissertation as "survival curves". Statistical analysis of onset and offset survival curves were done with Kaplan-Meier survival analysis using the log-rank test (compared to 70 mM lidocaine). To determine concentration-dependent effects on the duration of motor block, non-gaussian recovery times were analyzed using the Kruskal-Wallis test with Dunn's *post-hoc* multiple comparisons test (compared

to normal saline). Recovery times were analyzed further using the unpaired Mann-Whitney test to determine differences between quaternary derivatives and 70 mM lidocaine. Potency values (EC50) were determined from concentration-response relationships fitted using non-linear regression analysis (equation for log[agonist] vs normalized response: y = 100/(1+10^(logEC50-x)) where EC50 is the concentration of agonist that gives a 50% response in the population of mice).

All data are presented as a sample size of n = 8, unless stated otherwise. Statistical tests were two-tailed and differences among groups were considered significant at $P < 0.05$, except for Mann-Whitney analyses wherein differences were considered significant at $P < 0.01$. Data were analyzed using Prism version 8 (GraphPad, San Diego, CA) unless stated otherwise.

Chapter 3: Results

3.1 Long-Lasting Sensory Blockade Produced by Quaternary Lidocaine Derivatives

3.1.1 Baseline Mouse Tail Flick Responses

There were no differences in baseline log[TFL] responses of naïve mice between experimental groups (Figure 8 A). The mean log[TFL] of naïve mice in experimental groups of QX-314, QX-572, and QX-222 were 0.08 (95% CI, 0.03 to 0.13), 0.08 (0.01 to 0.14), and 0.05 (0.01 to 0.10), respectively (one-way ANOVA; P = 0.68). The D'Agostino & Pearson test was used to assess whether the data come from a Gaussian distribution. A normal distribution of TFLs was not observed in QQ Plots of the continuous data; however, a $\log_{10}$ transformation of the continuous data confirmed a linear lognormal distribution (Figure 8 B). Continuous TFL responses were treated as the presence or absence of sensory block. I attributed sensory blockade with TFL responses > 4 s. From the baseline data, mean TFL in s [SD] of QX-314, QX-572, and QX-222 were 1.30 [0.56], 1.35 [0.64], and 1.21 [0.50], respectively. The cut-off value (4 s) was selected because it represented TFL responses that would be greater than 3 SDs from baseline responses.

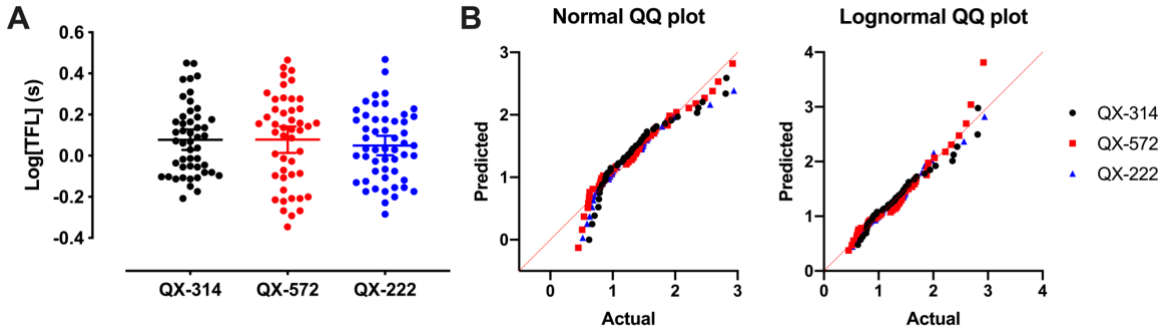


Figure 8 Baseline tail flick latencies of mice prior to QX compound injection. (A) There were no differences between baseline log[TFL] responses of mice (n = 147; one-way ANOVA; P = 0.68). Data presented with mean ± 95% CI. (B) QQ Plot showing TFL data does not fit a linear normal distribution (left). A logarithmic transformation of the data produces a linear lognormal distribution (right).

3.1.2 Dose-Response Relationships

To compare potencies, dose-response curves were constructed for QX-314, QX-572, and QX-222 in the mouse tail flick assay (Figure 9). Dose-response relationships were constructed based on observations of death, and the fact that LA-induced systemic toxicity –due to systemic absorption or intravascular injection– correlates with weight-based dose. Test concentrations were converted to dosages based on a 30 g mouse receiving 40 µL of injected solution. As shown in Figure 9 A, QX-314 had an estimated ED50 of 14 mg/kg (95% CI, 2 to 58) and an LD50 of 136 mg/kg (95% CI, 53 to 581). From the dose-response curve for QX-572 (Figure 9 B), the estimated ED50 was 9 mg/kg (95% CI, 5 to 15). An additional test concentration (560 mM) was added in order to determine the maximum efficacy of QX-222. The relative potency of QX-222 determined from the dose-response curve (Figure 9 C) was 42 mg/kg (95% CI, 17 to 99) with an LD50 of 936 mg/kg (95% CI, 512 to 2859). One animal in each of 30 and 280 mM (QX-314) and 280 mM (QX-572) groups was excluded from statistical analysis because the animal did not meet inclusion criteria (total number of animals excluded, n = 3). For ease of comparison, the EC50s of QX-314, QX-572, and QX-222 were: 35 mM (95% CI, 4 to 144), 18 mM (95% CI, 10 to 31), and 122 mM (95% CI, 50 to 282), respectively. In summary, the order of potency for sensory blockade was QX-572 > QX-314 > QX-222.

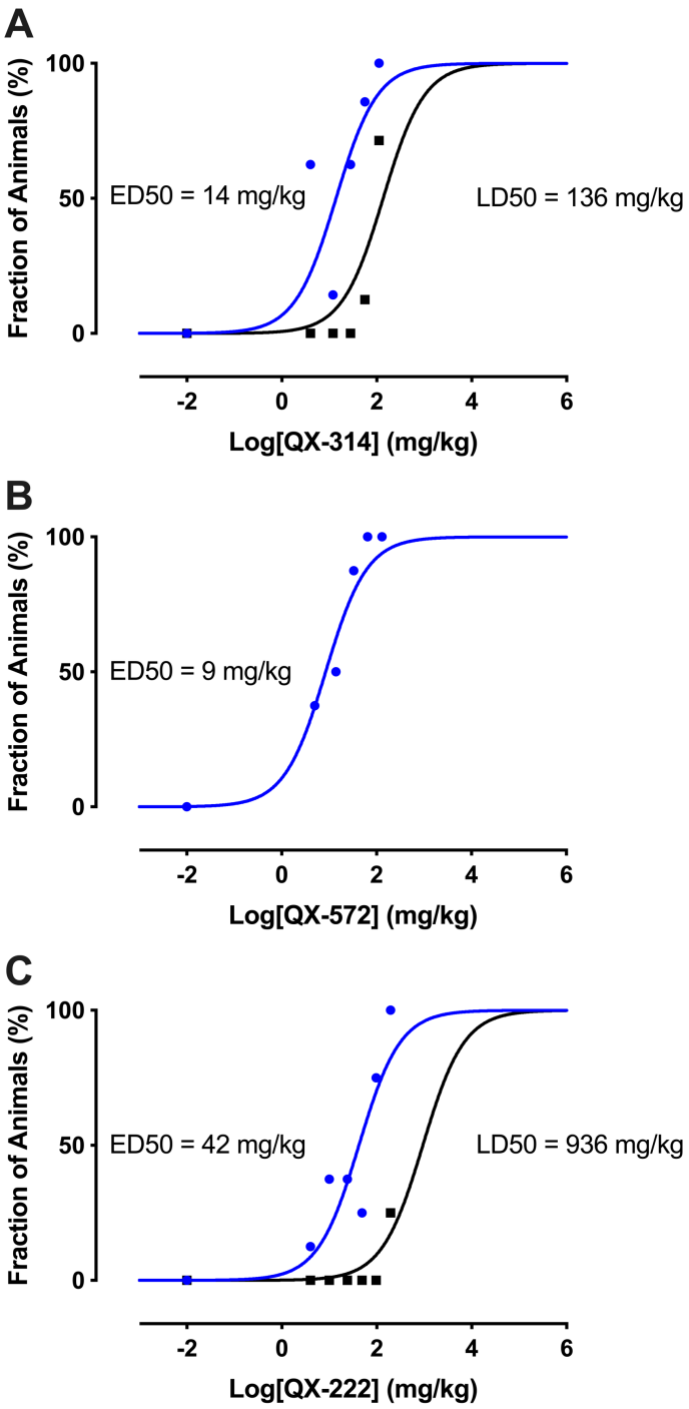


Figure 9 Dose-response relationships for sensory blockade. Dose-response curves for (A) QX-314 [$F_{(1,4)} = 0.27$; $P = 0.63$; $r^2 = 0.64$], (B) QX-572 [$F_{(1,4)} = 1.33$; $P = 0.31$; $r^2 = 0.95$], and (C) QX-222 [$F_{(1,5)} = 0.02$; $P = 0.88$; $r^2 = 0.79$]. Curves were fitted using non-linear regression analysis. Data points represent the fraction of animals/group with sensory blockade (blue) or that died (black). All groups, n = 8; except 30 and 280 mM QX-314, and 280 mM QX-572 (n = 7).

3.1.3 Onset of Sensory Blockade

Sensory blockade produced by the quaternary lidocaine derivatives, compared to 70 mM lidocaine, was associated with a delay to onset (Figure 10). The log-rank test indicated concentration-dependent differences between onset survival curves of QX-314 ($P < 0.001$), QX-572 ($P < 0.001$), QX-222 ($P < 0.001$) and 70 mM lidocaine. Saline treated mice in the QX-314 and QX-572 experiments did not indicate sensory block onset defined as two consecutive TFL responses > 4 s. However, sensory block onset was detected within 15 min of saline injection in 6 of 12 mice from experiments with QX-222. With 70 mM lidocaine, sensory block onset was observed within 5 min of injection in 7 of 8 animals. As demonstrated previously with QX-314 (Lim et al., 2007), QX-572 and QX-222 also produced sensory blockade with a delay to onset compared to lidocaine.

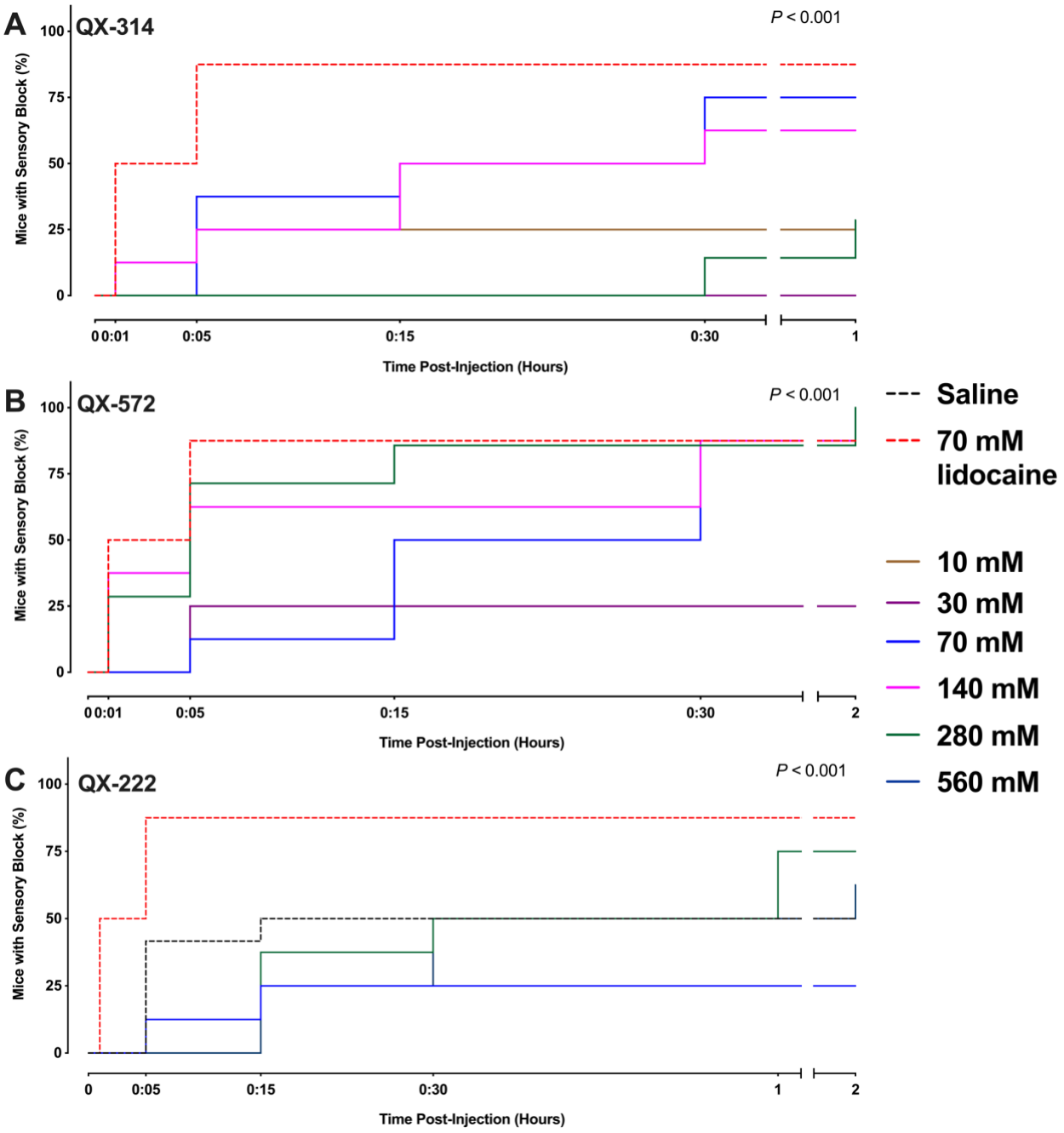


Figure 10 Onset of sensory blockade. Kaplan-Meier "survival curves" depicting the concentration-dependent slow onset of sensory block produced by (A) QX-314, (B) QX-572, and (C) QX-222. All *P* values were calculated using the log-rank test. Onset was defined as the first time point with two consecutive sensory block measurements. All groups, n = 8; except 30, 280 mM QX-314, 280 mM QX-572 (n = 7), and saline control group in QX-222 (n = 12).

3.1.4 Recovery and Irreversible Nerve Blockade

Recovery from sensory blockade due to QX-314 and QX-222 was reversible, in contrast to QX-572, which produced irreversible blockade (Figure 11). The log-rank test indicated concentration-dependent differences between offset survival curves of QX-314 ($P < 0.001$), QX-572 ($P < 0.001$), QX-222 ($P = 0.007$) and 70 mM lidocaine. All animals that displayed sensory nerve blockade due to QX-314 recovered during the course of the experiment (Figure 11 A). In contrast, 5 out of 8 animals that received 140 mM, and 3 out of 7 animals that received 280 mM QX-572 (Figure 11 B) did not recover from sensory nerve blockade [max. observation interval, 136 h]. With QX-222, 1 out of 8 animals that received 560 mM (Figure 11 C) did not recover from sensory nerve blockade, however, the last time point of assessment suggested that recovery was imminent [max. observation interval, 32 h]. Saline treated mice in the experiments with QX-314 and QX-572 were not included for analyses as sensory block onset was not detected. The offset of sensory block in 6 of 12 mice injected with saline in experiments with QX-222 occurred within 1 h. Full recovery from sensory blockade produced by lidocaine was observed in all animals. Unlike QX-314 and QX-222, QX-572 produced irreversible sensory blockade in the mouse tail flick assay.

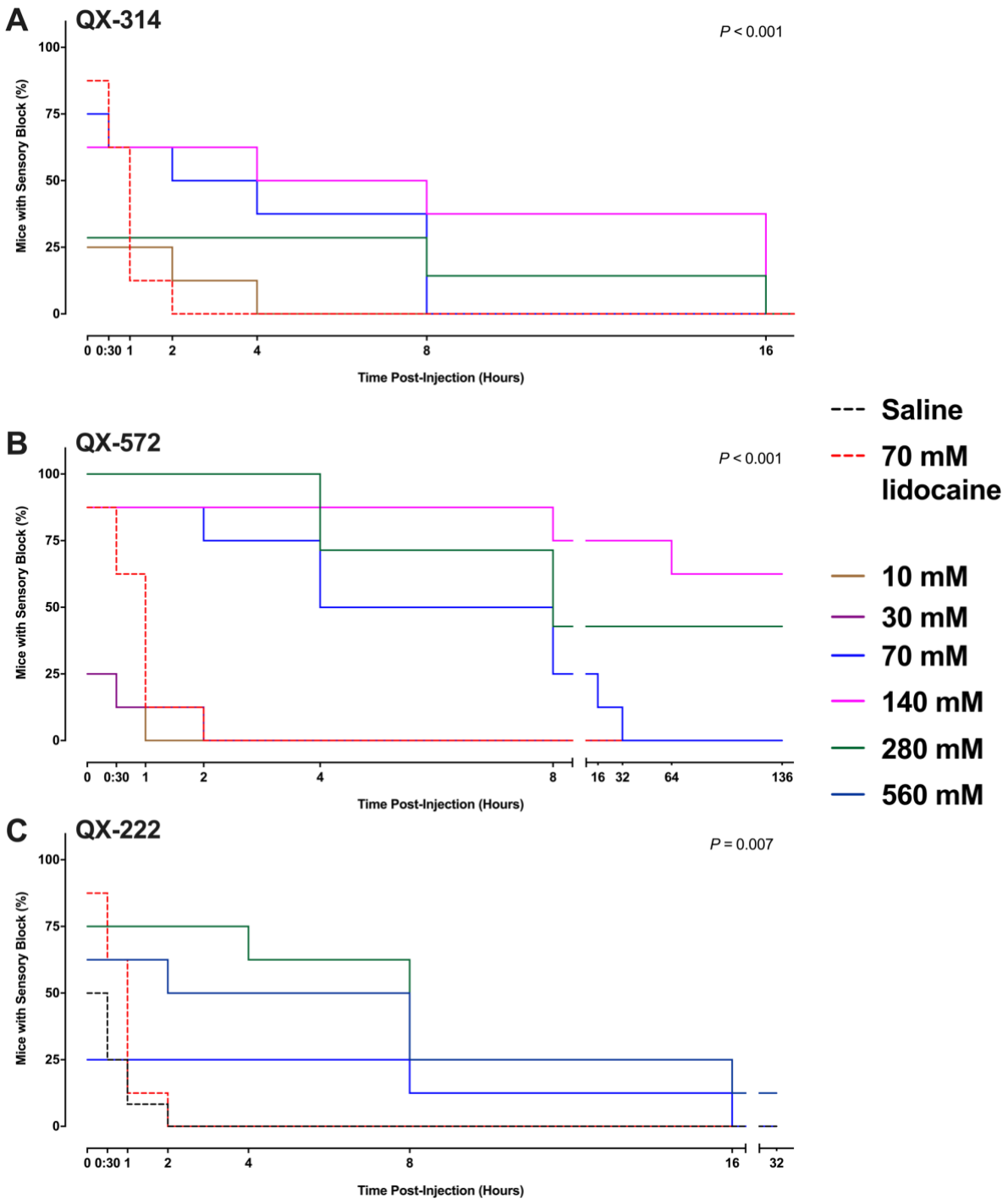


Figure 11 Offset of sensory blockade. Kaplan-Meier "survival curves" depicting the concentration-dependent slow offset of sensory block produced by (A) QX-314, (B) QX-572, and (C) QX-222. All *P* values were calculated using the log-rank test. Offset was defined as the first time point with two consecutive absence of sensory block measurements. See Figure 10 for group sizes.

3.1.5 Sensory Blockade Duration

QX-314, QX-572, and QX-222 concentration-dependently produced longer lasting sensory blockade compared to 70 mM lidocaine (Figure 12). Lidocaine produced sensory blockade that lasted for approximately 1 h post-injection. Figure 12 A shows the duration of sensory block produced by QX-314. Two-way repeated measures ANOVA of log[TFL] as a function of treatment and time confirmed differences in log[TFL] between QX-314 and lidocaine treated groups (Treatment: $P = 0.003$; Time: $P < 0.001$; Interaction: $P < 0.001$). I found that at 140 mM, QX-314 produced sensory blockade up to 8x longer than lidocaine (Difference in means, -0.320; 95% CI, -0.637 to -0.003; $P < 0.05$). Figure 12 B shows the duration of sensory block produced by QX-572. Two-way repeated measures ANOVA also confirmed differences in log[TFL] between QX-572 and lidocaine treatment groups (Treatment: $P < 0.001$; Time: $P < 0.001$; Interaction: $P < 0.001$). Notably, the duration of blockade due to QX-572 was twice that of lidocaine at 70 mM; higher concentrations produced irreversible blockade [max. observation interval, 136 h] (Difference in means, -0.655; 95% CI, -1.035 to -0.275; $P = 0.002$). Figure 12 C depicts the duration of sensory block produced by QX-222. Two-way repeated measures ANOVA confirmed differences in log[TFL] between QX-222 and lidocaine treated groups (Treatment: $P = 0.001$; Time: $P < 0.001$; Interaction: $P < 0.001$). A larger saline group size (n = 12) was required for blinding purposes as an additional test concentration was added (560 mM). I found that QX-222 at 560 mM produced sensory blockade up to 8x longer than lidocaine (Difference in means, -0.544; 95% CI, -1.042 to -0.045; $P = 0.03$). In summary, all quaternary compounds concentration-dependently produced long-lasting sensory blockade compared to lidocaine.

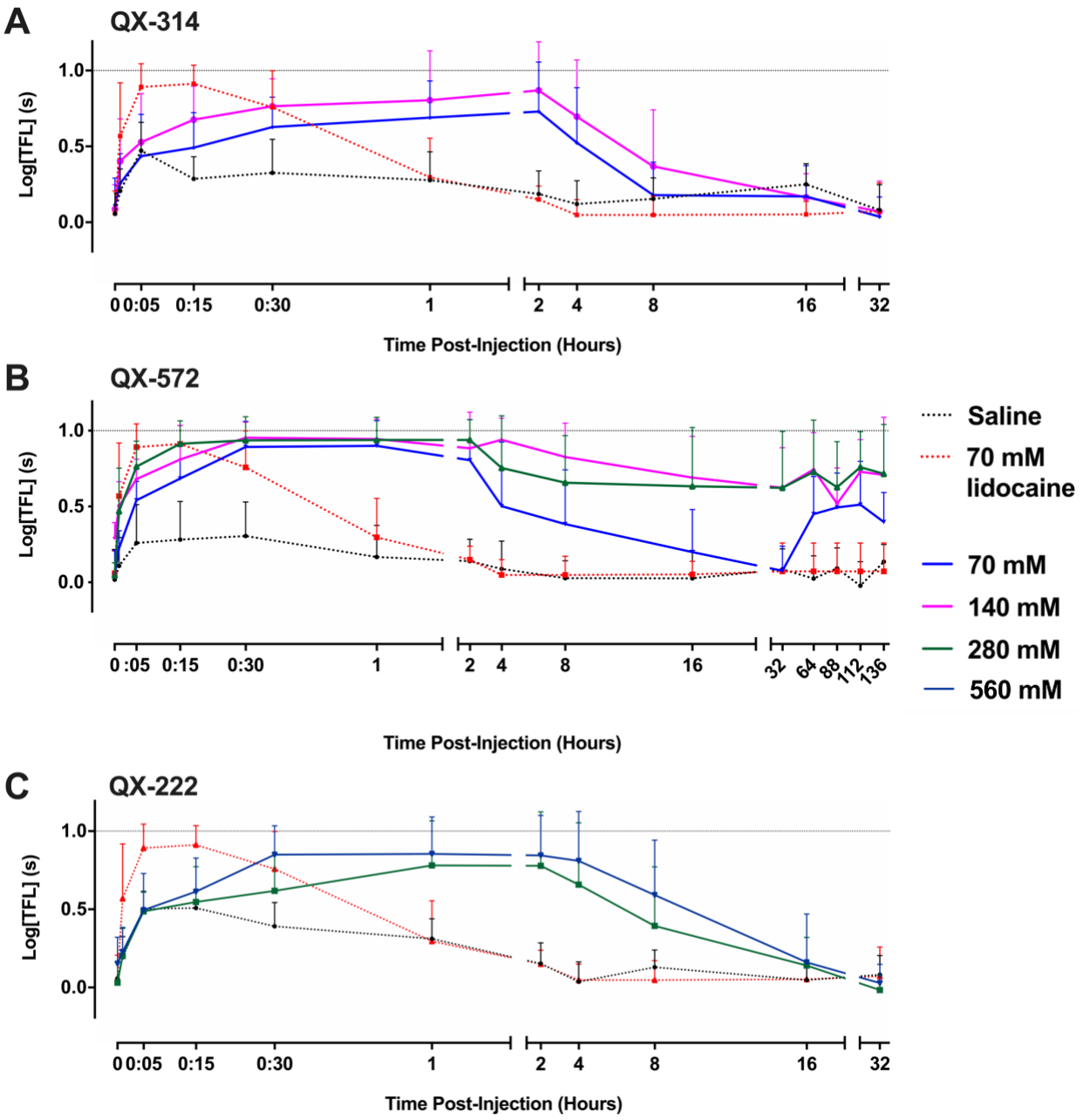


Figure 12 Long-lasting sensory blockade produced by quaternary lidocaine derivatives. Compared to 70 mM [~2%] lidocaine, sensory blockade produced by (A) QX-314 was up to 8x as long at 140 mM ($P < 0.05$) (B) QX-572 was up to twice as long at 70 mM ($P = 0.002$) and (C) QX-222 was up to 8x as long at 560 mM ($P = 0.03$). Group responses are presented as mean ± 95% CI. All groups, n = 8; except 30, 280 mM QX-314, 280 mM QX-572 (n = 7), and saline control group in QX-222 (n = 12).

3.2 Motor Blockade Produced by Quaternary Lidocaine Derivatives

3.2.1 Concentration-Response Relationships

Chi-square test for trend confirmed that QX-314, QX-572, and QX-222 concentration-dependently produced motor blockade in the mouse sciatic nerve blockade assay (Table 1). All mice (8 of 8) that were given 30 and 70 mM QX-314 were unable to use the treated left hind limb. Fisher's exact test confirmed that QX-314 produced motor blockade with similar efficacy (at ≥ 3 mM) compared to 70 mM lidocaine (P = 0.12). At 30 mM QX-572, 7 of 8 mice were unable to use the treated hind limb. QX-572 produced motor blockade with similar efficacy (at ≥ 3 mM) compared to 70 mM lidocaine (Fisher's exact test; P = 0.12). In contrast, only 70 mM QX-222 produced motor blockade comparable to lidocaine (Fisher's exact test; P = 0.28). At this concentration, 4 of 8 mice displayed motor blockade. Out of 8 mice that received 70 mM lidocaine, 7 were unable to use the treated hind limb to hang on to and walk along an inverted wire mesh.

Table 1 Sciatic nerve motor blockade in mice

Treatment	Concentration (mM)	Animals with Motor Blockade (%)	P value (Fisher's exact test)	P value (χ_2 test for trend)
Normal saline	0	1/24 (4%)	< 0.001	-
Lidocaine	70	7/8 (88%)	-	-
QX-314	3	3/8 (38%)	0.12	< 0.001
	10	3/8 (38%)	0.12	
	30	8/8 (100%)	0.99	
	70	8/8 (100%)	0.99	
QX-572	3	3/8 (38%)	0.12	0.04
	10	5/8 (63%)	0.57	
	30	7/8 (88%)	0.99	
QX-222	3	0/8 (0%)	< 0.01	0.01
	10	1/8 (13%)	0.01	
	30	2/8 (25%)	0.04	
	70	4/8 (50%)	0.28	

P values are from Fisher's exact test (compared to 70 mM lidocaine) or Chi-square test for trend.

To compare potencies among the quaternary derivatives, concentration-response curves were constructed for QX-314, QX-572, and QX-222 in the mouse sciatic nerve blockade assay (Figure 13). As shown in Figure 13 A, QX-314 had an estimated EC50 of 7 mM (95% CI, 2 to 20). From the concentration-response curve for QX-572 (Figure 13 B), the estimated EC50 was 4 mM (95% CI, 2 to 8). The relative potency of QX-222 determined from the concentration-response curve (Figure 13 C) was 77 mM (95% CI, 62 to 97). In summary, all quaternary agents concentration-dependently produced motor blockade in the mouse sciatic nerve blockade model, and the order of potency was QX-572 > QX-314 > QX-222.

The time courses of motor blockade are depicted in the form of group survival curves in Figure 14. For these studies, one animal from 70 mM QX-222 and two animals from 30 mM QX-314 groups were replaced due to toxicity and immediate death associated with inadvertent intravascular injection (total number of animals replaced, n = 3). At the concentrations tested in these studies, I observed no signs of macroscopic local tissue injury. Hence, further histological analyses of hind limb tissues were not performed.

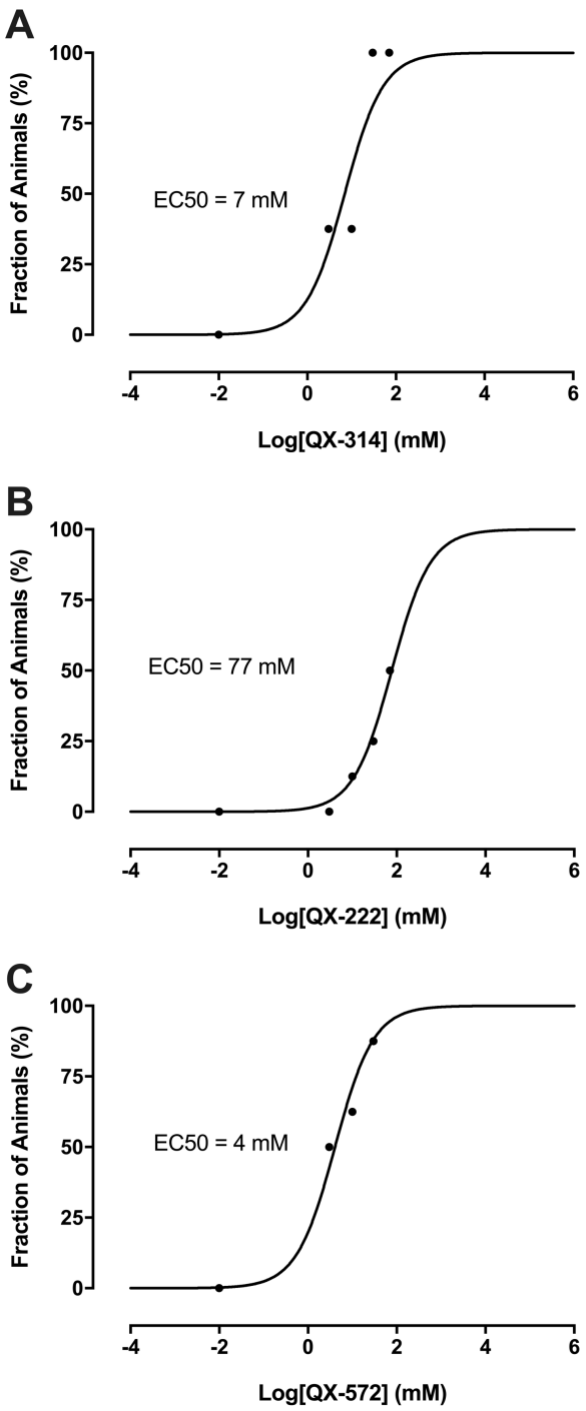


Figure 13 Concentration-response relationships for motor blockade. Concentration-response curves for (A) QX-314 [$F_{(1,3)} = 0.20$; $P = 0.69$; $r^2 = 0.69$], (B) QX-222 [$F_{(1,3)} = 1.59$; $P = 0.31$; $r^2 = 0.98$], and (C) QX-572 [$F_{(1,2)} = 2.00$; $P = 0.29$; $r^2 = 0.97$]. Curves were fitted using non-linear regression analysis. Data points represent the fraction of animals/group with motor blockade (black). All groups, n = 8.

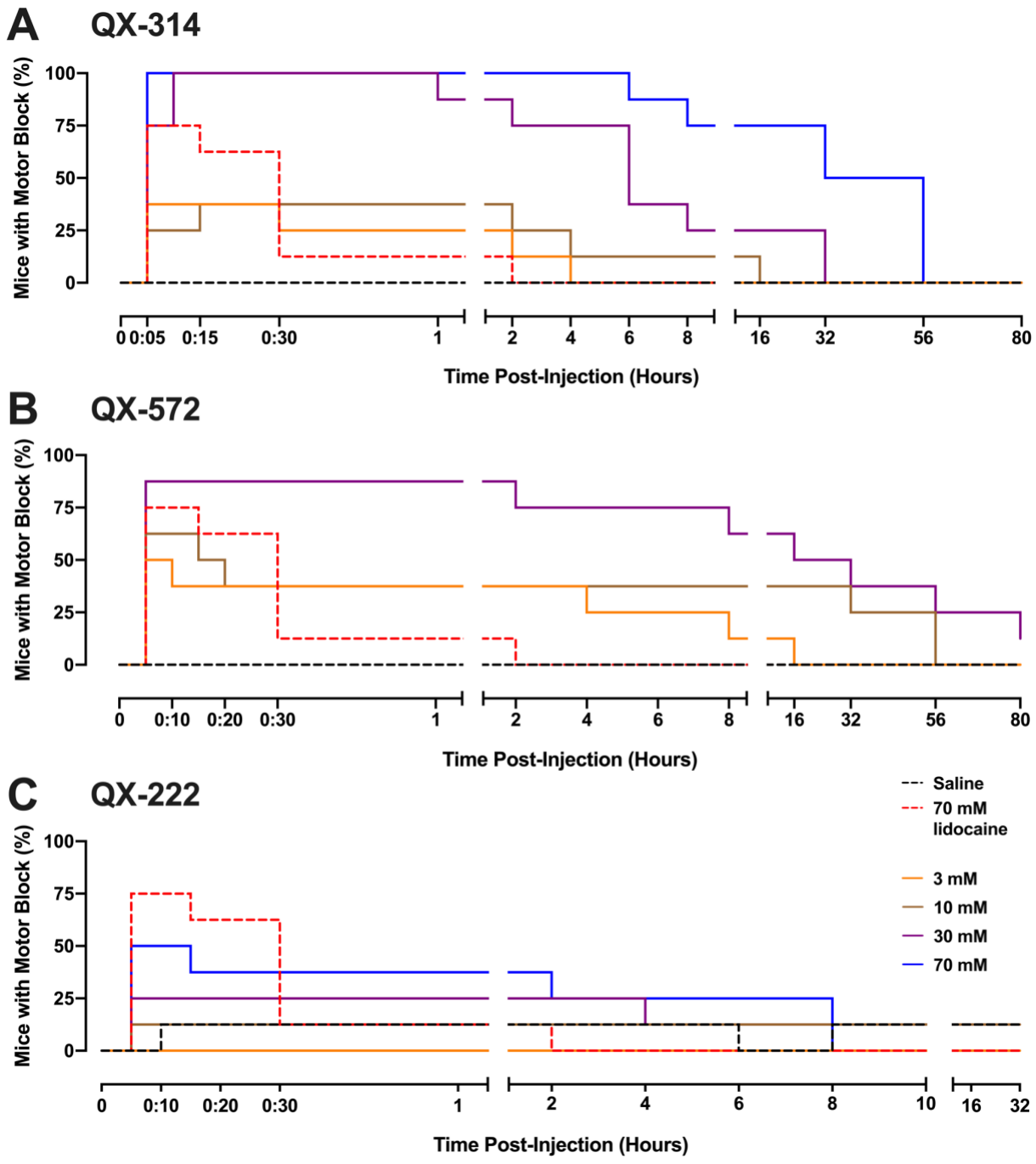


Figure 14 Time course of motor blockade. (A) QX-314, (B) QX-572, and (C) QX-222 concentration-dependently produce motor blockade in the mouse sciatic nerve assay. The fraction of animals with motor block responses are presented as Kaplan-Meier "survival curves". All groups, n = 8.

3.2.2 Onset of Motor Blockade

QX-572 and QX-222 produced motor blockade with rapid onset similar to lidocaine (Figure 15). In contrast, QX-314 produced motor blockade with a concentration-dependent delay to onset. The onset of motor block was defined as the first time point with consecutive motor block responses. As illustrated in Figure 15 A, all animals lost the ability to use the injected left hind limb within 10 min of injection with QX-314 (≥ 30 mM). In the 10 mM QX-314 group, there was an observed delay to onset. The log-rank test confirmed concentration-dependent differences between onset survival curves of QX-314 ($P < 0.001$) and 70 mM lidocaine. At all concentrations of QX-572 (Figure 15 B) and QX-222 (Figure 15 C), motor blockade occurred within 5 min of injection. The log-rank test found no difference between survival curves of QX-572 and 70 mM lidocaine ($P = 0.11$). The log-rank test detected differences between QX-222 and lidocaine survival curves ($P = 0.001$), however, caution must be taken when interpreting the result as only the 70 mM test group QX-222 was effective at producing motor blockade. Lidocaine rapidly inhibited motor function in 7 of 8 animals within 15 min of injection. Overall, motor block onset produced by QX-572 and QX-222 were similar to lidocaine at all tested concentrations.

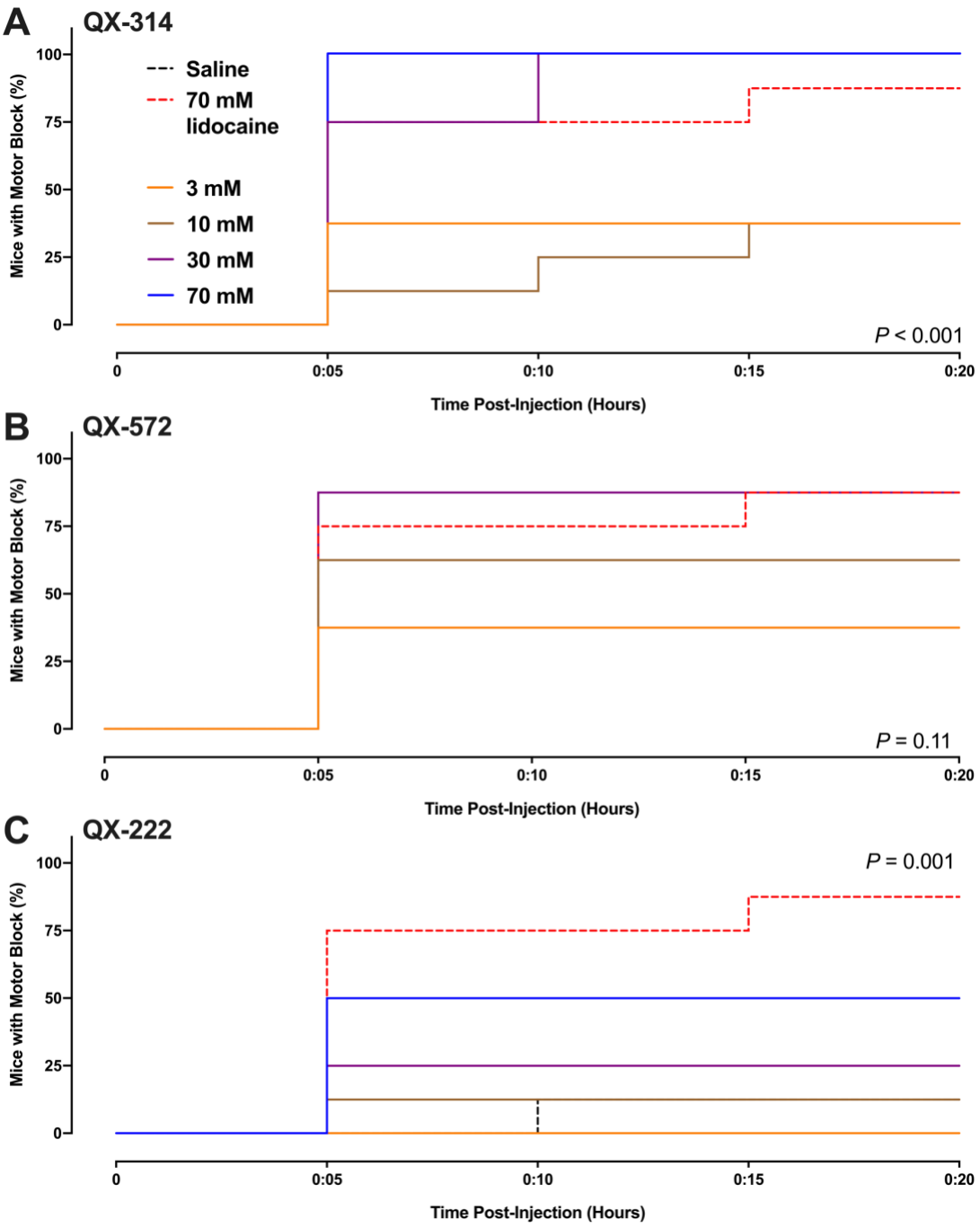


Figure 15 Onset of motor blockade. The fraction of animals with motor blockade over time are presented as Kaplan-Meier "survival curves". Onset was defined as the first time point with two consecutive motor block measurements. Log-rank survival analysis shows that (A) QX-314 produces concentration-dependent delay to motor block onset ($P < 0.001$). (B) QX-572 ($P = 0.11$) and (C) QX-222 ($P = 0.001$) produce motor blockade with similar onset to lidocaine. All groups, n = 8.

3.2.3 Recovery and Irreversible Nerve Blockade

All quaternary lidocaine derivatives produced motor blockade with a concentration-dependent delay to offset (Figure 16). The log-rank test confirmed concentration-dependent differences between offset survival curves of QX-314 ($P < 0.001$), QX-572 ($P = 0.01$), QX-222 ($P < 0.001$) and 70 mM lidocaine. Figure 16 A and B show delayed recovery from motor blockade produced by increasing concentrations of QX-314 and QX-572. Figure 16 C shows delayed recovery produced by 70 mM QX-222. All mice (7 of 8) that displayed motor blockade in the 70 mM lidocaine group regained motor function within 2 h of injection. With QX-572, 2 of 8 animals did not recover in the 30 mM concentration group [max. observation interval, 80 h]. Irreversible motor blockade was also observed in 1 animal from the 10 and 30 mM QX-222 treatment groups [max. observation interval, 32 h]. An unexpected finding occurred where 1 animal treated with saline showed signs of motor blockade and did not recover [max. observation interval, 32 h]. The offset of motor block produced by QX-314, QX-572, and QX-222 was delayed with increasing concentrations.

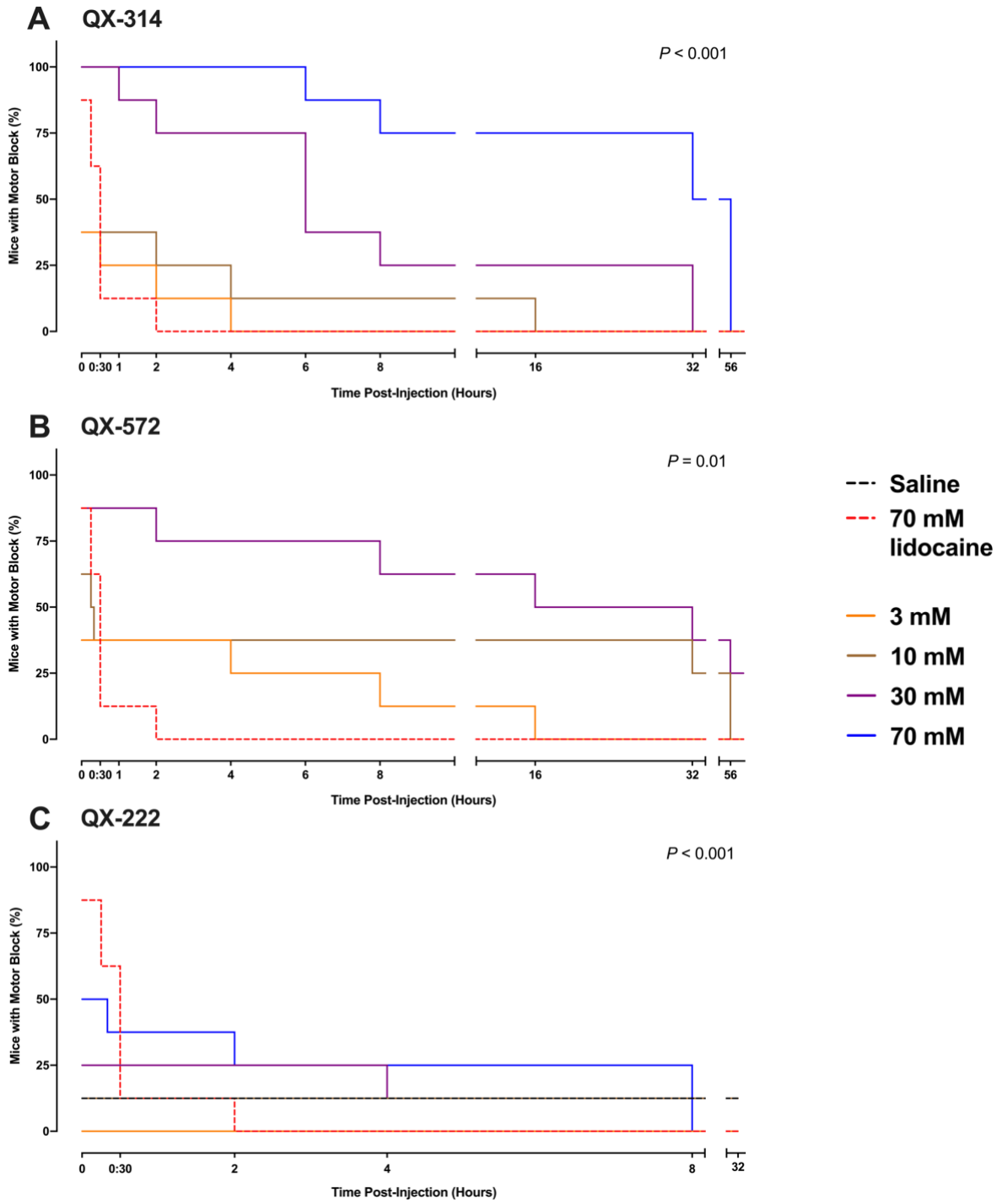


Figure 16 Offset of motor blockade. The fraction of animals with motor blockade over time are presented as Kaplan-Meier "survival curves". Offset was defined as the first time point with two consecutive absence of motor block measurements. Log-rank analysis confirms concentration-dependent differences between offset survival curves of QX-314 ($P < 0.001$), QX-572 ($P = 0.01$), QX-222 ($P < 0.001$) and 70 mM lidocaine. All groups, n = 8.

3.2.4 Motor Blockade Duration

QX-314 and QX-572, but not QX-222, produced motor blockade of long duration compared to lidocaine (Figure 17). At equimolar concentrations (70 mM), the median [IQR] duration of motor blockade produced by QX-314 was 44 h [14-56 h] compared to 0.5 h [0.25-0.5 h] with lidocaine ($P < 0.001$). No significant differences were detected between the duration of motor blockade at lower concentrations of QX-314 and lidocaine. QX-572 also produced longer-lasting motor blockade compared to lidocaine; at 30 mM, the median duration was 24 h [3.5-74 h] ($P = 0.008$). In contrast, QX-222 produced motor blockade of durations not longer than lidocaine, with a median duration at 70 mM of 10 min [0-390 min] ($P = 0.71$). It should be pointed out that this was the only concentration of QX-222 that was effective at producing motor blockade. For all comparisons regarding duration, mice that did not recover were assigned the last time point of assessment. In summary, unlike QX-314 and QX-572, QX-222 did not produce longer lasting motor blockade compared to lidocaine.

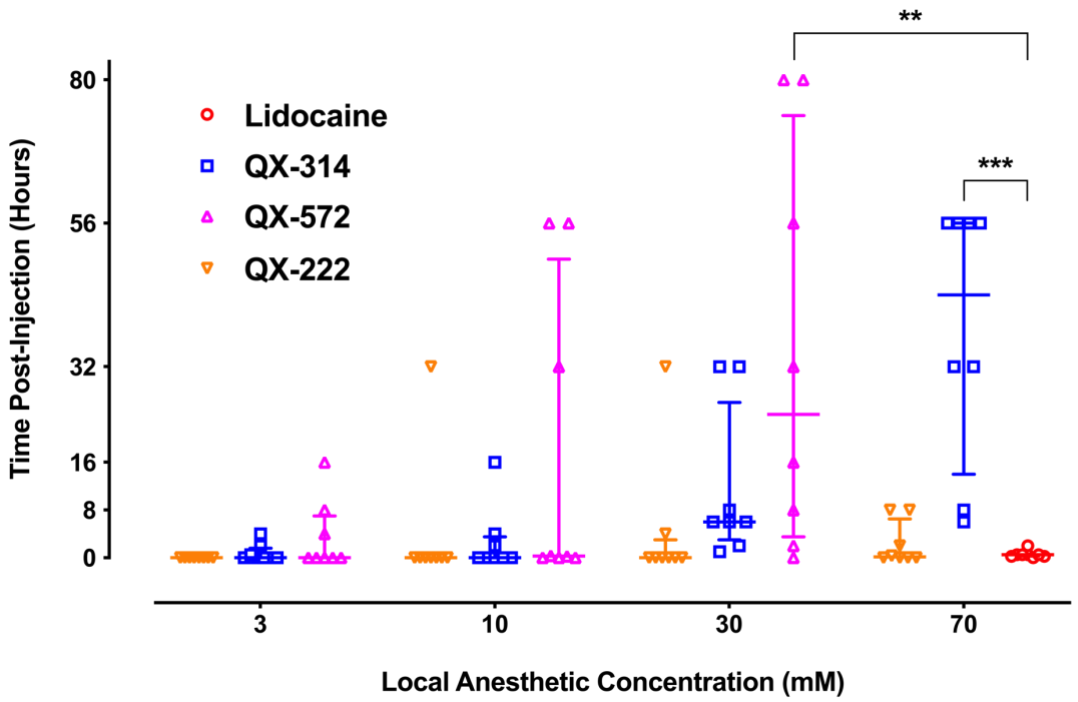


Figure 17 Duration of motor blockade produced by quaternary lidocaine derivatives. The median motor block duration of QX-314 at 70 mM was up to 88x longer than lidocaine (Mann-Whitney test; $P < 0.001$). QX-572 at 30 mM was up to 48x longer than lidocaine (Mann-Whitney test; $P = 0.008$). The duration of motor block produced by QX-222 at 70 mM was not longer than lidocaine (Mann-Whitney test; $P = 0.71$). Mice without motor block were assigned duration times of 0 hours. Horizontal bars are medians ± [IQR]. All groups, n = 8.

3.3 Toxicity Produced by Quaternary Lidocaine Derivatives

3.3.1 Local Tissue Toxicity

QX-314, QX-572, and QX-222 concentration-dependently produced local tissue toxicity in the mouse tail (Table 2). Tissue discolouration, edema, and ulceration were observed macroscopically at the site of injection with each quaternary derivative (≥ 70 mM; Figure 18). Notably, tissue injury consistent with ischemic necrosis was observed in tail tissues treated with 280 mM QX-572, but not with 280 mM QX-314 or QX-222 (Figure 19).

Table 2 Incidence of local tissue toxicity in the mouse tail flick assay

Outcome	Test Compound	Injection Concentration (mM)					
		10	30	70	140	280	560
Local Tissue Toxicity	QX-314	0/8 (0%)	0/7 (0%)	0/8 (0%)	1/8 (13%)	1/7 (14%)	-
	QX-572	0/8 (0%)	0/8 (0%)	6/8 (75%)	8/8 (100%)	7/7 (100%)	-
	QX-222	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	1/8 (13%)

The data are reported as the number of mice per group (%).

After 24 h post-injection, QX-314 and QX-222 produced diffuse myofibre degeneration similar to lidocaine. In contrast, QX-572 produced ischemic necrosis of myofibres and inflammation. Histopathological analyses revealed compartmental edema at the site of injection with all solutions including saline (Figures 20 and 21). Acute edema was observed with saline at 6 h and subsided by 24 h. The appearance of concentric, multinucleated myocytes at 6 h post-injection of 70 mM lidocaine, QX-314, and QX-222 indicated acute injury to myofibres. The extent of histological changes produced by QX-314 and QX-222 were comparable to lidocaine. By 24 h post-injection, acute injury to myofibres progressed into a degenerative process. In contrast to QX-314 and QX-222, at 6 h post-injection of QX-572, myocytes appeared circular with heavy hyalinization and neutrophil infiltration was detected (Figure 21). By 24 h post-injection, this inflammatory effect was more pronounced, and myocytes presented with vacuolation (Figure 21). Histological analyses did not indicate damage to neuronal somata due to injected solutions. In summary, QX-314, QX-572, and QX-222 produced differential patterns of local tissue toxicity.

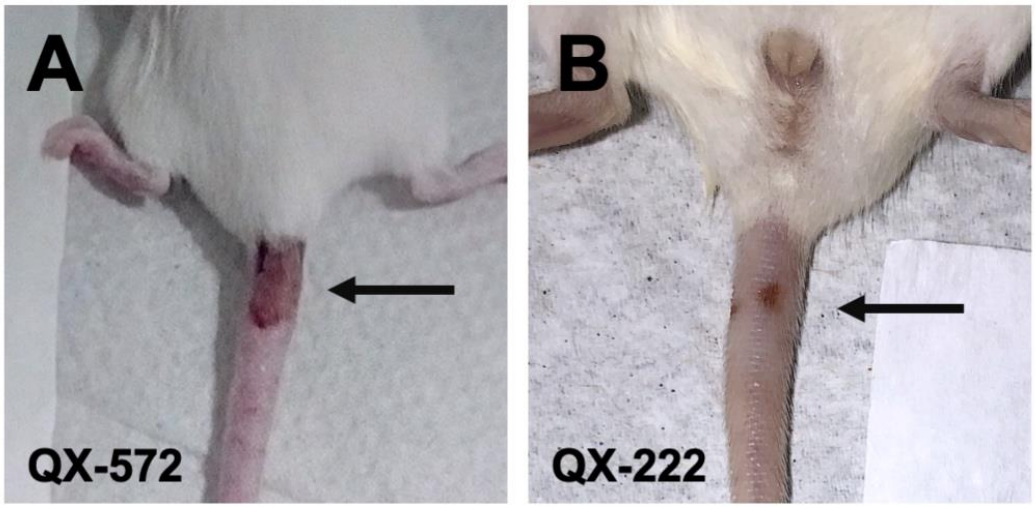


Figure 18 Macroscopic local tissue injury produced by QX-572 and QX-222. (A) Marked redness and ulceration after injection with 70 mM QX-572. (B) White, edematous tissue after injection with 560 mM QX-222. Images were taken at experimental endpoints. No images were collected for QX-314.

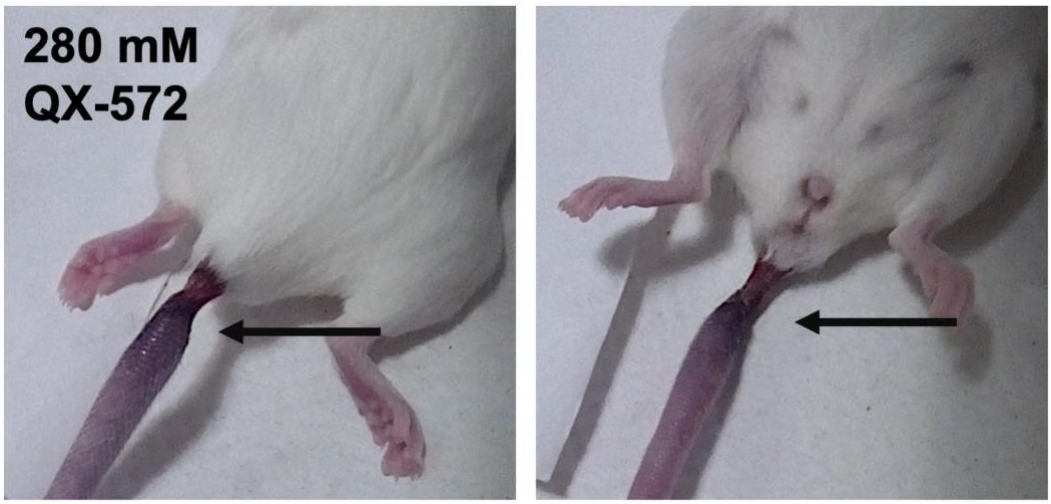


Figure 19 Ischemic necrosis of tail tissue produced by QX-572.
Images were taken 136 h post-injection. (Left/Dorsal; Right/Ventral).

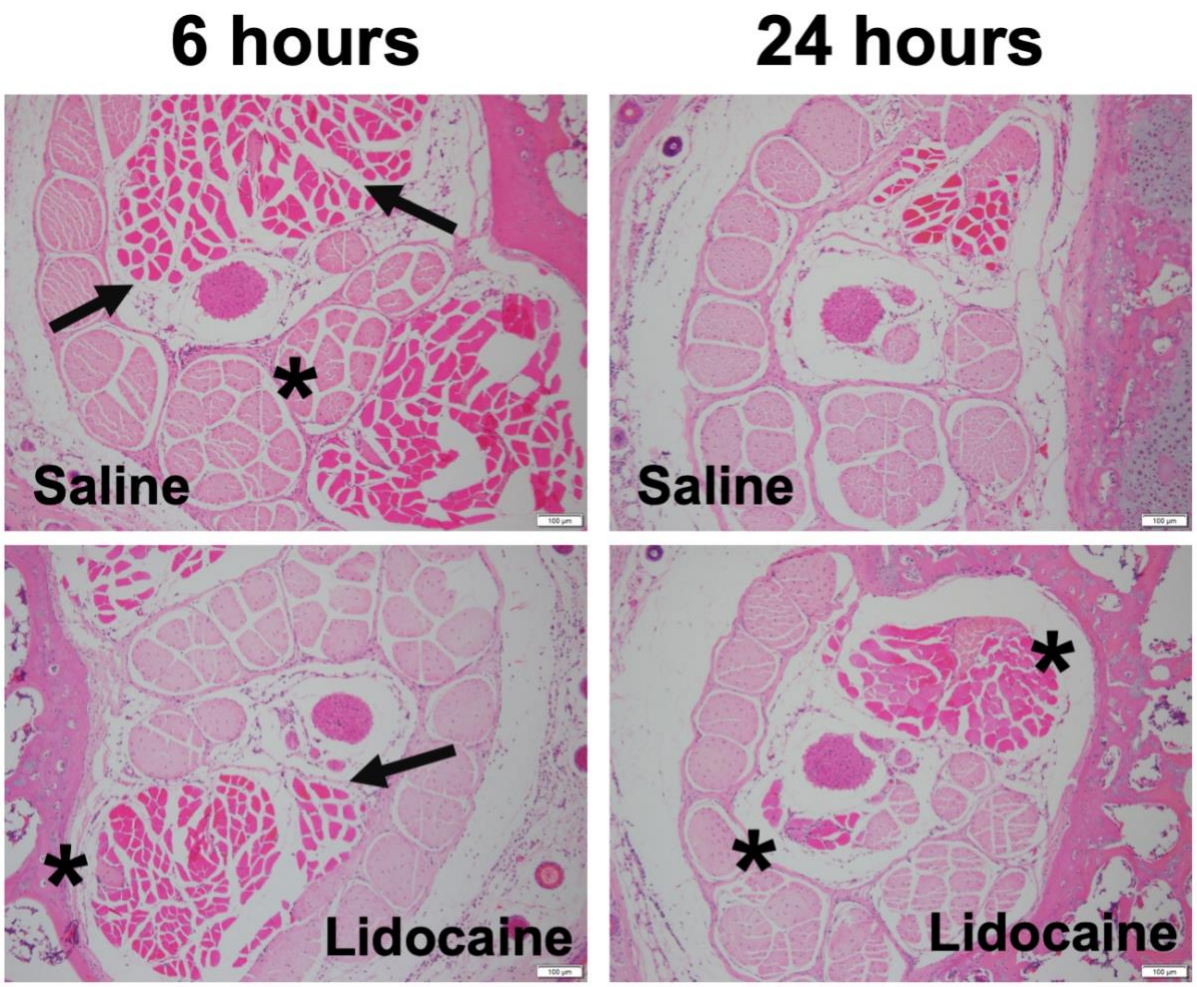


Figure 20 Histological changes in tail tissue after subcutaneous injection of saline & lidocaine. (Top) Myofibre bundles appear less edematous (arrows) after 24 h. Formalin fragmentation (asterisk) is an artifact of fixation. (Bottom) 70 mM lidocaine; moderate edema (arrow) and the appearance of concentric nuclei (asterisk) within myofibres. Persistent myofibre degeneration (asterisks) after 24 h as compared to saline. Cross-section images presented at 10x magnification with calibration bar 100 μm.

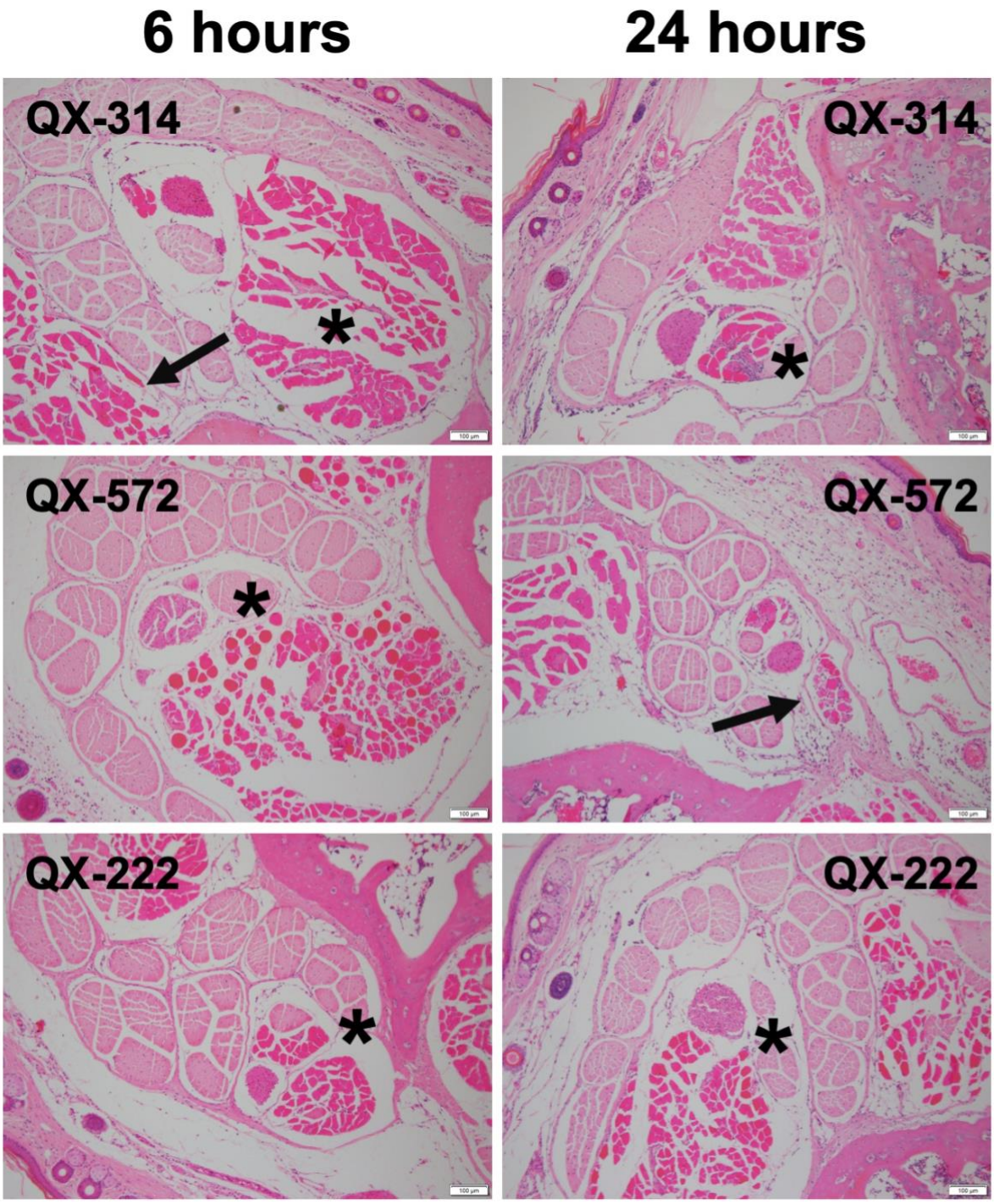


Figure 21 Histological changes in tail tissue after subcutaneous injection of quaternary agents. QX-314 and QX-222; moderate edema (arrow) with myofibre degeneration (asterisk) similar to lidocaine. QX-572; hyalinization of myofibres with distinct circular appearance (asterisk). Acute neutrophil infiltration and myofibre necrosis (arrow) after 24 h. Cross-section images presented at 10x magnification with calibration bar 100 μm. All concentrations are 70 mM.

3.3.2 Systemic Toxicity

QX-314 (≥ 140 mM) and QX-222 (≥ 560 mM), but not QX-572 (max., 280 mM) produced systemic toxicity that manifested in death (Table 3). Adverse reactions to test compound injection included jumping and excessive movement in animals, followed by convulsions, sedation, respiratory depression, and death. The behavioural observations occurred acutely (< 15 min) after peripheral administration of test compound at the base of the tail. No observed adverse reactions were seen with mice injected with saline or 70 mM lidocaine.

Table 3 Incidence of death in the mouse tail flick assay

Outcome	Test Compound	Injection Concentration (mM)					
		10	30	70	140	280	560
Death	QX-314	0/8 (0%)	0/7 (0%)	0/8 (0%)	1/8 (13%)	5/7 (71%)	-
	QX-572	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/7 (0%)	-
	QX-222	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	2/8 (25%)

The data are reported as the number of mice per group (%).

LC-MS/MS analyses of mice plasma indicated that quaternary lidocaine derivatives are absorbed into systemic circulation faster than lidocaine after peripheral administration (Figure 22). Ten minutes after injection of 70 mM lidocaine, the median [IQR] concentration detected in mice plasma was 11.5 [11.31-11.70] μM/L. Ten minutes after the injection of the same concentration with quaternary compounds, higher median [IQR] plasma concentrations of QX-222 (31.9 [30.29-33.66]), QX-314 (23.10 [18.70-27.69]), and QX-572 (15.7 [14.83-16.81]) were detected (in μM/L). I also found greater absorption of QX-314 and QX-222 with high injected concentrations (280 mM) which was not observed with QX-572 (Figure 20). After injection with 280 mM QX-314, the median [IQR] plasma level increased to 101.9 [68.90-117.30] μM/L. Similarly, median

[IQR] plasma levels of QX-222 increased to 84.4 [81.41-87.90] μM/L after injection with 280 mM. In contrast, plasma levels of QX-572 after injection with 280 mM remained similar to that of 70 mM (18.0 [16.86-18.81] vs. 15.7 [14.83-16.81] μM/L). All plasma samples were collected at 10 min post-injection (see section 2.2.2). Due to the small sample sizes (n = 2-3/concentration) in LC-MS/MS experiments, statistical analyses were not conducted. High concentrations were selected for LC-MS/MS analyses based on concentrations that produced death during the tail flick assay. The low concentration was selected to compare systemic absorption that produced effective sensory nerve blockade in the tail flick assay. In summary, systemic absorption of quaternary agents, as compared to lidocaine, occurred more readily after peripheral injection in mice.

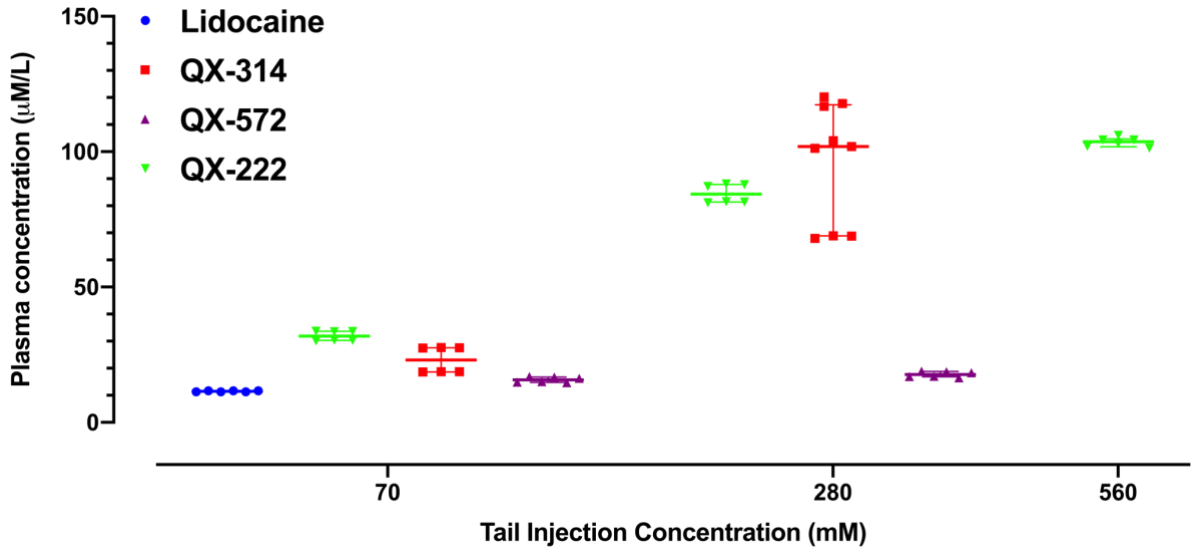


Figure 22 Plasma concentrations of quaternary lidocaine derivatives 10 min after tail injection. At an equimolar concentration, all quaternary lidocaine derivatives were detected in mice plasma at higher levels compared to lidocaine. At 280 mM, QX-222 and QX-314 were absorbed more readily compared to QX-572. Group responses are presented with median ± [IQR]. All groups, n = 2; except 280 mM QX-314 (n = 3). Plasma samples collected from each animal are analyzed and presented in triplicates.

3.4 LC-MS/MS Method Validation

3.4.1 Accuracy and Linearity

All calibration curves for lidocaine, QX-314, QX-572, and QX-222 were linear. The observed error (%) for lidocaine, QX-314, QX-572, and QX-222 was 5.9, 5.0, 6.8, and 5.9, respectively. Since the calibration curves were within the error allowance of 10%, they were considered accurate. Experimental results for each calibration standard and equations for calibration curves are presented in Figure 23.

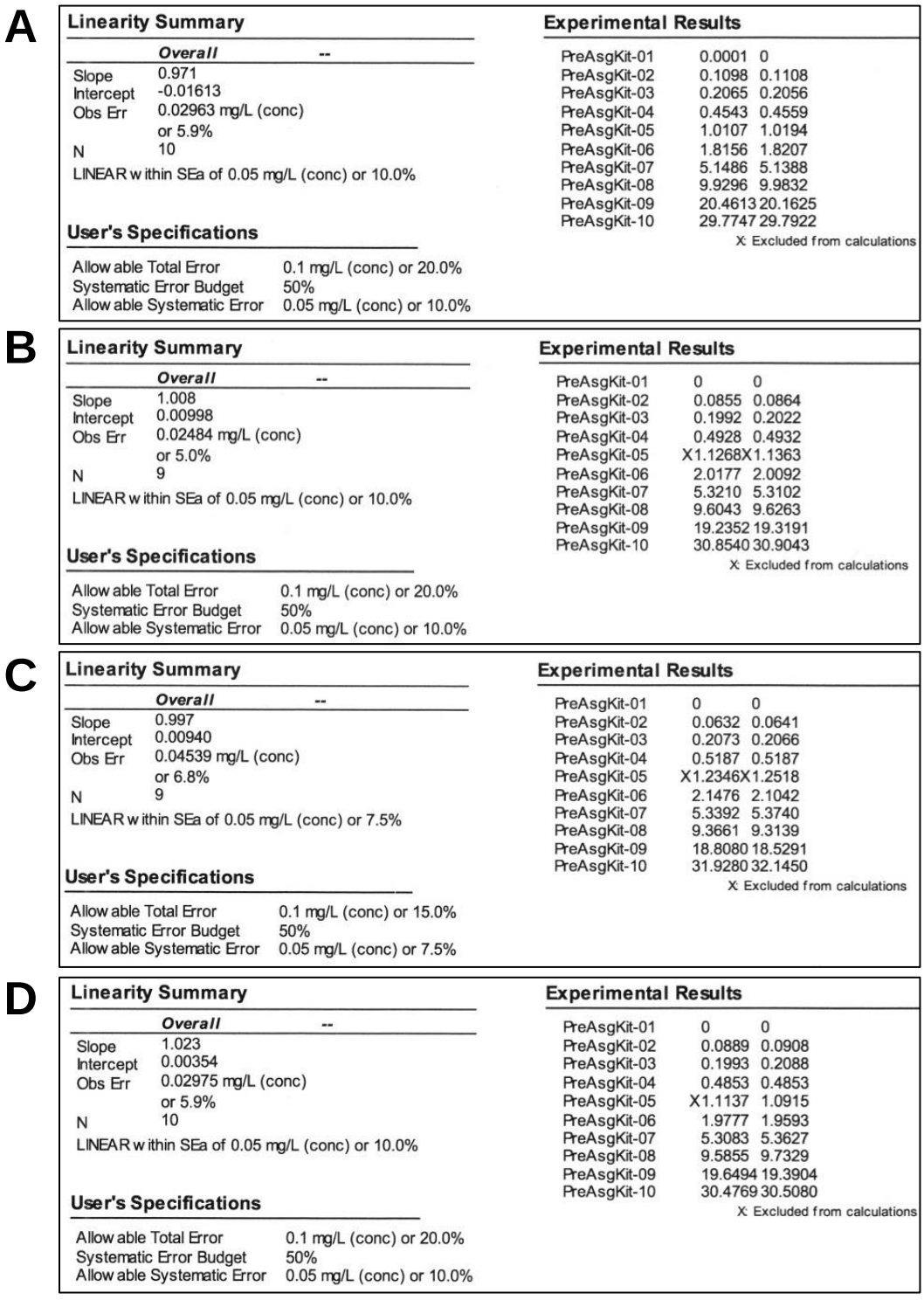


Figure 23 LC-MS/MS method validation: accuracy and linearity.
(A) Lidocaine, (B) QX-314, (C) QX-572, and (D) QX-222.

3.4.2 Sensitivity

The lowest amount of lidocaine, QX-314, QX-572, and QX-222 that could be quantified by the present LC-MS/MS method was (mg/L): 0.0197, 0.00034, 0.00155, and 0.0171, respectively. Sensitivity statistics are presented in Figure 24.

A

Sensitivity Statistics

2 SD Limit of Blank (95% Conf) 0.0197 mg/L
Manufacturer's Claim --
Passes --

Response Statistics

Concentration	Mean	SD
0	49029.84080	8836.11813
0.1	138814.23729	14249.17012

B

Sensitivity Statistics

2 SD Limit of Blank (95% Conf) 0.00034 mg/L
Manufacturer's Claim --
Passes --

Response Statistics

Concentration	Mean	SD
0	34.26017	16.21932
0.1	9567.19190	992.87970

C

Sensitivity Statistics

2 SD Limit of Blank (95% Conf) 0.00155 mg/L
Manufacturer's Claim --
Passes --

Response Statistics

Concentration	Mean	SD
0	76.13545	48.47491
0.1	6324.28323	635.35176

D

Sensitivity Statistics

2 SD Limit of Blank (95% Conf) 0.0171 mg/L
Manufacturer's Claim --
Passes --

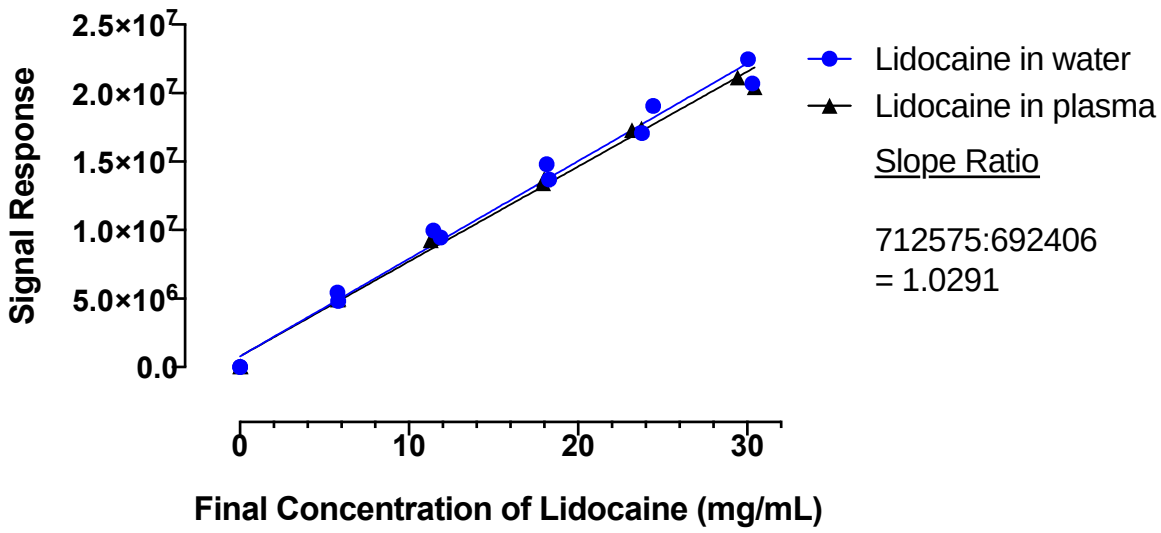
Response Statistics

Concentration	Mean	SD
0	291.25305	169.48731
0.1	2269.04844	300.19480

Figure 24 LC-MS/MS method validation: sensitivity.
(A) Lidocaine, (B) QX-314, (C) QX-572, and (D) QX-222.

3.4.3 Matrix Effects

The slope ratio obtained from lidocaine signal responses in plasma compared to water revealed a 3% ionization enhancement effect due to mice plasma (Figure 25). The same 3% ionization enhancement effect was observed for QX-314 (Figure 26) and QX-572 (Figure 27). The slope ratio for QX-222 indicated an ionization enhancement effect of 2% (Figure 28). The results obtained in matrix effects analyses demonstrated that the use of mice plasma did not significantly interfere with quantification of quaternary lidocaine derivatives.

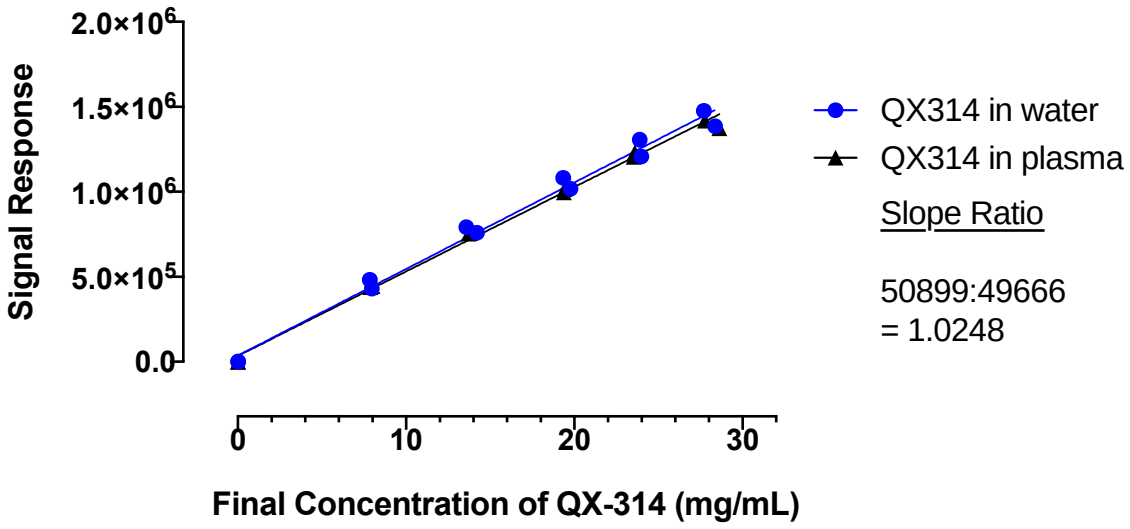


	Lidocaine in water	Lidocaine in plasma
Best-fit values		
Slope	712575	692406
Y-intercept	783514	789341
X-intercept	-1.100	-1.140
1/slope	1.403e-006	1.444e-006
Std. Error		
Slope	24687	19383
Y-intercept	449869	348108
95% Confidence Intervals		
Slope	657569 to 767581	649218 to 735595
Y-intercept	-218856 to 1785885	13707 to 1564975
X-intercept	-2.681 to 0.2889	-2.386 to -0.01883

Are the slopes equal?
F = 0.4113. DFn = 1, DFd = 20
P=0.5286

Concentration of Lidocaine-D10 in all samples = 0.001 mg/mL

Figure 25 LC-MS/MS method validation: lidocaine ionization enhancement.

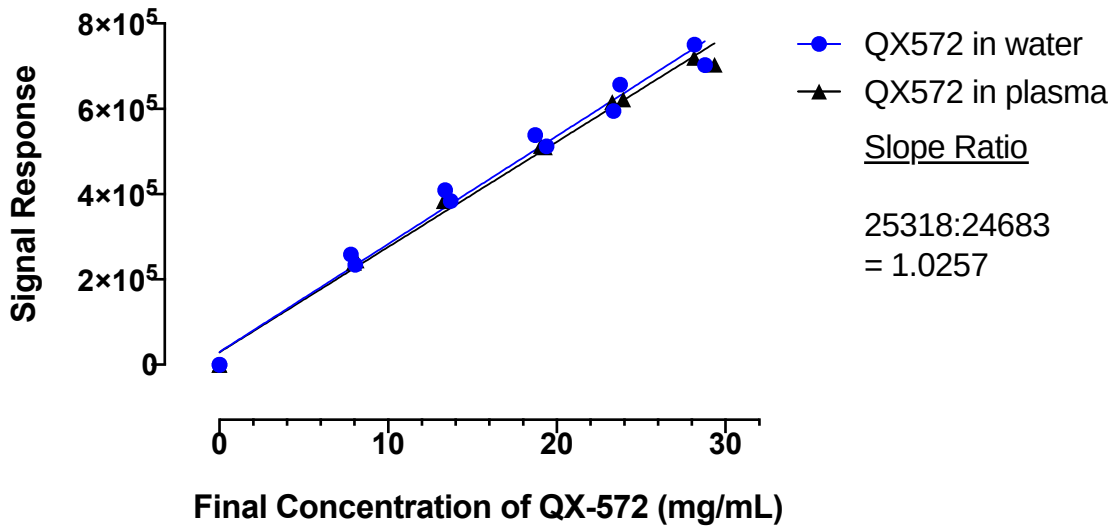


	QX314 in water	QX314 in plasma
Best-fit values		
Slope	50899	49666
Y-intercept	37297	35222
X-intercept	-0.7328	-0.7092
1/slope	1.965e-005	2.013e-005
Std. Error		
Slope	1614	1103
Y-intercept	29437	20027
95% Confidence Intervals		
Slope	47304 to 54495	47209 to 52123
Y-intercept	-28292 to 102886	-9401 to 79845
X-intercept	-2.153 to 0.5244	-1.679 to 0.1816

Are the slopes equal?
F = 0.3978. DFn = 1, DFd = 20
P=0.5354

Concentration of Lidocaine-D10 in all samples = 0.001 mg/mL

Figure 26 LC-MS/MS method validation: QX-314 ionization enhancement.

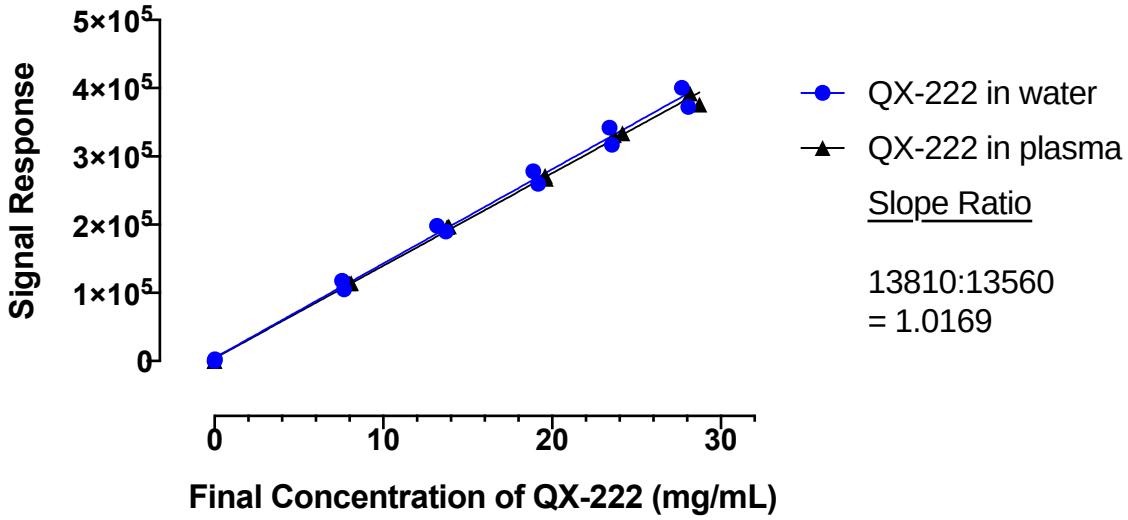


	QX572 in water	QX572 in plasma
Best-fit values		
Slope	25318	24683
Y-intercept	29868	29223
X-intercept	-1.180	-1.184
1/slope	3.950e-005	4.051e-005
Std. Error		
Slope	970.6	753.0
Y-intercept	17619	13742
95% Confidence Intervals		
Slope	23156 to 27481	23005 to 26360
Y-intercept	-9389 to 69126	-1396 to 59842
X-intercept	-2.949 to 0.3458	-2.576 to 0.05347

Are the slopes equal?
F = 0.2683. DFn = 1, DFd = 20
P=0.6102

Concentration of Lidocaine-D10 in all samples = 0.001 mg/mL

Figure 27 LC-MS/MS method validation: QX-572 ionization enhancement.



	QX-222 in water	QX-222 in plasma
Best-fit values		
Slope	13810	13560
Y-intercept	4814	4023
X-intercept	-0.3486	-0.2967
1/slope	7.241e-005	7.375e-005
Std. Error		
Slope	372.9	211.2
Y-intercept	6688	3878
95% Confidence Intervals		
Slope	12979 to 14640	13089 to 14030
Y-intercept	-10088 to 19716	-4618 to 12665
X-intercept	-1.506 to 0.6952	-0.9627 to 0.3308

Are the slopes equal?
F = 0.3429. DFn = 1, DFd = 20
P=0.5647

Concentration of Lidocaine-D10 in all samples = 0.001 mg/mL

Figure 28 LC-MS/MS method validation: QX-222 ionization enhancement.

3.4.4 Autosampler Stability

QX-314 and QX-572 were excluded from autosampler stability analyses because calculated concentrations were 0 mg/L in all samples. Analyses of the concentrations for lidocaine and QX-222 in four samples over three trials indicated autosampler intra- and inter-stability at room temperature (Figure 29). Kruskal-Wallis with Dunn's multiple comparisons test found no differences between three trials at room temperature for both lidocaine ($P = 0.21$) and QX-222 ($P = 0.99$). No significant differences were found in concentrations of lidocaine (median difference [IQR]: 0.0080 [0.0079-0.0080]; Wilcoxon signed-rank test, $P = 0.13$) or QX-222 (median difference [IQR]: 0.0180 [(-)0.0637-0.0488]; $P = 0.88$) after five days of storage at room temperature. Freezing of samples also did not affect autosampler stability (Figure 30). Wilcoxon signed-rank test found no significant differences in concentrations of lidocaine (median difference [IQR]: 0.0080 [0.0080-0.0081]; $P = 0.13$) or QX-222 (median difference [IQR]: 0.0535 [(-)0.1067-0.0657]; $P = 0.88$) after one freeze-thaw cycle.

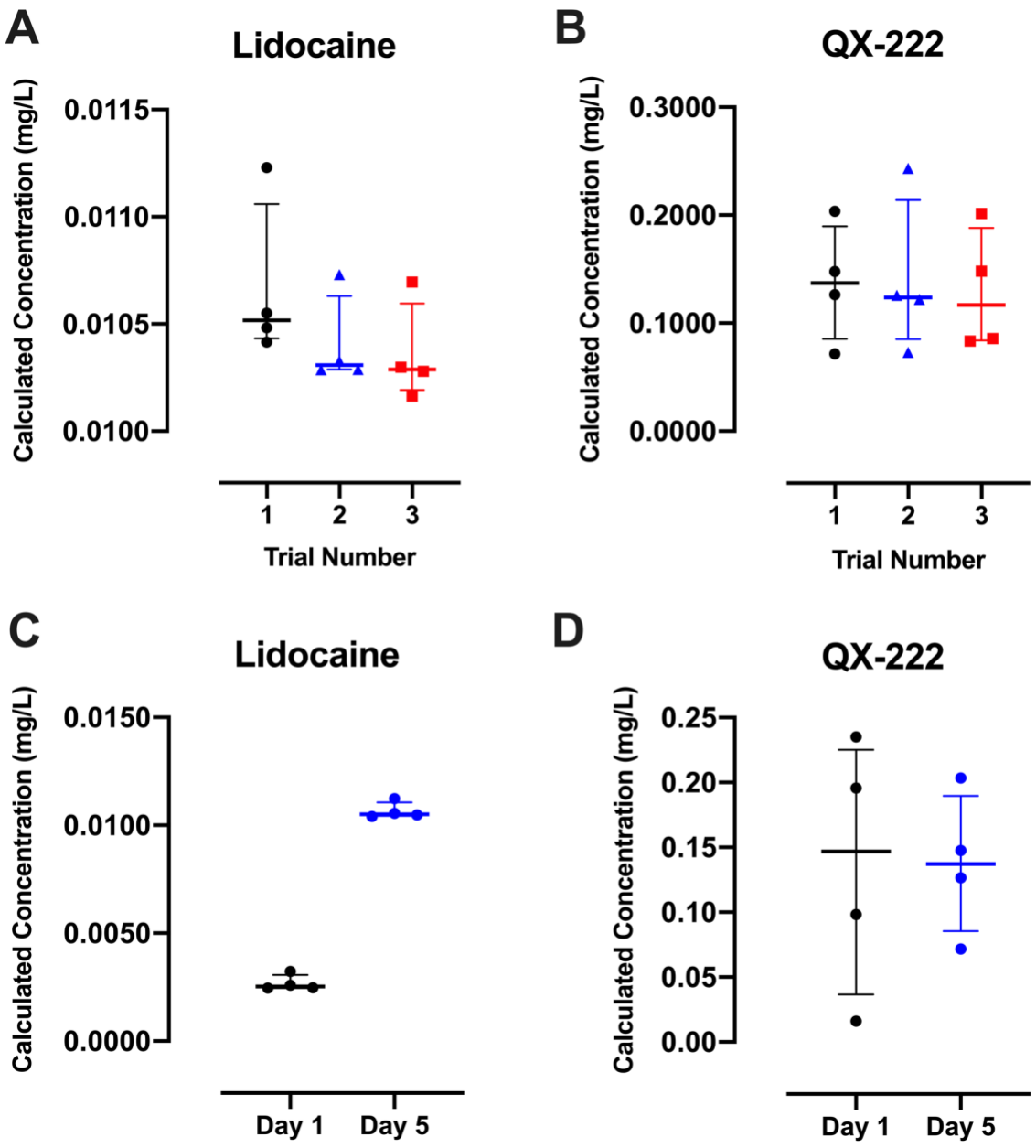


Figure 29 LC-MS/MS method validation: autosampler stability at room temperature. Four samples were prepared from drug-free mice plasma. To determine intra-stability, three trials were run within the same day. Quantitation results were stable within three trials at room temperature for both (A) lidocaine (Kruskal-Wallis test; $P = 0.21$) and (B) QX-222 (Kruskal-Wallis test; $P = 0.99$). To determine inter-stability, samples were kept in room temperature for five days. Quantitation results for (C) lidocaine (Wilcoxon signed-rank test; $P = 0.13$) and (D) QX-222 (Wilcoxon signed-rank test; $P = 0.88$) were stable. Horizontal bars are median ± [IQR].

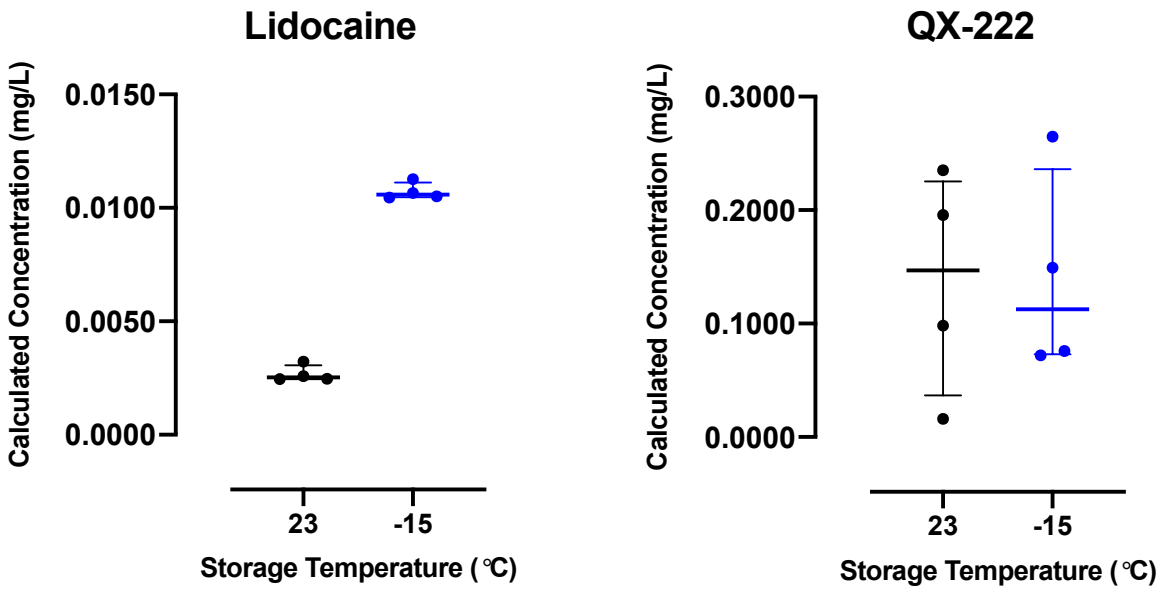


Figure 30 LC-MS/MS method validation: effect of freezing on autosampler stability. To determine the effect of freezing, drug-free mice plasma was frozen at -15° C and restored at room temperature 23° C. Four LC-MS/MS samples were prepared and autosampler stability was assessed. Quantitation results were stable after one freeze-thaw cycle for lidocaine (Wilcoxon signed-rank test; $P = 0.13$) and QX-222 (Wilcoxon signed-rank test; $P = 0.88$). Horizontal bars are median ± [IQR].

Chapter 4: Discussion

4.1 Summary of Results

The results presented in this thesis demonstrate that quaternary lidocaine derivatives concentration-dependently produce sensory and motor blockade. I found that QX-572 and QX-222 both produce long-lasting sensory blockade similar to QX-314. However, unlike QX-314 and QX-572, QX-222 does not produce motor blockade of extended duration. I also determined that the order of potency is QX-572 > QX-314 > QX-222 (Table 4). Collectively, these results support the primary hypothesis that QX-572 produces long-lasting local anesthesia. The relative absence of long-lasting motor blockade, however, does not support the hypothesis that QX-222 produces long-lasting local anesthesia but indicates a potentially useful property of sensory/motor separation.

The present results also demonstrate that quaternary lidocaine derivatives possess different toxicity profiles. Concentration-dependent local tissue damage presented as ischemic necrosis with QX-572, and myofibre degeneration with QX-314 and QX-222. These findings refute the secondary hypothesis that QX-572 and QX-222 possess more favorable local toxicity profiles relative to QX-314. Additionally, I found that the local injection of QX-314 and QX-222, but not QX-572, produces dose-dependent systemic toxicity. The absence of systemic toxicity combined with evidence of slower systemic absorption indicates that relative to QX-314, QX-572 may possess a more favorable systemic toxicity profile.

Table 4 The comparative efficacy and safety of QX-314, QX-572, and QX-222 in mice

Assessment		Lidocaine	QX-314	QX-572	QX-222
Sensory Block					
EC50 (mM)		69[a]	35 (4 to 144)	18 (10 to 31)	122 (50 to 282)
ED50 (mg/kg)		25[a]	14 (2 to 58)	9 (5 to 15)	42 (17 to 99)
LD50 (mg/kg)		300[b]	136 (53 to 581)	-	936 (512 to 2859)
T.I.		~12	~10	-	~22
Motor Block					
EC50 (mM)		-	7 (2 to 20)	4 (2 to 8)	77 (62 to 97)
Murine Plasma Concentrations					
70 mM	μM/L	11.5 [11.3-11.7]	23.1 [18.7-27.7]	15.7 [14.8-16.8]	31.9 [30.3-33.7]
	μg/mL	2.7 [2.65-2.74]	6.9 [5.6-8.3]	5.5 [5.2-5.9]	8.2 [7.8-8.6]
280 mM	μM/L	-	101.9 [68.9-117.3]	18.0 [16.9-18.1]	84.4 [81.4-87.9]
	μg/mL	-	30.5 [20.6-35.1]	6.3 [5.9-6.5]	21.7 [20.9-35.1]

Mean potency values are presented with (95% CI). T.I. = Therapeutic index; LD50/ED50. Median [IQR] plasma concentrations were calculated, using LC-MS/MS, from blood samples (n = 2/3) collected 10 min after subcutaneous tail injection.
[a]From Wang et al. (2010)
[b]From Buckett and Marwick (1975)

4.2 Speculations on Structure-Activity Relationships

As discussed in 1.3.2.1, the general molecular structure of clinically useful LAs consists of an aromatic group, a short alkyl chain containing an ester or amide bond, and an amino group (de Jong, 1994). Because the aromatic group confers lipophilic characteristics and the amino group confers hydrophilic characteristics, LAs are amphipathic compounds. These three components work in concert to give LAs their pharmacological properties (Tetzlaff, 2000). That said, the relationship between pharmacological effects and molecular structure is complex. Clinically relevant LAs are classified according to the type of chemical bond in the short alkyl chain. Since QX-314, QX-572, and QX-222 all possess amide bonds with the same alkyl chain distance to the

quaternary nitrogen, this component of the general structure provides little insight into their pharmacological differences. Nevertheless, there are other structural differences between these quaternary agents. In the following sections, I describe two possible structure-activity relationships (Figure 31) that may explain the observed differences in potency, duration, onset, and offset of sensory and motor nerve blockade produced by QX-314, QX-572, and QX-222.

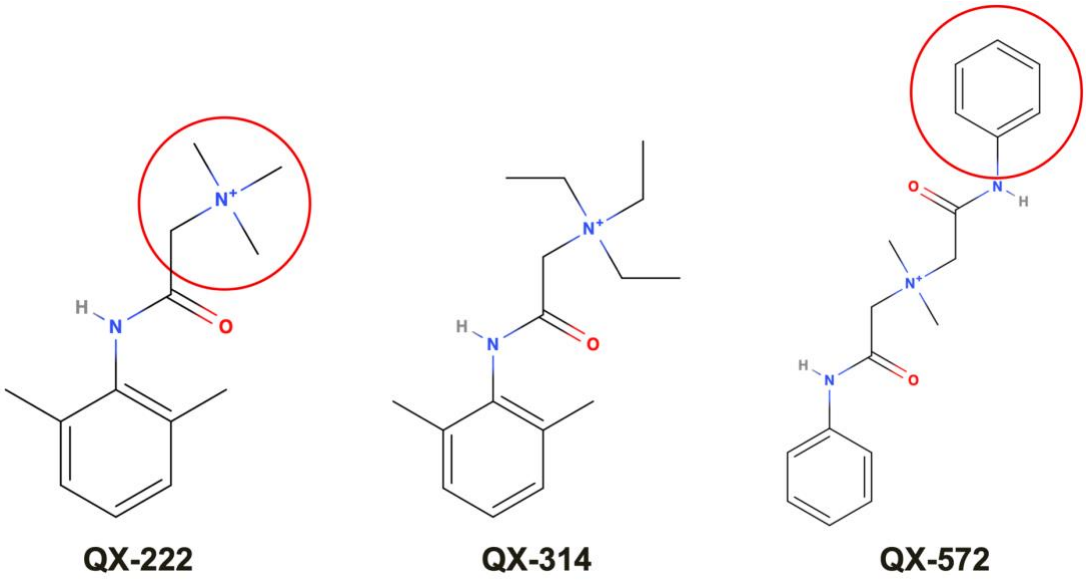


Figure 31 Chemical structure differences of quaternary lidocaine derivatives.

4.2.1 The Substituted Amine Nitrogen

Out of the three quaternary compounds, QX-314 and QX-222 are the most similar. The difference between their molecular structures are the alkyl substituents found on the quaternary amine. QX-314 possesses N-ethyl substitutions on the quaternary amino group whereas QX-222 possesses N-methyl substitutions. As a result, there is a decrease in steric bulk around the quaternary nitrogen of QX-222. This limits the extent of water solvation, preventing stabilization of the ion, and therefore; decreases lipid solubility (Patrick, 2013). It has been well established in the scientific literature that lipid solubility is the driving biochemical factor that determines LA

potency (Arthur & Strichartz, 1987). Manipulation of the substituted amine nitrogen is also observed with tertiary compounds like bupivacaine and ropivacaine. In fact, the higher degree of alkyl substitution on the amine tail of bupivacaine results in a more lipid soluble and thus, more potent compound. The present finding that QX-314 is more potent than QX-222 corroborates the notion that an increase in the chain length of alkyl substitutions on the amine nitrogen increases local anesthetic potency (de Jong, 1994; Tetzlaff, 2000).

Consistent with other HARC findings (Lim et al., 2007), I found that peripheral injection of QX-314 produces robust sensory and motor blockade of long duration. Moreover, I found that the duration of sensory and motor nerve blockade produced by QX-314 was longer than that of QX-222. Even though it was possible to achieve equivalent long-lasting sensory blockade with QX-222, high concentrations were required to produce this effect. Indeed, at the low concentrations tested in the motor blockade study, QX-222 failed to produce long-lasting effects relative to lidocaine. It is likely that the shorter duration of nerve blockade produced by QX-222 is the result of decreased lipid solubility and potency. On the topic of QX-222, it has also been suggested that compounds with low lipid solubility possess weak LA effects due to an irregular distribution around nerve trunks (Franz & Perry, 1974). Since QX-222 is likely the least lipid soluble of the quaternary agents as a result of N-methyl substitutions, I speculate that irregular distribution, in addition to decreased potency, may be responsible for the short duration of motor block observed in this study and others (Hu et al., 2014). While it is possible for QX-222 to produce motor blockade of long duration with high injected concentrations, this may increase the risk of toxicity and thus, limit its clinical potential.

The biochemical factors that dictate onset of nerve blockade by LA agents are the pK_a, concentration, and lipid solubility (Arthur & Strichartz, 1987; de Jong, 1994). Most clinically used

LAs are weak bases with pK_a values in the range of 7-9 (Tetzlaff, 2000). The speed of blockade onset for tertiary anesthetics decreases with increasing pK_a since more physiological buffering is required (Benzon et al., 1993). However, due to the quaternary nature of QX-314, QX-572, and QX-222, it is plausible that pK_a has little influence on quaternary LA onset as physiological buffering systems will not reach the predicted ion-concentration ratios for deprotonation. The pK_a values for QX-572 and QX-222 have yet to be established, however, QX-314 has a pK_a value of 9.81 (Gliklich & Hoffman, 1978) which corroborates the present suspicion.

I found a concentration-dependent delay to sensory and motor block onset produced by QX-314, consistent with a previous report from HARC (Lim et al., 2007). The onset of block was faster as the concentration of QX-314 increased. This supports the suggestion that more quaternary compound diffuses over the critical length of a myelinated nerve fibre and therefore, more drug is available to reach the binding site. A concentration-dependent delay to sensory block onset was also observed for QX-222, however, motor block onset occurred much more rapidly. Reasons for the faster onset of motor block observed with QX-222 are unknown. According to Hille (1977b), uncharged and charged local anesthetics bind to the same receptor site whereby access occurs through lipid fenestrations (lipophilic pathway) or openings in activated sodium channels (hydrophilic pathway) (Catterall, 2012). It was thought that quaternary compounds could only reach the binding site through the hydrophilic pathway due to their permanent charge. The delayed onset observed in the sensory block experiments leads me to speculate that they may also reach the binding site through lipid fenestrations (Rud, 1961). However, if lipid solubility was a major factor in determining onset, the agent expected to have the slowest onset would be QX-222. This was not observed in the motor block experiments. New advancements in molecular imaging will

help to determine whether it is indeed possible for charged compounds to navigate through lipophilic fenestrations given alterations to amino acid residues (Gamal El-Din et al., 2018).

Consistent with a previous report from HARC, there was a concentration-dependent delay to nerve blockade offset produced by QX-314 (Lim et al., 2007). I did not observe irreversible nerve blockade with QX-314, however, it has been reported at concentrations lower than those used in this study (Sagie & Kohane, 2010). This discrepancy may be due to differences among assays, and criteria used for assessing nerve recovery. Recovery from nerve blockade produced by QX-222 was also associated with a concentration-dependent delay to offset. In previous *in vitro* studies, offset times in the order of lidocaine > QX-222 > QX-314 were reported which are consistent with present findings (Yeh & Tanguy, 1985). With QX-222, three animals displayed signs of irreversible blockade. It is difficult to explain reasons for irreversible blockade because two incidences were observed using very low concentrations (10 and 30 mM) and one occurred at the highest concentration (560 mM). The variability of these observations suggest that trauma and irreversible damage may have occurred as a result of direct intraneural administration. Unexpected findings occurred in this study wherein six animals treated with saline exhibited responses for sensory nerve block, although, these responses were transient. Reasons for unexpected sensory block responses to saline are unclear. They may have been the result of stress-induced analgesia from restraint during assessment or physical impingement of the nerve from injection pressure. Overall, it seems that recovery from nerve blockade is not influenced by the degree of N-alkyl substitution found on either QX-314 or QX-222.

4.2.2 The Aromatic Residue

Notwithstanding the permanent cationic charge, little structural comparisons can be drawn between QX-572 and its two smaller quaternary relatives. Compared to QX-314 and QX-222, QX-572 possesses an extended substitution on the quaternary amide. This extended region contains a carbonyl amide bond, and perhaps more importantly, an additional aromatic moiety. Normally, lipid solubility is influenced by the co-operative effects between nitrogen atoms located in the aromatic residue and substituted amine (Tripsa et al., 1986). As a result of the additional aromatic moiety, it is conceivable that there would also be an increase in lipid solubility. Moreover, the extended region containing a second carbonyl amide bond would likely increase steric bulk, further decreasing the interactions between water molecules and the charged amine (Hille, 1977a; Patrick, 2013). My speculation about increased potency due to lipid solubility has also been suggested by others who found that QX-572 was more potent than QX-314 (Frazier et al., 1970). This structure-activity relationship would indeed corroborate the superior potency of QX-572 observed in this study.

Long-lasting sensory blockade produced by QX-572 supports the notion that highly lipophilic local anesthetic agents will have a longer duration of action (Katzung, 2017). Using molecular imaging, ionic interactions between cationic amine groups of LAs and anionic residues of VGSC have been identified (Gamal El-Din et al., 2018). Stabilizing hydrophobic interactions were found between the aromatic potion of lidocaine and residues lining the central cavity of the sodium channel. Due to the additional aromatic group in QX-572, I surmise that increased hydrophobic interactions are responsible for its long sensory block duration. Although the present findings show that QX-314 produced the longest duration of motor block, I did not investigate QX-572 at an equimolar concentration. I suspect that the duration of motor block produced at an

equivalent concentration would supersede that of QX-314, however, high concentrations of QX-572 would likely increase the risk of toxicity. Interestingly, and in contrast to the present study, Shao and colleagues (2015) found no occurrence of motor blockade when QX-572 was administered perineurally to the sciatic nerve. Their studies were performed under different experimental conditions whereby the pH of injected solutions was not adjusted to physiological levels. Consequently, this may have limited the concentration of QX-572 available for diffusion across peripheral nerves. Mechanisms that explain increased duration associated with lipid soluble LAs are unclear, it may be the result of partitioning to myelin sheaths or non-specific binding to membrane surface proteins (Covino & Vassallo, 1976). Further modification of the additional aromatic substitution may provide insight into these mechanisms.

I found a concentration-dependent delay to sensory block onset for QX-572. However, similar to QX-222, motor block onset occurred much more rapidly. At the low concentrations tested, motor block onset was similar to 2% lidocaine. Reasons for discrepancies between sensory and motor block onset are unclear. In the literature, one study using rabbit vagus nerve fibres found that LAs of the amide family blocked C fibres with similar onset while A fibres were blocked proportional to lipid solubility (Wildsmith et al., 1987). This suggests that differences between nerve fibre populations may affect the rate of nerve blockade onset. Indeed, this would explain differences observed for QX-572; however, it does not support the findings with QX-222. Nonetheless, my *in vivo* findings are consistent with those from Gintant and colleagues (1983) where onset of block was most rapid with QX-572, followed by QX-222, and slowest with QX-314. Overall, more investigation into the effects of lipid solubility on block onset are necessary.

Similar to QX-314 and QX-222, recovery from nerve blockade produced by QX-572 was associated with a concentration-dependent delay to offset. Importantly, I observed irreversible

nerve blockade in both sensory and motor block assays. QX-572 is the bulkiest out of the quaternary lidocaine derivatives. It is plausible that after diffusion across the membrane, QX-572 becomes trapped within the nerve leading to prolonged duration. Moreover, since stabilizing hydrophobic interactions are made between the aromatic group and residues lining the central cavity of the sodium channel (Gamal El-Din et al., 2018), one could make the argument that the additional aromatic substitution, like that on QX-572, would increase these stabilizing interactions. Altogether, steric effects and increased stabilizing interactions may explain the irreversible nerve blockade produced by QX-572.

4.3 Toxicity of Quaternary Lidocaine Derivatives

4.3.1 Local Tissue Toxicity: Myotoxicity

Peripheral injection of QX-314, QX-572, and QX-222 produced concentration-dependent local tissue toxicity. Macroscopic damage presented as tissue discolouration, edema, and ulceration. This is consistent with reports of connective tissue damage produced by QX-314 and QX-222 when injected near the sciatic nerve of rats (Shankarappa et al., 2012). In the present study, the order of severity was QX-572 > QX-314 > QX-222 > lidocaine. Histological examination confirmed diffuse myofibrillar degeneration and incidences of inflammatory cell infiltration. Importantly, QX-572 produced ischemic necrosis which was not observed at equivalent concentrations of QX-314 or QX-222. Compared to myocytes that were exposed to QX-314 and QX-222, those exposed to QX-572 appeared darker and more circular. The darkened appearance is likely due to increased hyaline production as a result of growth factors released during myofibre repair (Carosio et al., 2011), and the circular appearance suggests greater insult to the sarcolemma. Also unique to QX-572 was the presence and infiltration of inflammatory cells.

Initially, few neutrophils were observed near damaged myofibres, however, this later became a more pronounced inflammatory response. It is difficult to extrapolate potential mechanisms of myotoxicity based on macroscopic and histologic observations. However, there is agreement in the literature that LA-induced muscle necrosis is primarily due to intracellular dysregulation of Ca^{2+} homeostasis (Zink & Graf, 2004). This hypothesis was based on the observation that both LAs and non-anesthetic agents, known to increase myoplasmic intracellular Ca^{2+}, produce similar myonecrosis (Benoit et al., 1980). Future experiments that use Ca^{2+} imaging may help to visualize the effects of quaternary agents on molecular mechanisms that regulate intracellular Ca^{2+}.

I suspect that differences in local tissue toxicity produced by quaternary agents are attributable to increased lipid solubility. This speculation is supported by the finding that lipid soluble LAs induce necrosis while less lipid soluble LAs induce apoptosis (Onizuka et al., 2011). It should be noted that Onizuka and colleagues (2011) found lidocaine to be more toxic than QX-314; however, human leukemia cell lines were used in their study which may not reflect *in vivo* toxicological phenomena. My speculation about the influence of lipid solubility also is supported by the fact that bupivacaine is more effective than ropivacaine in increasing intracellular Ca^{2+} in skeletal muscle (Zink et al., 2003). In summary, my findings do not support the hypothesis that QX-572 and QX-222 have overall more favorable local toxicity profiles relative to QX-314.

4.3.2 Local Tissue Toxicity: Neurotoxicity

LA agents, such as lidocaine, are known to produce time- and dose-dependent neurotoxicity (Lirk et al., 2014; Werdehausen et al., 2009). In humans, the clinical presentation of neurotoxicity after spinal anesthesia ranges from paresthesia and transient neurologic symptoms to irreversible nerve blockade seen with cauda equina syndrome (Horlocker et al., 1997; Lambert

et al., 1994). Neurotoxicity may also occur after peripheral nerve blockade where large volumes of LAs are deposited perineurally (Hogan, 2008). Consistent with this, I observed irreversible nerve blockade after administration of a large volume of quaternary agent relative to the mouse tail and hind limb.

In both sensory and motor blockade studies, perineural injection of QX-222 and QX-572, but not QX-314, produced irreversible nerve blockade. At the cellular level, QX-222 at 70 mM did not produce axonal swelling and histological changes were comparable to lidocaine after 24 h. Combined with the fact that LA-induced neurotoxicity is concentration-dependent (Werdehausen et al., 2009), the irreversible motor blockade observed at low concentrations of QX-222 (e.g. 10 and 30 mM) likely do not represent neurotoxicity, but rather, nerve trauma from injection or intraneural injection. It is more conceivable, however, that irreversible sensory blockade occurred after injection with 560 mM QX-222. In contrast, 70 mM QX-572 produced microscopic tissue injury after 24 h that presented as ischemic necrosis with infiltration of inflammatory cells. While axonal swelling was also not observed, it is plausible that over a longer period of time, 30 mM QX-572 could also produce ischemic necrosis and inflammation. The ischemic effect of QX-572 on local tissue may induce oxidative neuronal injury, and initiation of apoptosis or necrosis leading to further irreversible nerve damage (Hogan, 2008). Indeed, this speculation is supported by the finding that high concentrations of QX-572 (e.g. 140 and 280 mM) produced marked macroscopic tissue necrosis after 136 h. Compared to QX-222 and QX-572, perineural injection of QX-314 in the mouse tail or hindlimb did not produce irreversible nerve blockade. Moreover, similar to the other quaternary agents, QX-314 did not produce axonal swelling. The lack of behavioural and histological change due to QX-314 may not reflect an absence of neurotoxicity as previous reports

from the HARC have demonstrated marked irritation and toxicity after intrathecal injection (Schwarz et al., 2010).

Several cellular pathways have been identified that are associated with LA-induced neurotoxicity. Lidocaine-induced neurotoxicity in particular has been extensively studied in the scientific literature. Lidocaine is neurotoxic to dorsal root ganglion neurons after prolonged *in vitro* exposure (Gold et al., 1998). It depolarizes neuronal membranes which leads to activation of voltage-gated calcium channels and increased intracellular Ca^{2+}. This may lead to downstream activation of the p38 mitogen-activated protein kinase (MAPK) pathway which is associated with proinflammatory cytokine release and neuronal apoptosis (Haller et al., 2006; Lirk, Haller, Myers, et al., 2006). Because infiltration of inflammatory cells and myocyte necrosis was observed histopathologically in the present study, I suspect that QX-572 may induce neurotoxicity through the MAPK pathway. However, since Ca^{2+} is an important regulator of cell homeostasis, it is likely that LA-induced Ca^{2+} dysregulation may lead to the activation of multiple pathways including the caspase apoptotic pathway (Johnson et al., 2004; Lirk, Haller, Hausott, et al., 2006; Verlinde et al., 2016). Finally, another potential pathway is the activation of transient receptor potential vanilloid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1) channels. Several studies have demonstrated that LAs induce Ca^{2+} influx by activating TRPV1 (Leffler et al., 2008) and TRPA1 (Leffler et al., 2011). Since QX-314 has been found to activate TRPV1 (Rivera-Acevedo et al., 2011), it may be possible that QX-572 and QX-222 also activate these cation channels to induce Ca^{2+} dysregulation, apoptosis, and subsequent neurotoxicity.

Neurotoxicity produced by quaternary agents may be influenced by lipid solubility. From my structure-activity predictions, an increase in lipid solubility may lead to membrane disruption if the molecule gets trapped within the membrane. Additionally, lipophilic agents may also interact

with cellular organelles such as the endoplasmic reticulum or mitochondria. Both are important regulators of cell signalling pathways involving apoptosis. It is important to mention that the neurotoxic profiles of quaternary compounds must be interpreted with caution as transmission electron microscopy was not used to assess neurotoxicity. Hematoxylin and eosin staining is inferior to this technique and therefore, not suitable to determine neuropathology. Nonetheless, the present observations of irreversible blockade do not support my hypothesis that QX-572 and QX-222 have more favorable local toxicity profiles relative to QX-314. Further study involving dose-response analyses of the neurotoxic effects produced by quaternary lidocaine derivatives will help to shed light on potential mechanisms involving neurotoxicity.

4.3.3 Systemic Toxicity

Previous reports from HARC have demonstrated that intravenously administered QX-314 produces systemic toxicity with greater potency than lidocaine (Cheung et al., 2011) and that intrathecal injection produces adverse behaviours leading to death in mice (Schwarz et al., 2010). From the point of view of systemic and spinal anesthesia, QX-314 evidently possesses a narrow therapeutic window compared to lidocaine. However, it was postulated that systemic toxicity due to absorption from peripheral administration may be favourable as quaternary compounds likely possess slower absorption kinetics.

Here, I found that peripheral, perineural injection of QX-314 and QX-222, but not QX-572, produced dose-dependent systemic toxicity in mice. Moreover, I found that QX-314 is more potent than QX-222. Adverse behaviours displayed by animals reflected a brief period of excitability followed by seizure activity, sedation, respiratory depression and ultimately, death. Surprisingly, and in contrast to intrathecal injection, death occurred within 15 min of injection,

which suggested rapid systemic absorption of QX-314 and QX-222. It is possible that absorption into peripheral blood vessels occurs more readily for smaller and less lipid soluble quaternary agents such as QX-314 and QX-222. This would imply that the extent of systemic absorption is influenced by molecular structure.

Indeed, this suspicion was confirmed by LC-MS/MS quantitation of quaternary agents in mouse plasma 10 min after peripheral injection. These pharmacokinetic experiments revealed higher plasma levels of QX-314 and QX-222 compared to QX-572 after an equimolar injection. Additionally, systemic levels of QX-572 did not increase to the same extent as QX-314 or QX-222 regardless of a fourfold increase in the injection concentration. It should be noted that despite not observing adverse systemic reactions to QX-572, my assessments were limited to a maximum concentration. Thus, adverse reactions should not be ruled out at higher concentrations.

Absorption kinetics for quaternary lidocaine derivatives are unknown. However, tertiary LAs produce biphasic effects on smooth muscles surrounding peripheral blood vessels (Blair, 1975). At low doses, tertiary LAs produce vasoconstriction and at high doses they produce vasodilation. It is possible that biphasic effects are also produced by QX-314 and QX-222 since I detected greater plasma concentrations in the systemic circulation using high concentrations. Because this mechanism does not fit with QX-572, it is possible that this effect on smooth muscles and absorption kinetics is quaternary agent-specific.

4.4 QX-222 and QX-314 versus Ropivacaine and Bupivacaine

The observed differences between QX-222 and QX-314, upon comparison to the scientific literature, curiously resemble two well-established differences between the clinical LAs, ropivacaine and bupivacaine. Despite discrepancies between human and animal studies with

regards to the order of potency between them (Wang et al., 2001), there is general agreement that ropivacaine provides two clinical advantages over bupivacaine (Kohane et al., 1998).

First, in terms of nerve block, ropivacaine confers greater sensory-motor separation than bupivacaine (Feldman & Covino, 1988; Zaric et al., 1996). An important question is what biochemical property of ropivacaine provides this advantage. One observation is that ropivacaine possesses a tertiary N-alkyl substitution that is one carbon shorter than bupivacaine, as previously mentioned. This renders ropivacaine less lipid soluble compared to bupivacaine, and thus less likely to penetrate large myelin nerve fibres involved in motor function (McClellan & Faulds, 2000). To my surprise, unlike QX-314, QX-222 did not produce motor blockade of long duration. Moreover, analogous to ropivacaine and bupivacaine, the structural difference between QX-314 and QX-222 is also attributable to the length of the N-alkyl substituents. Where N-methyl groups are found on QX-222, N-ethyl groups are found on QX-314. These pharmacological and structural similarities suggest that QX-222 may also confer better sensory/motor nerve separation in terms of long-lasting blockade.

Second, ropivacaine is less cardiotoxic than bupivacaine (Groban et al., 2001; Wang et al., 2001). Aside from inhibiting neuronal conductance, LAs dose-dependently produce toxic effects on cardiac automaticity, conductivity, and rhythmicity. For example, LAs prolong the QRS interval and produce myocardial depression (Block & Covino, 1981). Depending on the LA, the mechanism of LA-induced cardiotoxicity may be due to dysrhythmia or inadequate contractility (Butterworth, 2010). In the case of bupivacaine, cardiac arrythmias have been attributed to delayed atrioventricular conduction (Graf et al., 1997). This may be in part due to the fact that bupivacaine binds to voltage-gated sodium channels found in the heart for an extended duration (Clarkson & Hondeghem, 1985). Importantly, the reduced cardiotoxic effects of ropivacaine have been

attributed to stereoisomerism. From this perspective, bupivacaine exists in racemic form which contains both S (-) and R (+) enantiomers. In contrast, ropivacaine is produced in the pure S (-) enantiomeric form. Even though physiochemical properties of enantiomeric compounds are identical, LA enantiomers can have different affinities for binding sites and toxicities (Åberg, 1972). Indeed, in one study comparing R (+) and S (-) bupivacaine [levobupivacaine], Valenzuela and colleagues (1995) found that R (+) bupivacaine bound inactivated voltage-gated sodium channels faster and with greater potency than its S (-) counterpart. In the present study, QX-314 and QX-222 dose-dependently produced death and raised the possibility of systemic toxicity. In support of this speculation, LC-MS/MS analysis confirmed increased plasma concentrations of QX-314 and QX-222 relative to lidocaine, which did not produce death. Although the mechanism of death was not determined in my study, these findings suggest that systemic toxicity produced by QX-314 and QX-222 manifests as cardiac toxicity. Since QX-314 was more potent than QX-222 at producing systemic toxicity, I suspect that similar to ropivacaine, QX-222 is less cardiotoxic than its bulkier quaternary relative.

One mechanism for bupivacaine-induced cardiotoxicity reported by Sztark and colleagues (1998) is decreased ATP synthesis in the mitochondria. These investigators went on to suggest that the LA effect on energy production was dependent on lipid solubility. If QX-314 and QX-222 indeed produce cardiac toxicity, the greater potency of QX-314 relative to QX-222 may be attributable to the increased lipid solubility of QX-314 from N-ethyl substitution. With regard to stereochemistry, stereoisomerism cannot contribute to the cardiotoxic effect of quaternary lidocaine derivatives as these compounds are achiral. Thus, they do not possess enantiomeric R (+) and S (-) forms. Combined with motor sparing effects, the reduced cardiotoxicity of QX-222 emphasizes its potential for clinical use. These advantageous properties would make QX-222 an

ideal candidate for structural modification in hopes to identify quaternary agents suitable for postoperative analgesia.

4.5 The LC-MS/MC Method: Lidocaine Analogues

LC-MS/MS is an indispensable tool for clinical and forensic toxicology (Maurer, 2004). LC-MS/MS methods are used to identify or quantify drugs, poisons, or metabolites in blood, plasma, serum, or urine (Maurer, 2007). The data from LC-MS/MS provides important information about the pharmacokinetic properties of current therapeutics or novel drugs (Maurer, 2006). Because I observed death upon peripheral injection, it was imperative to perform pharmacokinetic studies to determine the possibility of systemic toxicity. To the best of my knowledge, only one validated LC-MS/MS method exists to quantify quaternary derivatives (Zhang et al., 2017). However, this method was designed to measure QX-OH which is chemically distinct from the quaternary lidocaine derivatives in the present study. So, in an effort to characterize the absorption characteristics of QX-314, QX-572, and QX-222, I developed a novel LC-MS/MS method with the help of the Provincial Toxicology Centre at the BC Centre for Disease Control.

The “Lidocaine Analogues” method has several advantages. First, this method is able to quantify multiple quaternary lidocaine derivatives. If future quaternary derivative synergism studies were carried out, samples containing a mixture of QX-314, QX-572, and QX-222 could be processed using this method. Second, this method uses an internal standard that is not a therapeutic drug. Therefore, in the case of accidental drug exposure, there would be little risk of estimation bias during analysis (Maurer, 2005). Third, this method uses a deuterated internal standard labeled at 10 different positions. Normally, internal standards are labeled at 3-6 different positions.

Because there are additional labels, the risk of internal standard carry over is reduced (Bogusz, 1997). Finally, this method uses small sample and injection volumes which reduces the number of required animals.

The benefits of the novel LC-MS/MS method, “Lidocaine Analogues”, go beyond this dissertation. In the future, this method can be used to investigate the pharmacokinetic properties of other quaternary lidocaine derivatives. It is my hope that others will use this method to understand pharmacokinetic properties of other quaternary lidocaine derivatives.

4.6 Study Limitations

These studies are not without limitations. In the experiments investigating sensory blockade, I measured the continuous tail flick response and made the assumption that sensory blockade would occur after the cut-off time set at 4 s. Another assumption was that the duration of block is proportional to effect. Despite demonstrating that sensory blockade is concentration-dependent, the magnitude of this effect is not reflective in the quantal dose-response relationships. Thus, one limitation is that there is difficulty with resolving the estimation of sensory blockade using a quantal definition.

A second limitation is that only one assay was used for each investigation of sensory and motor blockade. The use of an additional sensory assay, like the Hargreaves test, would have the advantage that animals can serve as their own controls at every time point. In the Hargreaves test, mice are not restrained, decreasing the chance of stress-induced analgesia that may confound results (Deuis et al., 2017). An additional method that can be used to assess motor function is the Rotarod test (Stanley et al., 2005). This test has the advantage that measurements are not quantal

and instead continuous; however, this method requires animals to be trained on the apparatus which may lead to animal learning.

A third limitation is that sensory and motor blockade effects are elucidated from mixed peripheral nerves. For sensory blockade, the tail flick response is mainly spinally mediated and therefore, the influence of quaternary derivatives on higher order pain processing, e.g. in the cortex, may not be discerned from these results (Irwin et al., 1951). In contrast, for motor blockade, the use of the hind limb is likely cortically mediated. Overall, the difference between spinal and cortical modulation for behaviours in each assay, in addition to the site of injection, may explain the variation in sensory and motor blocking effects of quaternary agents.

A fourth limitation is that with respect to neurotoxicity, transmission electron microscopy was not used to evaluate nerve fibre damage. Compared to hematoxylin-eosin staining, this method is more suitable to determine neuropathology. Therefore, separate future studies on the neurotoxic profiles of quaternary compounds are warranted.

A fifth limitation is that I did not determine 1-octanol/water partition coefficients (logP) for each quaternary lidocaine derivative. To determine logP, one simple approach would be to dissolve the quaternary compound in a known volume of octanol and water, and then determine the concentration of the agent in each component. LogP values –which reflect the lipophilicity of a particular compound– for lidocaine, QX-314, and QX-572 are 0.295, -1.875, and -2.730, respectively (Gupta, 1998). To my surprise, these values suggest that the quaternary agents partition primarily in the hydrophilic layer in the classic 1-octanol/water partition experiment. Because of this discrepancy, the present assumptions on the lipid solubility profiles of quaternary lidocaine derivatives must be taken with caution.

Finally, a discrepancy exists in the range of concentrations tested between sensory and motor blockade studies. Consistency between the studies may have led to more robust concentration-response analyses and better indications about the duration of block produced by quaternary agents. Furthermore, concentration-response relationships should have been established for lidocaine. As seen with clinical studies, the use of ED50 or ED95 values instead of absolute concentrations seems to be more advantageous for determining therapeutic ratios. Nevertheless, I chose to limit the concentration range to minimize toxicity in animals.

4.7 Future Directions

It remains unclear how quaternary lidocaine derivatives transverse across biological membranes. Previous research has shown that it is possible for QX-314 to permeate through activated cation channels like TRPV1 or TRPM8 to produce nociceptive- or cold-specific analgesia, respectively (Binshtok et al., 2007; Ongun et al., 2018). However, earlier work from HARC (Lim et al., 2007) and current work presented here, demonstrates that exogenous activation of such channels is not required for local anesthetic effect. Furthermore and importantly, axons of motor neurons do not significantly express TRPV1 or TRPM8 channels. The use of molecular imaging techniques has been one solution to elucidate possible interactions between quaternary compounds and hydrophobic barriers. Indeed, recent molecular imaging techniques using X-ray crystallography have demonstrated that LAs are capable of navigating through lipid fenestrations to reach their binding site (Gamal El-Din et al., 2018). However, other interactions with membrane surface proteins may be possible. One possibility is that quaternary agents slip through endogenously activated channels. Thus, future studies that incorporate radiolabelled elements into

the molecular structures of QX-314, QX-572, or QX-222 will be one way to shed light on possible interactions with membrane surface proteins and barriers.

The mechanism by which quaternary agents produce long-lasting local anesthesia also remains unclear. Multiple *in vitro* studies clearly demonstrate that these compounds bind intracellularly to VGSC to elicit their effects. As mentioned above, endogenously activated ion channels may be one pathway that permits entry into the cell. Once inside, permanently charged QX derivatives will likely become trapped within the cell leading to prolonged access to the binding site and hence, long-lasting blockade. Upon injection, LAs have been found to disrupt membrane stability which, consequently, affects membrane proteins and enzymes (Seeman, 1972). Membrane stretching may lead to the activation of mechanoreceptors such as TRPV2 and 4 (Guler et al., 2002; Muraki et al., 2003; O'Neil & Heller, 2005). This mechanism may provide greater access for quaternary agents to the intracellular binding site for extended blockade. With QX-572, peripheral injection induced infiltration of neutrophils which are known to release pro-inflammatory cytokines and chemokines (Tecchio et al., 2014). Release of the inflammatory mediators, interleukin-17 (IL-17) and interferon gamma (IFNγ), could potentially lead to the activation of other receptors or intracellular signalling pathways for increased facilitated diffusion. However, most clinical LAs are known to have anti-inflammatory effects (Cassuto et al., 2006) which begs the question as to whether this also applies to quaternary lidocaine derivatives. Besides possible drug interactions with inflammatory cells, future research is needed to determine the extent of plasma protein binding of quaternary lidocaine derivatives since potency and duration are also influenced by this biochemical property. Specifically, one should investigate the binding affinities for albumin, lipoproteins, or alpha-1-acid glycoproteins.

Additional work is needed to validate my speculations about the structure-activity relationships of quaternary lidocaine derivatives. But from a pragmatic point of view, understanding structure-activity relationships will require discrete modifications to structures. To the best of my knowledge, no *in vivo* studies have been performed on other quaternary lidocaine derivatives aside from QX-314, QX-572 and QX-222. Other related structures involve cyclization of the quaternary amine whereby quaternary haloalkylamines also produced long duration anesthesia (Ross et al., 1972). With the help of medicinal chemists, it would be worthwhile to see if the lipophilicity of QX-572 can be reduced. This may be accomplished if the length of the aromatic substitution on the quaternary amine is shortened. It would also be valuable to see if the lipophilicity of QX-222 can be increased such that it remains different from QX-314. This may be accomplished if the length of the N-alkyl substitutions are increased slightly. From there, similar *in vivo* studies comparing the anesthetic effects of such compounds may answer the question as to whether separation of long-lasting local anesthetic effect from toxicity is possible.

Finally, further toxicological studies are needed to elucidate the mechanism causing death. One of the most intriguing findings from the present study was that peripheral injection of QX-314 and QX-222 produced adverse events indicative of local anesthetic-induced systemic toxicity. The ensuing question is whether toxicity occurs as a result of ventricular arrhythmias leading to cardiac arrest, or CNS-mediated respiratory depression. One approach would be to incorporate animal electrocardiogram experimentation with respiratory monitoring. If QX-314 and QX-222 possess cardiotoxic effects, it would sensible to consider alternative LA delivery systems that reduce systemic absorption (Davidson et al., 2010). One possible solution may be the encapsulation of quaternary agents in liposomes (Boogaerts et al., 1993).

Chapter 5: Conclusion

In this thesis, I assessed the LA effects of three quaternary lidocaine derivatives and compared them to lidocaine. A series of laboratory experiments were conducted to determine peripheral nerve blocking effects of QX-314, QX-572, and QX-222. My objectives were to determine the order potency and investigate the duration of sensory and motor blockade. I found that the order of potency among these quaternary agents is QX-572 > QX-314 > QX-222. Moreover, I found that QX-314, QX-572 and QX-222 dose-dependently produce longer lasting sensory blockade compared to lidocaine. However, only QX-314 and QX-572 produce longer lasting motor blockade compared to lidocaine. These results support my primary hypothesis that QX-572 produces long-lasting local anesthesia. However, I cannot conclude the same for QX-222.

I also characterized toxicity profiles for each quaternary agent by assessing behavioral and local tissue toxicity following perineural injection. QX-314 and QX-222 dose-dependently produced adverse behaviors that were indicative of systemic toxicity. In my attempt to understand the behavioral toxicity results, I developed a novel LC-MS/MS method to quantify lidocaine and quaternary derivatives in mouse plasma. Quantitative results from LC-MS/MS confirmed increased systemic absorption of QX-314, QX-222, and QX-572 relative to lidocaine. With regard to local tissue toxicity, all quaternary lidocaine derivatives dose-dependently produced macroscopic tissue injury. I observed tissue discoloration, edema, and ulceration of tissues at the injection site. Histological examination revealed that QX-314 and QX-222 produced myofibre degeneration while QX-572 produced ischemic necrosis and inflammation. Collectively, these results do not support my secondary hypothesis that QX-572 and QX-222 possess more favorable toxicity profiles relative to QX-314.

The observed differences in potency and toxicity produced by QX-314, QX-572, and QX-222 are likely due to structure-activity relationships. The structure-activity relationship that explains the lesser potency of QX-222, may be the substituted amine nitrogen. It is possible that quaternary N-methyl substituents reduce potency by reducing the lipophilicity of QX-222. The structure-activity relationship that explains the greater potency of QX-572 may be the presence of an additional aromatic residue. I conclude that differences in quaternary anesthetic structure give rise to distinct potencies and toxicities. For this reason, maximizing the duration of anesthetic effect may come at the cost of toxicity.

Although the present findings do not unreservedly support the use of QX-314, QX-572, or QX-222 for the treatment of acute postoperative pain, they add to our current understanding of local anesthetic pharmacology. The contents of this dissertation demonstrate that contrary to traditional pharmacologic dogma, QX-572 and QX-222 also possess LA activity. Equally important, this exploratory work shows that long duration local anesthesia is possible with a group of quaternary compounds. The speculated structure-activity relationships will help to guide others design new quaternary compounds. If quaternary lidocaine derivatives could be designed such that they produce long lasting nociceptive-specific anesthesia with reduced toxicity, this would considerably improve the overall management of acute and chronic pain. Indeed, future experiments with the goal to find balance between potency and toxicity may require new quaternary compounds. In the event that pharmacokinetic studies are required, the new LC-MS/MS method may serve to foster rewarding interdisciplinary research opportunities. Finally, the clinical impact of this work will not be immediate, but hopefully, my efforts will stimulate enthusiasm for further study.

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