The oxime HI-6:

Determination of pharmacokinetics and the effect of atropine co-administration in guinea pigs and domestic swine

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By

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ABSTRACT

Chemical warfare agents including organophosphorus nerve agents (NA) continue to be a significant threat to both military and civilian populations. The current Canadian Armed Forces (CAF) treatment of NA poisoning includes administration of the oxime HI-6 (used to reactivate inhibited acetylcholinesterase) in combination with atropine contained in an autoinjector, with a benzodiazepine also being administered. Two salts of HI-6 are currently available: HI-6 2Cl (1-[[[4-(Aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyiminio)methyl] pyridinium dichloride (MW 376.22 g/mol) and HI-6 DMS (1-[[[4-(Aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyiminio)methyl] pyridinium dimethanesulfonate (MW 477.49 g/mol). Currently HI-6 is available to the Canadian Armed Forces under a special access program. In order to attain licensure of HI-6 numerous studies must be carried out in animal models to ensure its safety (tolerability and toxicity), efficacy and pharmacokinetics prior to human clinical trials. The present experiment aimed to determine and compare the pharmacokinetic parameters of HI-6 in two animal models under various conditions including: direct comparison of salts (HI-6 2Cl compared to HI-6 DMS), comparison of routes of administration (intramuscular compared to intravenous), comparison of effect of anaesthetic, comparison of different concentrations of HI-6, determination of the effect of atropine sulphate co-administration and evaluation of calculated pharmacokinetic parameters when infusing HI-6. Serial plasma samples were collected and HI-6 levels were quantified using a HPLC method. In all studies a significant difference was reported for absorption/distribution parameters when comparing salts. Additionally the absorption/distribution parameters when comparing routes of administration were significantly different however all other parameters were similar. Significant differences in calculated parameters were reported when examining the effect of anaesthetic on the pharmacokinetics of HI-6. Similar to previous ascending dose studies, differences were reported for the absorption/distribution kinetics. Co-administration of HI-6 with atropine sulphate did not have significant effect on the pharmacokinetics of HI-6. The determined pharmacokinetic values for both salts were accurate for the determination of an infusion rate to reach and maintain a target plasma concentration. Finally the calculated animal model pharmacokinetic data was compared to previously published human clinical trial data and the calculated pharmacokinetic values were found to be similar.

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LIST OF ABBREVIATIONS

% HI-6$_u$	percent HI-6 in urine
*	significant difference at p< 0.05
2Cl	dichloride
2-PAM	pralidoxime
ACh	acetylcholine
AChE	acetylcholinesterase
ADME	absorption distribution metabolism excretion
AS	atropine, atropine sulphate
AST	aspartate aminotransferase
AUC$_\infty$	area under the curve
b	y-axis intercept
BCME	bis(chloromethyl) ether
C	sample concentration
Ca	calcium
CAF	Canadian Armed Forces
CCAC	Canadian Council on Animal Care
CFHS	Canadian Armed Forces Health Services
Cl	total body clearance
Cl	chloride

Cl_r	renal clearance
C_{max}	maximum observed concentration
CNS	central nervous system
C_o	initial concentration
CO_2	carbon dioxide
C_p	plasma concentration
CREA	creatine
C_u	urine concentration
CWA	chemical warfare agents
D	dose
$D/At_{1/2}$	distribution/absorption half life
DMS	dimethanesulfonate
DND	Department of National Defense
DRDC	Defense Research Development Canada
ECG	electrocardiography
EEG	elcetroencephalography
$Et_{1/2}$	elimination half life
GA	tabun
GABA	γ-aminobutyric acid
GB	sarin
GD	soman

GF	cyclosarin
GGT	gamma-glutamyltransferase
GI	gastrointestinal
GLP	Good Laboratory Practices
HCO_3^-	bicarbonate
Hgb	hemoglobin
HI-6	(1-[[[4-(Aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyiminio)methyl] pyridinium
HI-6 2Cl	(1-[[[4-(Aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyiminio)methyl] pyridinium dichloride
HI-6 DMS	(1-[[[4-(Aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyiminio)methyl] pyridinium dimethanesulfonate
HPLC	high pressure liquid chromatography
IM	intramuscular
IV	intravenous
K	potassium
LD_{50}	median lethal dose
m	slope of the line
Mg	magnesium
NA	nerve agent
Na	sodium
NaCl	sodium chloride

NATO	North Atlantic Treaty Organization
nc	not calculated
nr	not reported
ns	no significant difference
OP	organophosphorous, organophosphates
P2S	pralidoxime mesylate
PAR	peak area ratio
Phos	phosphate
PK	pharmacokinetic
PNS	peripheral nervous system
R^2	coefficient of determination
SAP	Special Access Programme
SC	subcutaneous
SD	standard deviation
SSA	steady state anaesthesia
t	time
T_{max}	time to maximal concentration
UREA	urea nitrogen
V_d	volume of distribution
λ	rate constant

1. Introduction

1.1 History of Chemical Warfare

Throughout history there is evidence demonstrating that toxic chemicals have been used in wars, conflicts, malicious poisonings and executions. World War I introduced the modernization of chemical warfare when agents such as chlorine, phosgene and mustard agents were deployed against the allied forces. Organophosphorous nerve agents (NA) were first discovered in 1936 when a German chemist (Dr. Gerhard Schrader) working on developing novel pesticides isolated tabun (GA) and later, sarin (GB) (Holstege, Kirk, & Sidell, 1997; Szinicz, 2005). On-going research during World War II led to the discoveries of soman (GD) and cyclosarin (GF) (Salem, Ternay, & Smart, 2010). During the Cold War era both the United States and the Soviet Union amassed stockpiles of "G" agents while adding the newly discovered V agents such as VX and Russian VX (Wiener & Hoffman, 2004).

The most notable use of NA in modern time was during the Iran-Iraq War where it is estimated that one million Iranians were exposed to chemical warfare agents including tabun, sarin and sulphur mustard. It has been reported that an estimated 50, 000 deaths were directly attributable to NA exposure and an additional 100, 000 people were listed as suffering from chronic chemical injuries from exposure to the vesicant agent mustard (Firouzkouhi, Zargham-Boroujeni, Nouraei, Yousefi, & Holmes, 2013). More recently the terrorist attacks in Matsumoto and Tokyo, Japan in 1994 and 1995 respectively, saw the release of sarin gas and VX killing a total of 19 people and resulting in several hundred others being admitted to local hospitals for mild to moderate poisoning (Nozaki et al., 1995; Okumura et al., 1996; Yanagisawa, Morita, & Nakajima, 2006; Yanagisawa et al., 1995).

Chemical warfare agents including NA continue to be a significant threat to both military and civilian populations as evidenced most recently from reports on Syria's chemical warfare potential (Dolgin, 2013; Nicoll, Delaney, & Dung, 2013).

1.2 Organophosphorous Compounds

Organophosphorous (OPs) compounds are a class of highly toxic chemicals that include nerve agents and pesticides. Organophosphorous NAs are often further divided into two categories; the G-agents (GA, GB and GD) which are highly volatile and the V-agents including VX, which possess low volatility (Sawyer et al., 2011). All organophosphorous NAs and pesticides share a similar general structure. A central phosphorous atom shares two single bonds with alkyl groups (with one of them being an O-alkyl group), a double bond usually with an oxygen atom (may also be a sulfur group) and finally a single bond linking a leaving group, such as fluoride (labeled X), Figure 1.1 (Cannard, 2006; Kellar, 2006).
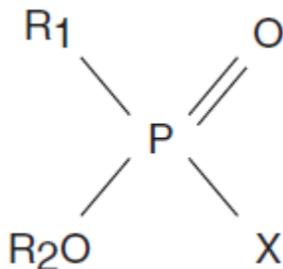
Figure 1.1 General structure of organophosphorous nerve agent.

The G series agents as shown in Figure 1.2 are all derivatives of phosphonic acid with either a cyanide or fluoride substituent and varying degrees of volatility, while VX has a sulphur substituent (Watson et al., 2009).
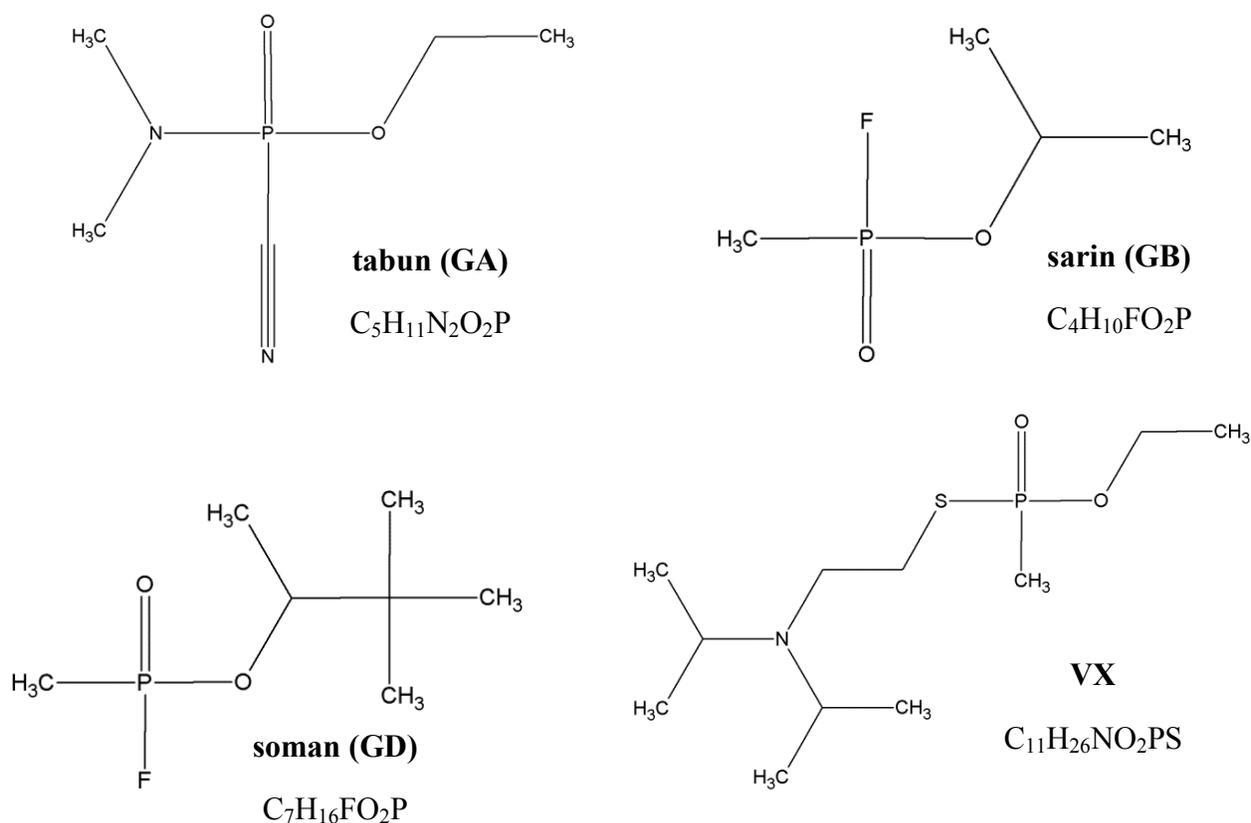
Figure 1.2 Chemical structures of G series organophosphorous nerve agents and VX.

NA can be absorbed by all routes, including inhalation, ingestion and dermal absorption. Their toxicity is not limited to an acute phase, as chronic effects such as abnormal EEG and ECG traces in addition to psychological issues have been reported in victims of the Tokyo subway incident (Nishiwaki et al., 2001; Okumura et al., 2005; Yanagisawa, et al., 2006).

1.2.1. Mechanism of Action of OPs

All organophosphates (OP's) exert their actions through the inhibition of acetylcholinesterase (AChE), which inhibits acetylcholine (ACh). AChE is a carboxylesterase enzyme that is found throughout the body: in particular on red blood cell membranes, neuromuscular junctions and cholinergic synapses. Acetylcholinesterase is responsible for the breakdown of ACh into acetate

and choline within the extracellular fluid which terminates its physiological effects at post-synaptic receptors. Acetylcholine is a neurotransmitter that binds with nicotinic and muscarinic receptors. Within the peripheral nervous system (PNS) ACh plays a role in skeletal muscle contraction in addition to the regulation of cardiac rhythm. In the central nervous system (CNS) ACh plays a role in learning and memory and its mis-regulation has been linked to Alzheimer's disease and other forms of dementia.

Under normal physiological conditions the action of ACh at the neuromuscular junction is terminated by cleavage mediated by AChE. The choline moiety of ACh binds to the anionic site of AChE while the acetyl groups binds with the esteric AChE site. The choline moiety is then cleaved off through hydrolysis resulting in acetic acid and choline (which is recycled) and free AChE is again available to react with additional ACh (Taylor, 2011).

In the presence of irreversible anticholinesterase agents, such as organophosphorous NA, hydrolysis of ACh is inhibited by the direct inhibition of the active site of AChE. Nerve agents form a bond with the serine hydroxyl group of the esteric AChE binding site, resulting in liberation of the NA leaving group to form a stable phosphorylated AChE, thus disabling its ability to bind and hyrdolyse ACh. Inhibition of AChE leads to an overt stimulation of cholinergic receptors and results in a severe cholinergic crisis. This interaction and inhibition may become permanent if the agent-enzyme complex has undergone the process of aging: a process whereby the NA-AChE complex is no longer capable of being reactivated as a result of the formation of a covalent bond (through dealkylation) between the NA and AChE (Soukup et al., 2010). Nerve agent aging rates (time for 50% of cholinesterases to age) range from as little as 2 minutes for soman and in excess of 40 hours for VX, necessitating the need for a rapid and efficacious treatment in the case of GD poisoning or unknown NA exposure (Sidell, 1974b; Sidell & Groff, 1974).

1.2.2. Signs and Symptoms of OP Poisoning

The clinical signs and symptoms of organophosphorous insecticide and NA poisoning are

generally attributable to ACh accumulation as a result of AChE inhibition (Marrs, 1993). Depending on the route of exposure, the onset of symptoms can occur anywhere from seconds to hours following exposure. Typically, initial signs following an inhalation exposure include salivation, lacrimation, urination, defecation, GI distress and emesis; all consequences of increased cholinergic stimulation of muscarinic receptors (Eyer, 2003). As toxicity progresses, signs and symptoms include tightness in the chest, shortness of breath, muscle fasciculations (attributed to overt nicotinic stimulation), increased sweating, salivation and lacrimation, as well as GI effects including nausea, vomiting, cramps, diarrhea and involuntary defecation and/or urination. Signs of severe toxicity also include respiratory muscle paralysis and centrally mediated symptoms such as seizures and respiratory depression (Wiener & Hoffman, 2004). Death is usually the result of respiratory failure attributed to increased respiratory secretions in combination with bronchospasm and diaphragm paralysis (Chauhan et al., 2008; McDonough & Shih, 1997).

1.2.3. Toxicity of OP Nerve Agents

The toxicity of organophosphorous NA has been reported for several animal models.   However, only guinea pig and swine data are presented for comparison here. VX has the most potent estimated median lethal dose (LD_{50}) in humans following percutaneous exposure at 0.14 mg/kg followed in toxicity by GD (5 mg/kg) and GB (14.3 mg/kg) (Chauhan, et al., 2008). The percutaneous LD_{50} for VX in anaesthetized swine is reported to be 79.6 µg/kg (Bjarnason et al., 2008), while the intravenous LD_{50} values reported for GB and GD are 10.1 µg/kg (Sawyer, Mikler, Tenn, Bjarnason, & Frew, 2012) and 4.6 µg/kg (McKenzie et al., 1996) respectively. Similarly, in guinea pigs VX is the most potent nerve agent with a 9 µg/kg LD_{50} when administered subcutaneously followed by soman (28 µg/kg) and sarin (42 µg/kg) (Krewski et al., 2013).   The period between NA exposure and the manifestation of clinical signs of poisoning is highly variable between routes of administration. Clinical symptoms have been reported as early as 30 seconds following inhalation exposure and up to as long as 18 hours following percutaneous exposure (Wiener & Hoffman, 2004). The toxicity of NAs combined with their

varied latency period and the potential to intoxicate individuals without detection necessitates an efficacious and broad spectrum treatment.

1.3. Treatment of Organophosphorous Poisoning: Oximes

Oximes are a class of chemical compounds belonging to the imines that share the same basic structure as shown in Figure 1.3.
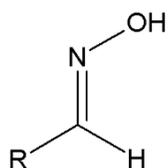
Figure 1.3 General oxime structure. R represents an organic side chain.

Oximes are used therapeutically for the treatment of organophosphorous pesticide and NA poisoning. Pralidoxime (2-PAM), obidoxime and HI-6 represented in Figure 1.4 are all examples of oximes used for treatment of NA poisoning.
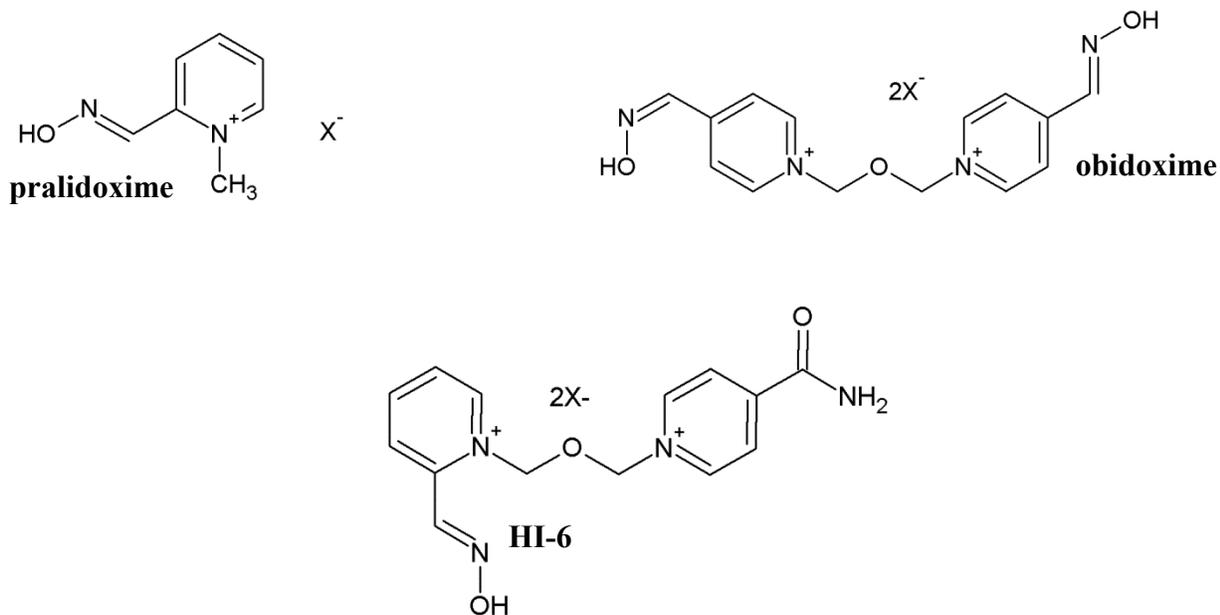
Figure 1.4 Examples of oximes used for treatment of NA poisoning.

The initial discovery of oximes for the treatment of NA poisoning can be linked to Irwin B. Wilson's discovery of pralidoxime over 50 years ago (Alston, 2005) and Wilson and Ginsburg's supportive findings in the 1950's that in fact 2-PAM was an efficacious reactivator of phosphorylated cholinesterases. Several additional oximes including trimedoxime, methoxime and obidoxime were all investigated following 2-PAM. However, all presented limited efficacy against some of the agents and none represented a truly broad spectrum antidote.

1.3.1. Mechanism of Action of Oximes

Oximes reactivate phosphorylated cholinesterase through displacement of the phosphoryl moiety due to their high affinity for the enzyme and strong nucelophicity (Jokanović & Stojiljković, 2006). The oxime-enzyme reaction proceeds as a two-step reaction. The oxime is orientated in the anionic (active) site of AChE via the positively charged quaternary nitrogen group found on the oxime (Wiener & Hoffman, 2004). The phosphate group of the nerve agent (bound in the esteric site) undergoes a nucleophilic reaction by the oxime in turn displacing the nerve agent from the enzyme (reactivating the enzyme) and forming a phosphorylated oxime (Eyer & Worek, 2007). Liberation of AChE allows for the hydrolysis of ACh and therefore prevents further unchecked cholinergic stimulation at muscarinic and nicotinic receptors.

The rate of AChE reactivation depends on the structure of the phosphoryl moiety bound to the enzyme (in other words it depends on the NA present), the concentration of the oxime present at the active site and the rate of aging (Milatović & Jokanović, 2009). Additionally, NA inhibited enzymes may be reactivated at varying rates and with varying efficacies depending on the structure of the oxime relative to the structure of the enzyme nerve agent complex (Lundy, Raveh, & Amitai, 2006). Consequently, not all oximes are equally effective against all nerve agents.

1.3.2. Current Treatments

For many years obidoxime and pralidoxime have been used by militaries worldwide as their

oximes of choice. The problem with obidoxime and pralidoxime is that they are weak reactivators of soman-inhibited AChE and thus provide very limited protections in the event of soman intoxication (Lundy, et al., 2006). HI-6 was originally synthesized in Germany (Hagedorn, Gündel, Hoose, & Jenter, 1976) and has been found to be not only effective against agents such as sarin or VX, both of which can be managed with administration of either obidoxime or pralidoxime, but also effective against cylclosarin, which is more resistant to reaction by pralidoxime and obidoxime (Lundy, et al., 2006).

The current Canadian Armed Forces (CAF) treatment of NA poisoning includes administration of an oxime (HI-6 DMS, 636 mg) to reactivate AChE in combination with atropine (2 mg, an acetylcholine muscarinic receptor antagonist) contained in an auto-injector. Up to three auto-injectors may be administered within 30 minutes of NA exposure until the casualty is able to receive additional medical attention. Auto-injector treatment may need to be supplemented with an anticonvulsant such as diazepam; this is also administered intramuscularly. HI-6 is administered to reverse the inhibitory effect of the NA on AChE so that free ACh can be broken down and decrease the excessive cholinergic stimulation. Atropine (AS), a competitive antagonist for muscarinic acetylcholine receptors is administered to directly inhibit ACh binding to muscarinic receptors and thus reduce symptoms including: miosis, salivation, lacrimation, diarrhea, bronchorrhea and may help control bradycardia. Co-administration of atropine is critical as its immediate actions significantly reduce respiratory secretions and constriction, as well as limiting overt CNS stimulation. Finally, a benzodiazepine such as diazepam may be administered to end the propagation of convulsions that result due to cholinergic depolarization of neurons centrally. Diazepam achieves this by hyperpolarizing neurons within the CNS through its enhancement of γ-aminobutyric acid (GABA, major inhibitory neurotransmitter of CNS) at gabaergic receptors (Eddleston, Buckley, Eyer, & Dawson, 2008).

1.3.2. Future Treatments

Currently, the treatment of NA poisoning relies on the administration of HI-6/atropine auto-injectors. Although auto-injector administration is an effective treatment, the need for additional secondary treatment is apparent due to the latency period and NA depot created by dermal

exposure to NAs, as well as our understanding of the implications of pesticide poisoning and the sustained treatment that is required (Johnson et al., 2000; Pawar et al., 2006; Tush & Anstead, 1997). Initial auto-injector administration of HI-6 may not fully mitigate NA poisoning due to an inability to sustain and maintain an optimal plasma concentrations from auto-injector doses alone. Additionally, NA compounds are highly lipophilic and may form depots that persist much longer than anticipated, leading to re-inhibition and a secondary cholinergic crisis (Eyer, 2003) again supporting the need for an option of sustained treatment where the dose can be adjusted accordingly.

The Canadian Armed Forces Health Services (CFHS) and Defense Research Development Canada (DRDC) within the Department of National Defense (DND), are developing an intravenous formulation of HI-6 to allow for sustained delivery and optimized plasma concentrations of HI-6 to allow for continuous and optimal treatment following initial auto-injector administration.

1.4. HI-6

HI-6 is a bispyridinium aldoxime first synthesized in Germany by Ilse Hagedorn and Irmo Stark in 1966 (Hagedorn, et al., 1976). It is comprised of an oxime moiety (as shown in Figure 1.3) attached to a quaternary nitrogen pyridinium ring. Two salts of HI-6 are currently available: HI-6 2Cl (1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyiminio)methyl] pyridinium dichloride (MW 376.22 g/mol) and HI-6 DMS (1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyiminio)methyl] pyridinium dimethanesulfonate (MW 477.49 g/mol) as shown in Figure 1.5.
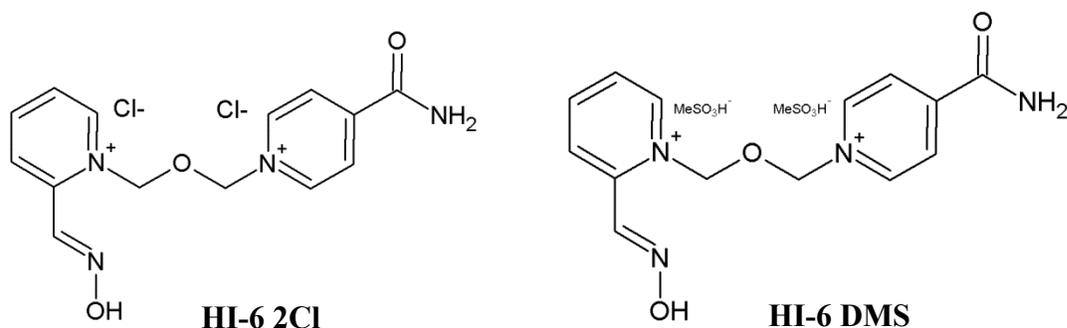
Figure 1.5 Chemical structures of HI-6 2Cl and HI-6 DMS.

Originally HI-6 was synthesized using a dichloride (2Cl) salt. However the alternative salt dimethylsulfonate (DMS) was selected for synthesis in order to increase the solubility of HI-6 in solution (Thiermann, Seidl, & Eyer, 1996). Additionally the manufacturing process for HI-6 2Cl requires BCME (bis(chloromethyl) ether) which is highly carcinogenic and a regulated chemical that is no longer allowable in manufacturing processes in most countries (Broomfield, Hackley, Hahn, Lenz, & Maxwell, 1981; Yang, Yoon, Seong, Park, & Jung, 2003), while HI-6 DMS can be synthesized without carcinogenic intermediates (Kuca et al., 2008).

Of currently fielded oximes, HI-6 has been shown to be the most efficacious treatment of NA poisoning for soman, sarin and VX (Bajgar, Fusek, Kuca, Bartosova, & Jun, 2007; Jokanović & Stojiljković, 2006; Koplovitz & Stewart, 1994). Additionally, HI-6 2Cl has a low toxicity compared to other available oximes as reported by Bartosova et al. (2006) and Jokanović and Sotjiljković (2006). Currently, no published data is available comparing the toxicity of HI-6 DMS to other available oximes. Preliminary unpublished data in rats suggests that both HI-6 2Cl and DMS have similar toxicity levels. The acute toxicity values found in literature suggest that 2-PAM is two times more toxic than HI-6 (Clement, 1981). Comparing the dosage licensed for use for 2-PAM: 1800 mg in man (3 x auto-injectors containing 600 mg each) while taking into consideration the acute toxicity difference between 2-PAM and HI-6 and assuming that HI-6 is as efficacious and tolerable if not more than 2-PAM, the dose of HI-6 could in theory be two times larger (3600 mg in man) than that administered for 2-PAM. This difference in toxicity and

ultimately tolerable dosage amounts, could potentially provide even greater protection than the levels currently being tested for HI-6. This difference in toxicity and the potential dosing limitations underscores the need to understand the pharmacokinetics of HI-6 over a large concentration range.

1.4.1 Absorption of HI-6

Oximes in general have a low lipophilic character (Karasova, Pavlík, Chladek, Jun, & Kuca, 2013) due to their bis-pyridinium structure and ionized form in solution making them poorly and slowly absorbed compounds that do not readily cross the blood brain barrier. Oral formulations of HI-6 have been evaluated in dogs (Maksimović, Jovanović, Kovačević, & Bokonjić, 1987) and man (Jovanovic, Maksimovic, Joksovic, & Kovacevic, 1990), and were found to have poor absorption from the gastrointestinal tract and low bioavailability (Voicu, Bajgar, Medvedovici, Radulescu, & Miron, 2010). Poor oral absorption can most likely be attributed to the structure of HI-6, although Jovanovic and coworkers (1990) also suggested that first pass metabolism may be involved. Plasma protein binding of HI-6 has been reported to be less than 5% (Ligtenstein & Kossen, 1983) indicating that a large percentage of HI-6 is available for therapeutic action. Poor oral absorption and the therapeutic need for rapid administration of HI-6 have led to the development of intramuscular and intravenous formulations. Although highly soluble in water and saline, HI-6 in solution does not remain stable and has been considered impractical to store for any length of time in solution (Nyberg et al., 1995) thus the requirement for wet/dry autoinjectors (Spöhrer, Thiermann, Klimmek, & Eyer, 1994) or an intravenous product that can easily be reconstituted for administration. Several studies have compared the rates of absorption and bioavailability of HI-6 following intramuscular and intravenous administration in rats (Simons & Briggs, 1985), rabbits (Lukey, Woodard, Clark, & McCluskey, 1989) and dogs (Simons & Briggs, 1983). All of these studies indicated that virtually 100% of an intramuscularly administered dose is absorbed, suggesting that equimolar doses administered either IV or IM will result in equivalent bioavailability and absorption.

1.4.2 Distribution of HI-6

The efficacy of HI-6 following NA exposure is entirely dependent upon its plasma concentration and the distribution of the oxime throughout the CNS, PNS and neuromuscular junctions. Following IM administration HI-6 is rapidly distributed from the site of injection and transported throughout the body via the bloodstream (Karasova, et al., 2013). The transport of oximes across membranes throughout the body occurs via passive transport and is subject to structure of various membrane lipids and proteins (Herkert, Freude, Kunz, Thiermann, & Worek, 2012).

Studies examining the distribution of HI-6 in rats following an IM injection indicated that the highest levels of HI-6 were found in the kidneys with decreasing amounts present in cartilage, plasma, liver, heart, lung, diaphragms and within the CNS (Lundy, Hand, Broxup, Yipchuck, & Hamilton, 1990). The limited amounts of HI-6 found within the CNS (brain and cerebrospinal fluid) is not surprising due to the polarity of HI-6. The volume of distribution of HI-6 approximates that of the extracellular fluid (Clement, Bailey, Madill, Tran, & Spence, 1995), reinforcing the inability of HI-6 to readily cross membranes.

1.4.3 Metabolism of HI-6

There has been limited examination of the metabolism or biotransformation of HI-6. HI-6 is known to release large amounts of cyanide *in vitro*. A study examining the concentrations and elimination of cyanide following HI-6 administration in dogs reported that cyanide liberation from HI-6 is the result of chemical degradation and not a metabolic process (Eyer, Kawan, & Ladstetter, 1987). Only 4% of all of the HI-6 administered resulted in chemically liberated cyanide with the majority of HI-6 (80%) eliminated unchanged in urine and the remainder metabolized.

Another study examined the biotransformation of HI-6 in rats, dogs and rhesus monkeys, and indicated that no metabolites of HI-6 could be found in the brain, liver, kidney or diaphragm from 30 minutes up to 24 hours following IM administration of HI-6 (Ecobichon, Comeau, O'Neil, & Marshall, 1990). However, metabolites were found in urine samples as previously reported, indicating possible biotransformation as a result of an enzymatic mechanism, most

likely hydrolysis. 2-PAM is one of the metabolites found in urine samples following HI-6 administration. This catabolic product is not thought to be due to liver metabolism, as autoradiograph studies showed that HI-6 concentrations below plasma levels were detected in the liver (Garrigue et al., 1990) indicating that little HI-6 is taken up by the liver. Total metabolism accounted for less than 10% of administered HI-6 and does not influence its therapeutic activity.

1.4.4 Elimination of HI-6

Elimination of HI-6 from the body appears to be mainly due to urinary excretion; Clement, et al. (1995) reported that renal clearance of HI-6 approached the glomerular filtration rate with approximately 60% of HI-6 excreted unchanged in urine within the first 24 hours following administration, supporting the conclusion that HI-6 is not reabsorbed. Although glomerular filtration accounts for the majority of HI-6 elimination, a small amount can also be accounted for by tubular secretion, a process that is facilitated by the polar nature of oximes and has been reported for the elimination of 2-PAM, an oxime similar to HI-6 (Shek, Higuchi, & Bodor, 1976). Autoradiography studies found less than 4% of HI-6 administered in fecal matter within 24 hours of administration, indicating that the biliary route in not a likely excretion route of HI-6 (Garrigue, et al., 1990).

1.4.5 HI-6 2Cl vs HI-6 DMS

Two studies have directly compared the pharmacokinetic profiles of equimolar doses of HI-6 2Cl and HI-6 DMS when administered in swine. The authors of these studies reported that at equimolar doses of the two HI-6 formulations, identical pharmacokinetic profiles were observed (Karasová et al., 2013; Lundy et al., 2005). 2-PAM, a similar oxime, is also available in a chloride or mesylate salt form. The solubility differences between HI-6 2Cl and HI-6 DMS have been well documented and lead one to postulate that there may be additional physiochemical differences such as the pKa and pH of solution that may lead to varied absorptive and distributive properties of each salt. For this reason additional studies were undertaken to

examine the differences between HI-6 2Cl and HI-6 DMS under various administration conditions and will be discussed in detail later in this thesis.

1.4.6 IV vs. IM Administration

Numerous studies have been conducted examining the pharmacokinetics of HI-6 2Cl when administered either IV or IM. However, very few have directly compared routes of administration within the same study. Of the studies conducted, very limited pharmacokinetic parameters were examined and only in one study were equimolar doses between administration routes compared (Baggot, Buckpitt, Johnson, Brennan, & Chung, 1993; Karasová, et al., 2013; Lukey, et al., 1989; Nyberg, et al., 1995).

As most of the efficacy data to date has been conducted using the 2Cl salt in order for regulatory agencies to accept that both HI-6 salts are similar, it is necessary to clearly understand the pharmacokinetics of HI-6 2Cl and DMS when administered both IM and IV. This is critical information that is required to support the development of an extended care product for treatment of NA exposure using titrated infusions of HI-6.

1.4.7 HI-6 and Anaesthesia

Often pharmacokinetic studies are carried out using anaesthetized animals not only for ethical reasons, but also due to the increased safety and ease of sample collection for those involved in the study. It is important when using these animal studies for the preclinical evaluation of oximes for treatment of NA poisoning to understand if anaesthesia alters the pharmacokinetics or pharmacodynamics of the oxime.

Two studies have examined the change in the pharmacokinetics of HI-6 2Cl when administered to conscious or anaesthetized rhesus monkeys (Clement, Lee, Simons, & Briggs, 1990) and dogs (Klimmek & Eyer, 1986). Dogs administered HI-6 2Cl, IM while anaesthetized with thiopental-Na and chloralose exhibited a significantly faster absorption rate, a decreased maximal concentration, longer elimination half-life and decreased renal clearance of HI-6 compared to

conscious dogs administered an equimolar dose (Klimmek & Eyer, 1986). The authors suggested that the decrease in the elimination of HI-6 could be attributed directly to the cardiovascular effects of the anaesthetic. Rhesus monkeys anaesthetized with methoxyflurane and administered HI-6 2Cl, IM showed similar changes in the pharmacokinetics of HI-6 as those reported for dogs: the elimination half-life was longer in anaesthetized monkeys and clearance was reduced compared to conscious animals (Clement, et al., 1990). Examination of the effect of anaesthetic on both HI-6 2Cl and DMS is necessary to further understand the role anaesthetic may play on the determination of critical pharmacokinetic parameters and how this may translate to treatment in conscious humans.

1.4.8 Dosage differences

Health Canada's Special Access Programme (SAP) is the program that allows the Canadian Armed Forces to access HI-6 for use. The SAP allows practitioner's access to pharmaceutical products that are not licensed for sale in Canada to treat patients with life-threatening conditions, where conventional treatments may not be suitable.

The standard dosages of HI-6 allowed for use in an autoinjector under the Special Access agreement were chosen not based on human therapeutic or toxicity data, but rather due to the solubility restrictions of the confined volume of the autoinjector (Clement, et al., 1995; Kušic, Boškovic, Vojvodić, & Jovanović, 1985; Thiermann, Seidl, et al., 1996). The HI-6 2Cl autoinjector contains 500 mg while the DMS autoinjectors contains 633 mg (molar equivalent of 2Cl). Lundy, et al. (2005) proposed that due to the fact that the largest safe dose of HI-6 in man is estimated to be substantially greater than the dose administered in an autoinjector, larger doses of HI-6 DMS (due to its better solubility) may be used to better counteract the effects of NA poisoning. The pharmacokinetics of HI-6 at these higher doses should therefore be understood.

Three ascending dose studies have been conducted: one examining HI-6 DMS in swine (633 mg and 1899 mg) (Lundy, et al., 2005) and two examining HI-6 2Cl in either rats (10-50 mg/kg) (Maksimović, 1979) or humans (62.5, 125, 250 and 500 mg) (Clement, et al., 1995). All three studies reported no significant difference between pharmacokinetic parameters calculated

between ascending doses, suggesting that the pharmacokinetics of HI-6 is not altered even at doses as high as 1899 mg of HI-6 DMS (three times the dose of one autoinjector). In the human clinical trial there were indications that time to maximal concentration and absorption half-life increased as doses were increased whereas, elimination half-life and clearance decreased as doses were increased.

Further investigations of ascending dose studies need to be conducted as the highest dose examined (1899 mg) has only been evaluated for HI-6 DMS administered IV in one group of four animals in one study.

1.4.9 HI-6 Co-administration with atropine sulphate

An understanding of the extent of atropine sulphate (AS) bioavailability and its interaction with the oxime it is being co-administered with, is critical to achieve the most favorable therapeutic outcome when treating NA poisoned casualties. Several studies have examined the effect an oxime (in particular 2-PAM) has on the absorption of AS when co-administered. These studies suggest that when co-administered at the same site, 2-PAM significantly reduces the absorption of AS (Sidell, Magness, & Bollen, 1970), whereas co-administration of the mesylate salt of pralidoxime resulted in improved AS absorption (Holland, Parkes, & White, 1975; Holland & White, 1971). This difference in absorption patterns of AS when co-administered with two different salts of pralidoxime is thought to be due to osmotic forces that directly affect the absorption of AS at the site of injection (Sidell, 1974a). Pralidoxime mesylate (P2S) has a much lower osmolarity than that of pralidoxime chloride, supporting this proposed theory on AS absorption and indicating that the HI-6 salts whose osmolarity are more similar to P2S should not affect AS absorption when co-administered (Mikler, Stewart, Bohnert, Patel, & Loon, 2009).

Studies conducted in rhesus monkeys reported that co-administration of atropine with HI-6 2Cl did not have any effect on the pharmacokinetics of HI-6 (Clement, et al., 1990). Dogs administered HI-6 2Cl alone or with AS did not show significant differences in the rates of atropine absorption (Thiermann, Radtke, Spohrer, Klimmek, & Eyer, 1996a). However, plasma concentrations of HI-6 in the treatment group where HI-6 and AS were co-administered, were higher than those treated with HI-6 only (Jovanović, Kovačević, & Maksimović, 1992).

Currently there is inadequate evidence to indicate clearly whether or not co-administration of AS attenuates HI-6 absorption when administered IM or IV. The pharmacokinetic interaction between HI-6 and AS needs to be further examined in support of licensure work, particularly since all studies examining HI-6 and AS conducted this far have only used the dichloride salt.

1.4.10 Historical HI-6 Pharmacokinetic Data

The pharmacokinetics of HI-6 2Cl have been studied in several animal models including mice (Clement, Simons, & Briggs, 1988), rats (Ligtenstein & Kossen, 1983; Simons & Briggs, 1983; van Helden, van der Wiel, Zijlstra, Melchers, & Busker, 1994), guinea pigs (Busker, Zijlstra, Philippens, Groen, & Melchers, 1996a), dogs (Baggot, et al., 1993; Simons & Briggs, 1983; Spöhrer, et al., 1994), swine (Bohnert, Vair, & Mikler, 2010; Lundy, et al., 2005; Nyberg, et al., 1995), sheep (Moore, Hayward, Steve T, & Lukey, 1991) and monkeys (Clement, et al., 1990). Bohnert, et al. (2010) and Lundy et al. (2005) have published preliminary data on the pharmacokinetics of HI-6 DMS in guinea pigs and swine. However, the data presented is limited and needs to be extended to include an infusion scenario.

Clinical trials have been conducted in humans for both HI-6 2Cl (Clement, et al., 1995; Jovanovic, et al., 1990; Kušic, et al., 1985) and HI-6 DMS (Morelli & Jensen, 2010).

The following is a brief summary of previously determined pharmacokinetic parameters of HI-6 2Cl and HI-6 DMS administered either IM or IV where available for guinea pigs, swine and humans.

Table 1.1 Reported pharmacokinetic parameters for HI-6 2Cl administered IM in guinea pigs, swine and humans. Data presented are the range of values reported. T_{max} represents time to maximal concentration, V_d represents volume of distribution and *Cl* represents total body clearance. Guinea pigs were administered 350 µmol/kg (or approximately 130 mg/kg) of HI-6 2Cl and 15mg/kg AS, IM (Busker, Zijlstra, Philippens, Groen, & Melchers, 1996b). Swine were administered either an autoinjector of HI-6 2Cl (500mg) and AS (2mg) (Göransson-Nyberg et al., 1995) (numbers reported on first line), or 500 mg of HI-6 2Cl IM using a syringe and 22G 1" needle (Lundy, et al., 2005). Humans were administered various doses (62.5, 125, 250 or 500 mg) of HI-6 2Cl IM (Clement, et al., 1995).

	T_{max} (min)	Elimination Half-Life (min)	V_d (L/kg)	*Cl* (ml/min)
Guinea Pig	20[1][a]	24 – 26[a]	0.09 – 0.14[a]	nr
Swine	8 – 12[b]	53 – 76[b]	0.28 – 0.47[b]	nr
	7 – 11[c]	64 – 98[c]	0.46 – 0.60[c]	98 – 130[c]
Human	28 – 36[d]	64 -78[d]	0.22 – 0.28[d]	232 – 297[d]

nr, represents not reported

[1] *Note.* [a]Values from "Comparison of the Efficacy of Single or Repeated HI-6 Treatment Following Soman Poisoning in Guinea Pigs and Marmosets," by Busker, et al., 1996, Toxicology, 112(3), p.183-194. [b]Values from "Treatment of Organophosphate Poisoning in Pigs: Antidote Administration by a New Binary Autoinjector," by Göransson-Nyberg, et al., 1995, Archives of Toxicology, 70(1), p.20-27. [c]Values from "The Pharmacokinetics and Pharmacodynamics of Two HI-6 Salts in Swine and Efficacy in the Treatment of GF and Soman Poisoning," by Lundy, et al., 2005, Toxicology, 208(3), p.399-409. [d]Values from "The Acetylcholinesterase Oxime Reactivator HI-6 in Man: Pharmacokinetics and Tolerability in Combination with Atropine," by Clement, et al., 1995, Biopharmaceutics and Drug Disposition, 16(5), p.415-425.

Table 1.2 Reported pharmacokinetic parameters for HI-6 DMS administered IM in swine and humans. Data presented are the range of values reported. T_{max} represents time to maximal concentration, V_d represents volume of distribution and Cl represents total body clearance. Swine were administered 633 mg of HI-6 DMS, IM using a syringe and 22G 1" needle (Lundy, et al., 2005). Humans were administered 636 mg of HI-6 DMS, IM using a syringe and 22G 1" needle (Morelli & Jensen, 2010). At this time no studies could be found that examined the pharmacokinetics of 636 mg of HI-6 DMS, IM using a syringe and 22G 1" needle (Morelli & Jensen, 2010) of HI-6 DMS administered IM in guinea pigs.

	T_{max} **(min)**	**Elimination Half-Life (min)**	V_d **(L/kg)**	Cl **(ml/min)**
Swine	7 - 11[2e]	77 – 88[e]	0.44 – 0.54[e]	113 – 135[e]
Human	40 – 60[f]	67 – 85[f]	0.52 – 0.54[f]	340- 362[f]

Currently the only data that has been published on the pharmacokinetics of either HI-6 2Cl or HI-6 DMS when administered IV was conducted in guinea pigs. Although Lundy, et al. (2005) examined HI-6 DMS administered IV to swine, the pharmacokinetic parameters were not reported but rather only a statement made that the pharmacokinetics were similar to that of an IM injection.

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[2] *Note.* [e]Values from "The Pharmacokinetics and Pharmacodynamics of Two HI-6 Salts in Swine and Efficacy in the Treatment of GF and Soman Poisoning," by Lundy, et al., 2005, Toxicology, 208(3), p.399-409. [f]Values from "Phase I, Open-Label, Fed, Single-dose, Pharmacokinetic, Safety and Tolerance Evaluation of Intramuscularly Administered HI-6 Dimethanesulfonate," by Morelli, G. & Jensen, M.L., 2010, Directorate Health Services Operations, AA81840.

Table 1.3 Reported pharmacokinetic parameters for HI-6 2Cl and HI-6 DMS administered IV in guinea pigs. Data presented are the mean value ± SD. Guinea pigs were administered and IV bolus of HI-6 2Cl (41.5 mg/kg) or HI-6 DMS (52.6 mg/kg).

	HI-6 2Cl	**HI-6 DMS**
Elimination Half-life (min)	47.36 ± 9.70[3]	53.74 ± 14.68
V_d (L/kg)	0.260 ± 0.036	0.355 ± 0.054
***Cl* (ml/min/kg)**	3.96 ± 1.06	4.77 ± 1.22

Although a large amount of research has been conducted on the pharmacokinetics of HI-6 2Cl in numerous species there are several gaps in understanding the profile of HI-6. A direct comparison between HI-6 2Cl and HI-6 DMS has not been completed under equimolar dose administration via the same route in the same species. Little is understood about the effect of atropine co-administration on the pharmacokinetics of HI-6 2Cl or DMS. Additionally the role that anaesthetic plays in the pharmacokinetics of HI-6 has not been examined for the anaesthetic isoflurane (a commonly used inhaled anaesthetic for both animal models and human use clinically). Finally the pharmacokinetics determined for both HI-6 2Cl and DMS need to be evaluated when applied to dosing calculations for extended intravenous infusions of HI-6.

1.5. Experimental Rationale

Due to the extremely toxic nature of chemical warfare agents (CWA) it is unethical to perform testing on humans and thus suitable animal alternatives are used for research and development purposes. This is true not only for CWAs but unlicensed pharmaceutical products used for the

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[3] *Note.* The data presented is from "Development and validation of a sensitive HPLC method for the quantification of HI-6 in guinea pig plasma and evaluated in domestic swine", by S. Bohnert, C. Vair and J. Mikler, 2010, *Journal of Chromatography B, 878,* p.1412. Reproduced with permission.

treatment of nerve agent exposure as is the case for the oxime HI-6.   Currently HI-6 is available to the Canadian Armed Forces under the SAP. In order to attain licensure of HI-6 numerous studies must be carried out in animals to ensure its safety (tolerability and toxicity), efficacy and pharmacokinetics prior to human clinical trials.

Determining the pharmacokinetic parameters of HI-6 is critical not only for licensure purposes, but also for its appropriate use and dosing when used in a clinical setting due to NA exposure. It is important to examine at least two animal models. This would include: a small animal model to ensure that initial dosing estimates are appropriate and non-toxic. A large animal model is also required for use in order to examine the properties of the compound in a model as close to humans as possible, and logical for that particular compound. Examining multiple routes of administration of the compound is important for determining an accurate pharmacokinetic profile, as different administration routes may elicit drastically different properties that would require dosing regimens to be altered accordingly. Similarly, it is important to understand the effects that anaesthetic may play on the pharmacokinetics of HI-6: the intended use of HI-6 is in conscious subjects and as such it is important to understand if anaesthesia (as is used in most of the animal studies conducted) alters the pharmacokinetic profile of HI-6 in a clinically relevant manner. Finally it is important to understand if co-administration of atropine with HI-6 changes the pharmacokinetics of HI-6, particularly when considering the possibility of extended therapies of HI-6 (such as an infusion) when atropine may not be given continuously or at all depending on the clinical symptoms the casualty is manifesting. This thesis describes work carried out to characterize and understand the pharmacokinetic parameters of HI-6 under different conditions, to facilitate its eventual licensure.

1.6. Animal Models

1.6.1. Guinea Pigs

The guinea pig has been selected as the small animal of choice when studying the effects of NA and/or their treatments because guinea pigs have relatively low levels of carboxylesterase when

compared to mice (Maxwell & Koplovitz, 1990) and rats (Gordon, Leadbeater, & Maidment, 1978). Carboxylesterase acts as an endogenous scavenger of NAs and thus higher levels of carboxylesterase require a larger dose of NA to produce an equivalent toxicity. Although carboxylesterase levels in guinea pigs are not as low as swine or primates, guinea pigs represent the best rodent model for OP and oxime studies (Wetherell, Price, Mumford, Armstrong, & Scott, 2007). Additionally guinea pigs represent a widely accepted model by North Atlantic Treaty Organization (NATO) member countrires for use when investigating NAs and oximes.

1.6.2. Domestic Swine

Domestic swine were selected as a suitable larger scale animal model when examining the effects of nerve agents and oxime for several reasons. Serial blood sampling is easily accomplished in a swine model allowing for highly accurate pharmacokinetic profile determination. Additionally, swine share many anatomical similarities with humans and can easily be monitored for physiology and other clinical chemistry and hematology parameters that can be correlated to human clinical results (Critser, Laughlin, Prather, & Riley, 2009; Douglas, 1972; Hannon, Bossone, & Wade, 1990). There is a clear understanding on the kinetics of AChE and NAs in swine and how this can be related to humans (Aurbek, Thiermann, Szinicz, Eyer, & Worek, 2006; Worek et al., 2008) making the domestic swine model very useful for bridging the gap between small animal studies and clinical trials for oxime licensure. Finally, there is a growing body of published work examining the use of domestic swine exposed to NA and their treatment while under anaesthesia (Dorandeu et al., 2007).

2. Hypotheses

1. The pharmacokinetics of HI-6 2Cl and DMS are similar to one another;
2. The elimination pharmacokinetics of HI-6 are similar when administered wither IV or IM;

3. The pharmacokinetics of HI-6 are different when administered to anaesthetized or non-anaesthetized models;

4. The pharmacokinetics of HI-6 are similar when different doses of HI-6 are administered; and

5. Co-administration with atropine will have no effect on the pharmacokinetic profile of HI-6 (2Cl and DMS) in either guinea pigs or domestic swine.

3. Objectives

1. Determination of the pharmacokinetic profile of HI-6 when:
   a. Administered to anaesthetized or non-anaesthetized guinea pigs intramuscularly;
   b. Administered by different routes (intramuscular or intravenous) in guinea pigs and domestic swine;
   c. Administered at different doses (low and high) in domestic swine; and
   d. Co-administered with atropine sulphate in guinea pigs and domestic swine.

2. To assess the validity of the derived pharmacokinetic parameters of HI-6 by achieving a target plasma concentration of HI-6 over the course of an infusion; and

3. Comparison of the derived guinea pig and domestic swine pharmacokinetics of HI-6 to known human HI-6 pharmacokinetics.

4. Materials and Methods

4.1 Animal Care and Handling

All animal studies were conducted within the confines of protocols approved by the DRDC (Defense Research and Development Canada) Animal Care Committee in accordance with the Canadian Council on Animal Care (CCAC) guidelines. In conducting this research the following guidance documents published by the CCAC were adhered to: "Guide to the Care and Use of Experimental Animals" and "The Ethics of Animal Experimentation".

4.1.1 Guinea Pigs

Male Hartley guinea pigs 380 – 660g (five to six weeks old) of high health status were obtained from Charles-River (Saint-Constant, QC). Animals were acclimatized for at least 10 days prior to use and were housed in pairs in clear shoebox cages. All animals were housed at room temperature (16-24ºC) with a 12 hour light/dark cycle. Standard guinea pig chow (Lab Diet®, Richmond, IN) and tap water were available *ad libitum* in addition to carrot slices (20 – 40g) provided daily. Small red translucent shelters were placed in each cage for environmental enrichment.

Surgical cannulation was either performed by Charles-River prior to shipment or was carried out at DRDC Suffield. The following briefly describes the surgical procedure performed in-house. Following acclimatization and thirty minutes prior to surgery, buprenorphine (0.05 mg/kg) (McGill University, Montreal, QC) as a pre-analgesic and glycopyrrolate (0.04 mg/kg) (CDMV Western Distribution Centre, Calgary, AB) as an anticholinergic were administered subcutaneously (SC). While under anaesthesia (2-3% isoflurane in 100% oxygen) (Abbott Laboratories Ltd., Montreal, QC) two, 1-2 cm skin incisions, one to the right of the midline of the dorsal cervical area and the other at the midline of the dorsal cervical area just posterior of the shoulder blades were made. A tunnel was created between the two dorsal incisions using blunt dissection and the sampling end of the cannula was fed through leaving a short section exposed on the back to allow for blood sampling later. A third incision was made on the ventral surface of the animal halfway between the point of the shoulder and the mandible just off of midline on the right side. A tunnel was blunt dissected from the ventral incision to the dorsal and the cannula was fed through. The right jugular vein was isolated from its surrounding tissue and a ligature was fastened at the cranial end using silk sutures. A second piece of silk was placed under the caudal end of the exposed jugular but was not fastened. A small V-shaped incision was made in the jugular vein near the cranial end and the beveled end of the cannula was inserted 4-6 cm. The cannula was fastened in place with Tissumend II™ (Veterinary Products Laboratories, Phoenix, AZ) and ligatures caudal to the cannula insertion point. After the cannula was tested for patency, additional cannula length was fed under the skin of the caudal incision to allow for animal movement. Finally, all incisions were closed using simple interrupted sutures or skin suture staples. The cannula was filled with a locking solution (1:1 glycerol: heparinized

saline (500 IU/ml)) and the animal was placed on 100% oxygen until consciousness was regained. Four to six hours post-surgery an additional SC bolus of buprenorphine was administered. Following surgery animals were allowed to recover for a minimum of 48 hours prior to experimental use.

Catheters were checked and maintained for patency at a minimum of once every five days. A locking solution of heparinized glycerol (500 IU/ml) (SAI Infusion Technologies, Lake Villa, IL) was used for long term maintenance of catheters and heparinized saline (1 IU/ml, SAI Infusion Technologies) was used for short-term storage and flushing of catheters.

Guinea pig treatment groups were as follows. A total of 10 experimental groups (average of 6 animals per group) were used for the guinea pig studies and are shown in Table 4.1.

Table 4.1 Treatment groups for guinea pig experimental studies.

Treatment Group	HI-6 Salt	Anaesthetic	Administration Route	HI-6 Dose Administered	AS Dose Administered
1	2Cl	None	IM	120 µmol/kg (45 mg/kg)	0
2	DMS	None	IM	120 µmol/kg (57 mg/kg)	0
3	2Cl	Isoflurane	IM	120 µmol/kg (45 mg/kg)	0
4	DMS	Isoflurane	IM	120 µmol/kg (57 mg/kg)	0
5	2Cl	Isoflurane	IV bolus	110 µmol/kg (41.5mg/kg)	0
6	DMS	Isoflurane	IV bolus	110 µmol/kg (52.6 mg/kg)	0
7	2Cl	Isoflurane	IV infusion	0.35 µmol/min·kg	0
8	DMS	Isoflurane	IV infusion	0.49 µmol/min·kg	0
9	2Cl	Isoflurane	IV infusion	0.35 µmol/min·kg	4.4 µg/min·kg
10	DMS	Isoflurane	IV infusion	0.49 µmol/min·kg	4.4 µg/min·kg

AS represents atropine sulphate.

On the day of HI-6 administration animals included in Treatment Groups 1 and 2 were weighed and then placed in individual cages with access to water, guinea pig chow and carrots. Glycopyrrolate (0.04 mg/kg) was administered SC 20 minutes prior to control blood sample collection and every two hours (0.02 mg/kg) thereafter for the duration of the experimental period. Blood samples were collected as follows: using a 1 ml syringe with a 23G 1" needle attached, 0.2 ml of blood was drawn from the cannula. A second needle and syringe was then used to draw the 100 µL sample volume which was immediately transferred into an EDTA tube.

The initial 0.2 ml of blood withdrawn was then returned to the animal, followed by a 0.1 ml saline or heparinized saline flush. Thirty minutes after collection of the control blood sample HI-6 was administered (120 µmol/kg in 0.1 ml of saline) IM in the right *quadriceps femoris*. Blood samples were collected over the course of the six hour experimental period as follows: 2, 5, 10, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360 minutes, 24 hours and 48 hours post HI-6 administration. Following collection blood samples were centrifuged at 3000 x g for 10 minutes at 4ºC. Plasma aliquots were removed, snap frozen in liquid nitrogen and stored at -80ºC until HPLC (high pressure liquid chromatography) analysis could be completed. Throughout the course of the experimental period animals were monitored for irregular behavior, changes in physiology and signs of distress.

On the day of treatment animals belonging to treatment groups 3, 4, 5 and 6 were administered glycopyrrolate (0.04 mg/kg) SC, 20 minutes prior to induction with isoflurane. Prior to anaesthetic induction a control blood sample (100 µL) was collected into an EDTA tube via the indwelling catheter and stored on ice. Guinea pigs were weighed and then placed in an induction chamber primed with 5% isoflurane in 100% oxygen and transferred to a cone mask 3% isoflurane (100% oxygen carrier gas) once induced. A level of anaesthesia was established to ensure no response to a toe-pinch without attenuation of respiration rate and oxygen saturation (1.5-2.5% isoflurane). Following steady state anaesthesia (SSA; a period of at least 30 minutes with the animal at the same percent of isoflurane showing no response to toe-pinch or respiratory complications), HI-6 was administered either as an IV bolus (110 µmol/kg in 0.2 ml of saline via a *digital dorsal vein*); treatment groups five and six, or IM injection (120 µmol/kg in 0.1 ml of saline via the *quadriceps femoris*); treatment groups one through four, inclusive. Throughout the course of the six hour (IM groups) or eight hour (IV bolus groups) experimental periods, anaesthetic plane, heart rate, respiration rate and oxygen saturation were monitored to ensure appropriate levels of anaesthesia and monitored for signs of physiological distress. Glycopyrrolate (0.02 mg/kg) was administered SC every two hours following the initial loading dose. Blood samples for treatment groups 3 and 4 were collected in the same manner as described for treatment groups 1 and 2. Guinea pigs that received an IM injection of HI-6 were allowed to recover from anaesthetic at six hours with 100% oxygen supplementation as needed. Animals were observed throughout the recovery period for signs of normal behavior and activity

prior to being returned to their cage with access to feed and water. Supplemental fluids (0.9% NaCl) were administered SC as deemed necessary following anaesthetic recovery. This was based upon loss of body weight during the experimental period.

Following IV bolus administration of HI-6 blood samples were collected in the same manner as the IM treatment groups at the following time points: 5, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 300, 360, 420, 480 and 510 minutes, 24 hours and 48 hours post HI-6 administration. At eight hours animals receiving an IV bolus were allowed to recover from anaesthetic in the same manner as described above. The pharmacokinetic parameters determined for treatment groups 5 and 6 were used to calculate the IV maintenance infusion dose to maintain a target plasma concentration of 100 μM. The following equation was used to calculate the infusion dose in μmol/min·kg:

$$\textit{Infusion Maintenance Dose} = Cl \times C_p \qquad\qquad (4.1)$$

Where *Cl* represents clearance in ml/min·kg and C_p represents plasma concentration in μmol/ml.

Treatment groups 7 and 8 were administered a continuous IV infusion of HI-6 (via a *digital dorsal vein*) while under anaesthesia over an eight hour period. HI-6 2Cl was infused at a rate of 0.35 μmol/min·kg while HI-6 DMS was infused at a rate of 0.49 μmol/min·kg as calculated based on the pharmacokinetic parameters determined for treatment groups 5 and 6. Blood samples were collected and handled in the same manner as all other treatment groups at the following time points: 5, 30, 60, 120, 180, 240, 300, 360, 420, 480 and 510 min after the infusion start time (0 min) and every 24 hours thereafter for 7 days. Guinea pigs were administered glycopyrrolate SC prior to induction (0.04mg/kg) and every two hours thereafter (0.02mg/kg). Animals were monitored throughout the course of the experimental period as described for all other anaesthetized guinea pigs and were also recovered from the anaesthetic in the same manner.

Animals dosed with an infusion of HI-6 and AS were treated in the same manner as those given an infusion of HI-6 only, with the exception of AS addition. Treatment group 9 received a co-infusion of HI-6 2Cl (0.35μmol/min·kg) and AS (4.4μg/min·kg), whereas treatment group 10 received a co-infusion of HI-6 DMS (0.49μmol/min·kg) and AS (4.4μg/min·kg).

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Animal weights for all groups were tracked over the experimental period. At the end of the experimental period (48 hours for IV bolus and IM injections and 7 days for infusion groups) animals were induced with 5% isoflurane in 100% oxygen for a minimum of 5 minutes or until no response was observed to toe-pinch, and then euthanized by intracardiac injection of 1 ml (240 mg/ml) sodium pentobarbital (Bimeda-MTC Animal Health Inc., Cambridge, ON). A necropsy was completed following euthanasia to look for any gross pathology.

4.1.2 Domestic Swine

Male castrated York-Landrace Cross swine weighing 19 to 31 kg (10 to 12 weeks) of high health were purchased from a local supplier. Animals were acclimatized for at least one week prior to experimental use in a temperature controlled environment (15 to 25ºC) with a 12 hour light/dark cycle and housed in groups of six animals per pen. Standard Hog Grower Feed (Landmark Feeds, Medicine Hat, AB) was provided daily (500 g per animal) and tap water was available *ad libitum*. Bite-Rite (Ikadan System USA, Clinton, NC) a four finger chew toy was available in each pen for environmental enrichment.

On the day of treatment animals were placed in a custom transport sling and induced with 5% isoflurane in a carrier gas of 100% oxygen at a flow rate of 4 L/min for a minimum of five minutes, or until the animal's muscle tone was flaccid. Following induction animals were placed in the dorsal recumbent position on a heated operating table. At this time an intravenous line was established in the ear of the swine to allow for infusion of normal saline (0.9% NaCl) at a rate of 9.5 ml/kg/hr to compensate for any fluid loss while under anaesthesia. Animals were then intubated with a 6.5 mm i.d. cuffed endotracheal tube (Canada Teleflex Medical, Markham, ON). Isoflurane was reduced to 3% in 100% oxygen at a flow rate of 2 L/min following intubation. Animals were instrumented to monitor heart rate, arterial oxygen saturation, core and surface body temperatures, respiration rate, end-tidal CO_2 and blood pressure (via a direct arterial line in a branch of the saphenous artery). Physiological parameters were monitored throughout the course of the experiment with a Siemens SC 7000 clinical monitor (Draeger Medical Systems, Danvers, MA) and data was downloaded every 2 seconds. A second arterial line was established in a branch of the saphenous artery for blood sample collection and a Foley

catheter was surgically placed to monitor urinary output and collect urine samples. Upon completion of instrumentation, isoflurane was reduced to approximately 2% and supplemented with oxygen at 30% to achieve a non-responsive plane of anaesthesia, while maintaining stable physiological parameters. Animals were allowed to stabilize at the reduced level of isoflurane for a minimum of 30 minutes at the end of which the animal was said to have reached SSA. Once SSA was achieved control blood samples were collected. Blood samples were collected in the following manner: an initial blood volume of 5 – 8 ml was removed using the arterial blood sampling line and a 10 ml syringe attached to a three way stopcock. A second 10 ml syringe was then attached and the sample volume of 10 ml of blood was removed and immediately transferred into the appropriate blood collection tubes, the initial blood withdrawn was returned to the animal and the sampling line was flushed with saline until it ran clear. The bladder was voided of all urine using a 60 cc syringe attached to the Foley catheter and an aliquot of urine was retained for HPLC analysis.

A total of 11 experimental groups were used for the swine studies and are shown in Table 4.2.

Table 4.2 Treatment groups for domestic swine studies.

Treatment Group	HI-6 Salt	Administration Route	HI-6 Dose Administered	AS Dose Administered
A	2Cl	IM	53 µmol/kg (20 mg/kg)	0
B	DMS	IM	53 µmol/kg (25.4 mg/kg)	0
C	2Cl	IM	53 µmol/kg (20 mg/kg)	0.1 mg/kg
D	DMS	IM	53 µmol/kg (25.4 mg/kg)	0.1 mg/kg
E	2Cl	IV bolus	26.8 µmol/kg (10.1 mg/kg)	0
F	DMS	IV bolus	26.8 µmol/kg (12.7 mg/kg)	0
G	2Cl	IV bolus	4008 µmol (1509 mg)	0
H	DMS	IV bolus	4008 µmol (1899 mg)	0
I	2Cl	IV bolus	4008 µmol (1509 mg)	0.1 mg/kg
J	DMS	IV bolus	4008 µmol (1899 mg)	0.1 mg/kg
K	DMS	IV infusion	0.48 µmol/min·kg	0

Intramuscular injections (HI-6 in 3 ml of saline, Treatment Groups A through D) were administered using a 20G 1" needle attached to a 3 ml syringe into the side of the gluteus muscle. Intravenous bolus injections (HI-6 in 3 ml of saline for Treatment Groups E and F or HI-6 in 5 ml of saline for Treatment Groups G through I) of HI-6 were administered using a port in the IV line established in the ear. The infusion of HI-6 DMS was administered using an IV bag attached to the IV line established in the ear and controlled by an IV pump. The infusion

dose was calculated as described in equation **3.1** and was based on the pharmacokinetic parameters determined for Treatment Group F, HI-6 DMS IV bolus of 26.8 µmol/kg (12.7mg/kg).

Blood samples were collected over the course of the experimental period for HPLC (high pressure liquid chromatography) determination of HI-6 plasma concentrations, blood gas levels including pH and bicarbonate (HCO_3^-), hemoglobin (Hgb) levels, electrolyte levels including sodium (Na), potassium (K), chloride (Cl), calcium (Ca), magnesium (Mg) and blood chemistry levels including phosphate (Phos), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), urea nitrogen (UREA) and creatine (CREA).   Blood samples collected for HPLC analysis were centrifuged at 3000 x g for 10 minutes at 4ºC. Plasma aliquots were removed, snap frozen in liquid nitrogen and stored in an ultra-low freezer until HPLC analysis could be completed. Blood samples collected for blood gas levels were analyzed within 2 minutes of collection using a Stat Profile® pHOx Analyzer (Nova Biomedical, Waltham, MA). Hematology samples were analyzed within two minutes of collection using a HemaTrue Analyzer (Heska, Loveland, CO). Samples for electrolyte levels and blood chemistry were collected in serum separation tubes which were allowed to sit at room temperature for 30 minutes and then spun at room temperature at 3500 x g for 10 minutes. Serum was removed and transferred to the Dri-Chem 4000 Chemistry Analyzer (Heska, Loveland, CO) for blood chemistry analysis. Electrolyte levels were determined from serum samples using an EasyLyte analyzer (Medica, Bedford, MA). Urine samples were collected over the course of the experimental period for determination of HI-6 concentrations using HPLC analysis. Immediately following urine sample collection samples were transferred to a 4 ml cryogenic vial and snap frozen in liquid nitrogen. Timelines for blood and urine sample collection for IM and IV bolus treatment groups and the infusion group are shown in Figures 3.1 and 3.2 respectively.

Figure 4.1 Blood and urine sample collection timeline for domestic swine administered IV bolus or IM injection of HI-6.

Blood and urine samples were collected following HI-6 administration over the course of the experimental period for animals treated with an IV bolus (Treatment Groups E through J) or IM injection (Treatment Groups A through D) of HI-6. SSA represents steady state anaesthesia (period of at least 30 minutes at a stable level of anaesthetic where the animal is in a non-responsive plane of anaesthesia while maintaining stable physiological parameters). The following indicate the type of analysis to be completed for each sample indicated per time point: HPLC (plasma for high pressure liquid chromatography), BG (blood gas analysis), Hgb (hemoglobin determination), E (electrolyte level determination), Chem (clinical chemistry analysis) and Urine for HPLC analysis. Blood samples were collected and handled as described in section 4.1.2.

HI-6 Infusion (0 min)

SSA	1m	3m	5m	10m	15m	30m	45m	1h	1h 30m	2 h	2h 30m	3h	3h 30m	4h	4h 30m	5h
HPLC 1	HPLC 2	HPLC 3	HPLC 4	HPLC 5	HPLC 6	HPLC 7	HPLC 8	HPLC 9	HPLC 10	HPLC 11	HPLC 12	HPLC 13	HPLC 14	HPLC 15	HPLC 16	HPLC 17
BG + Hgb						BG + Hgb		BG + Hgb		BG + Hgb		BG + Hgb		BG + Hgb		BG + Hgb
E + Chem														E + Chem		
Urine						Urine			Urine		Urine		Urine		Urine	

Infusion End

5h 20m	5h 40m	6h	6h 20m	6h 40m	7h	7h 20m	7h 40m	8h	8h 15m	8h 30m	8h 45m	9h
HPLC 18	HPLC 19	HPLC 20	HPLC 21	HPLC 22	HPLC 23	HPLC 24	HPLC 25	HPLC 26	HPLC 27	HPLC 28	HPLC 29	HPLC 30
	BG + Hgb		BG + Hgb		BG + Hgb		BG + Hgb		BG + Hgb		BG + H	
								E + Chem				
Urine		Urine		Urine		Urine		Urine		Urine		Urine

Figure 4.2. Blood and urine sample collection timeline for domestic swine administered an infusion of HI-6.

Blood and urine samples were collected following an infusion of HI-6 DMS (Treatment Group K). SSA represents steady state anaesthesia (period of at least 30 minutes at a stable level of anaesthetic where the animal is in a non-responsive plane of anaesthesia while maintaining stable physiological parameters). The following indicate the type of analysis to be completed for each sample indicated per time point: HPLC (plasma for high pressure liquid chromatography), BG (blood gas analysis),

Hgb (hemoglobin determination), E (electrolyte level determination), Chem (clinical chemistry analysis) and Urine for HPLC analysis. Blood samples were collected and handled as described in section 4.1.2.

At the end of the experimental period (six hours for IV bolus and IM injections, and nine hours for infusion experiments) isoflurane levels were increased to 5%. After a minimum of five minutes at 5% isoflurane animals were euthanized by administration of 6 ml (540 mg/ml) sodium pentobarbital (Bimeda-MTC Inc., Cambridge, ON) intravenously. Following euthanasia a necropsy was conducted to examine for signs of gross pathology.

4.2 HI-6 Treatment Solutions

Two different salts of HI-6 were administered to both guinea pigs and domestic swine: HI-6 2Cl ([(Z)-[1-[(4-carbamoylpyridin-1-yl)methoxymethyl]pyridin-2-ylidene]methyl]-oxo-azanium dichloride) obtained from Pharmsynthez ZAO (Saint-Petersburg, Russia) and HI-6 DMS ([(Z)-[1-[(4-carbamoylpyridin-1-yl)methoxymethyl]pyridin-2-ylidene]methyl]-oxo-azanium dimethane sulfonate) obtained from BioQuadrant (Laval, QC, Canada). The molecular weights of HI-6 2Cl and DMS are 376.22 g/mol and 478.50 g/mol respectively. Powder forms of both salts were stored in amber glass bottles in a desiccator at 2-6ºC. HI-6 2Cl and HI-6 DMS used for these studies were in excess of 99% pure.

HI-6 treatment solutions were prepared based upon animal weight on the morning of treatment with the exception of the domestic swine treatment groups G through J which were all administered set molar quantities of HI-6 independent of body weight. On the morning of treatment HI-6 was weighed out ± 0.05 mg (from the calculated weight) and dissolved in saline (0.9% NaCl), filter sterilized (0.2 µm) and transferred to the appropriate administration device (syringe, syringe with needle attached or IV fluid bag). Treatment solutions containing both HI-6 and atropine sulphate (AS) (Sigma-Aldrich, St. Lois, MO) were prepared in the same manner as solutions containing HI-6 alone.

4.3 HPLC Method for Determination of HI-6

4.3.1 HPLC Apparatus

Gradient paired-ion chromatography was carried out using HPLC equipment and columns supplied by Agilent Technologies Canada Ltd. (Mississauga, ON). The HPLC equipment included 1100 Vacuum Degasser, 1200 Binary Pump, 1100 Autosampler, 1100 Thermostatted Column Compartment and an 1100 Variable Wavelength Detector all controlled by Agilent ChemStation for LC 3D Systems© (Rev. B.03.01) software. An Agilent Zorbax Analytical Guard Column (C18, 4.6 x 12.5mm, 5 μm) and a Rapid Resolution SB-C18 Column (4.6 x 75mm, 3.5 μm) were used.

4.3.2 HPLC Conditions

A gradient ion-pair high pressure liquid chromatography (HPLC) method was carried out as described by Bohnert, Vair, and Mikler (2010) with modifications. Briefly HI-6 (both salts) in guinea pig plasma, swine plasma and urine was resolved and detected under gradient conditions at 302 nm (UV light), 40ºC at a flow rate of 0.8 ml/min. A gradient mobile phase was used transitioning from 60:40 (Component A (A): Component B (B)) to 0:100 (A:B) over the course of each run. Component A consisted of water:PIC B7:triethylamine – 974:25:1. Component B consisted of component A mixed 1:1 with methanol. The total running time for a single sample analysis was 10.5 min, where the internal standard 2-PAM (2-[(hydroxyimino)methyl]-1-methylpyridinium chloride) (Sigma–Aldrich Ltd.) was resolved at 2.45 ± 0.1 min and HI-6 (2Cl or DMS) was resolved at 6.33 ± 0.1 min.

4.3.3 Preparation of Standards and Samples

Preparation of plasma standards and samples for both guinea pigs and swine was performed as described previously by Bohnert, Vair and Mikler (2010). Briefly, plasma standards were

prepared using pooled naïve guinea pig or swine plasma spike with varying concentrations of HI-6 (4-1000 µg/ml). Spiked plasma was then combined with 2PAM and trichloroacetic acid, vortexed and clarified through centrifugation. Supernatant was removed and combined with sodium hydroxide, vortexed and clarified through centrifugation prior to loading for HPLC analysis. Study plasma samples were combined with 2PAM and trichloroacetic acid in the same ratios as prepared standards and processed as described for standards. Urine standards for both HI-6 salts were prepared using pooled naïve swine urine spiked with HI-6 (2Cl or DMS as appropriate for the samples being analyzed) over a concentration range of 0-240 µg/ml (final total solution concentration of HI-6). Prepared urine standards and urine samples were combined with 0.10 mg/ml 2-PAM 1:1, filter centrifuged and transferred to a HPLC vial for analysis.

4.3.4 Method Validation

The method described by Bohnert, Vair and Mikler (2010) was validated for determination of HI-6 2Cl and HI-6 DMS in guinea pig plasma. Additional validation has since been completed for the quantification of HI-6 in swine plasma and urine. Linearity, accuracy, precision and selectivity have been determined for HI-6 in swine plasma and urine. The validation tests were designed based upon published principles and limitations presented by Bansal & DeStefano (2007), FDA (2001) and Viswanathan et al. (2007).

Linearity is a measurement of the ability of a method to consistently obtain results proportional to the amount of HI-6 in the sample being analyzed over a range of concentrations and is expressed as an R^2 value. Linearity was determined by plotting the HI-6:2-PAM ratio for each replicate against the sample concentration and analyzing this relationship by the least squares linear regression. Five replicate injections of five different concentrations of HI-6 in swine plasma and urine spanning the working concentration range of the method were used to determine the linearity of the method in swine plasma and urine. The method was linear if the R^2 was greater than 0.95.

Accuracy determines how closely the obtained result of HI-6 concentration matches the true value of any given sample and is expressed as a percent of relative error. Relative error was

determined by comparing the calculated concentration (observed) with the expected value for three different concentrations spanning the working range. The method was said to be accurate if the relative error of the mean calculated concentration over all five replicates was within ± 15% of the expected value.

Precision measures how closely independent repeated measurements of the same concentration of HI-6 agree with one another and is expressed as relative standard deviation. Relative standard deviation was determined by comparing the standard deviations divided by the mean of five replicates of three different concentrations spanning the working range of the method. The method was said to be precise if the peak areas of both HI-6 and 2-PAM were within ± 15% of the mean value.

Selectivity is the ability of a method to differentiate between internal standard (in this case 2-PAM) and analyte (HI-6) without interference from any endogenous or other components contained in the sample. Selectivity was evaluated by examining the retention times of the HI-6 and 2-PAM peaks in five separate extractions of each of the following samples: zero (sample only containing internal standard 2-PAM) and samples containing both 2-PAM and HI-6 (in varying concentrations). The method was said to be selective if the retention times for both 2-PAM and HI-6 were within 10% of one another in all injection cases.

4.3.5 Quantification of HI-6

Quantification of HI-6 for each sample was determined on an individual assay basis for all HI-6 salts in all matrices (guinea pig plasma, swine plasma and urine). Two standard curves spanning the working concentration range and a minimum of three replicates of three different concentrations of quality control samples specific to the salt and matrix being evaluated were included with all animal samples to be tested for each run. Chromatograms were integrated automatically as part of the ChemStation software upon the completion of each sample analysis. Integration settings were kept constant across all salts and matrices tested.

HI-6 sample concentrations were determined based upon the linear regression calculated from the standard curves that were generated using known concentrations of HI-6 and 2-PAM. Linear regression was calculated as described in equation **4.2**.

$$\log(PAR) = m * log(C) + b \tag{4.2}$$

Where:

PAR is the peak area ratio of HI-6 to 2-PAM;

C is the know sample concentration (as injected);

m is the slope of the line; and

b is the y-axis intercept of the line.

A logarithmic regression rather than a standard linear regression was used to linearize the relationship between the peak area ratio of HI-6:2-PAM and known concentration and normalize the distribution of data over the very large range of concentrations included in the standard curve.

The formula for linear regression was used to back calculate sample HI-6 concentrations in µg/ml based on the HI-6:2-PAM ratios determined following integration of peaks in ChemStation as shown in equation **4.3**.

$$Sample\ Concentration = exp\left[\frac{\log(PAR) - b}{m}\right] \tag{4.3}$$

Plasma (or urine) concentration of HI-6 in µg/ml was calculated from the sample concentration as shown in equation **4.4.**

$$HI - 6\ Concentration = \frac{Sample\ Concentraiton\ x\ Total\ Sample\ Volume}{Plasma\ or\ Urine\ volume} \tag{4.4}$$

Where:

Sample Concentration was determined using equation **4.3**;

Total Sample Volume refers to the total amount of solution that was placed into an HPLC vial for analysis. For plasma sample analysis the total volume was 210 µL and for urine sample analysis the total volume was 100 µL.

Plasma or Urine Volume refers to the volume of either plasma (35 µL) or urine (50 µL) in the   total sample volume.

Finally HI-6 concentration was converted to a µmol/L value as shown in equation **4.5.**

$$C_p = \left[\frac{HI-6\ Concentration\ x\ 10^{-6}}{HI-6\ Salt\ Molecular\ Weight} \right] x\ 10^9 \qquad (4.5)$$

Where:

C_p represents the concentration of HI-6 in plasma in µmol/L and the same formula with the designation C_u representing the concentration of HI-6 in µmol/L in urine.

Plasma concentrations were calculated on an individual animal basis per group and are presented in Section 4: Results, as the average concentration including standard deviations as generated by GraphPad Prism 5.04 (GraphPad Software, Inc., La Jolla, CA).

pH and osmolarity were also determined for all HI-6 solutions using a standardized pH meter and osmometer.

4.4 Pharmacokinetic Determination

Pharmacokinetic (PK) parameters were determined for both guinea pig and swine studies using PK Solutions software (Summit Research Services, Montrose, CO). A one-compartment model and first order rate of elimination were assumed based on several published reports (Busker, et al., 1996b; Clement, et al., 1995; Lundy, et al., 2005; Morelli & Jensen, 2010). In a one compartment model it is assumed that the body acts as a single homogenous compartment where immediately following administration, the drug distributes throughout all tissues in the body and equilibrates instantaneously between these tissues. Drug recirculation is not assumed to occur and a linear response is observed when examining the drug concentration-time profile on a

logarithmic scale (DiPiro, 2010). First order rate of elimination refers to the proportional removal of the administered drug compared to the serum drug concentration.

The method of residuals (curve stripping or feathering), which resolves a curve into a series of exponential terms corresponding to the elimination phase occurring during the time course of the drug in the blood, was used for the determination of Area Under the Curve (AUC_∞), Distribution/Absorption Half Life ($D/At_{1/2}$), Elimination Half Life ($Et_{1/2}$), Volume of Distribution (V_d) and Total Body Clearance (Cl) (Farrier, 2000) for each salt in both species examined. The exponential terms are then used to calculate pharmacokinetic parameters.

Elimination Half Life refers to the amount of time required for the plasma concentration of HI-6 to decrease by 50 percent. The Distribution/Absorption Half Life indicates the amount of time required for the concentration of HI-6 to absorb/distribute to 50 percent of the maximal concentration into the central compartment. Volume of distribution is a value that directly correlates the amount of drug in the body to the concentration of drug measured in the plasma at any given time. Area under the curve represents the area below the plasma concentration versus time curve and is indicative of the total amount of drug present following administration. Total body clearance refers to the amount of drug cleared from the plasma over a given period of time by renal, hepatic and biliary processes, while renal clearance refers solely to the amount of drug cleared over time by the kidneys. Maximal concentration represents the highest concentration of drug achieved in plasma and time to maximal concentration represents the length of time required to reach the maximal concentration. Initial concentration is the concentration of drug immediately following IV administration.

The following PK parameters were calculated for guinea pig treatment groups (1 through 4) administered an IM injection of HI-6: $Et_{1/2}$, V_d, Cl, AUC_∞, Maximum Observed Concentration (C_{max}) and Time to Maximum Concentration (T_{max}). Similarly $Et_{1/2}$, V_d, Cl, AUC_∞ and Initial Concentration (C_0) were determined for all guinea pig treatment groups (5 and 6) administered an IV bolus of HI-6.

$D/At_{1/2}$, $Et_{1/2}$, , V_d, Cl, Renal Clearance Cl_r, AUC_∞, C_{max}, T_{max} and percent HI-6 excreted in Urine (% $HI-6_u$) were calculated for all subjects included in swine treatment groups (A through D) administered an IM injection of HI-6. Similarly $D/At_{1/2}$, $Et_{1/2}$, V_d, Cl, Cl_r, AUC_∞, C_0 and

%HI-6$_u$ were determined for all swine treatment groups (E through J) administered an IV bolus of HI-6.

Calculated PK parameters were based on the exponential expression of 1st order kinetics using the standard equation for exponential decay:

$$lnC = lnC_0 - \lambda t \tag{4.6}$$

Where:

C represents the concentration of drug

C_0 represents the concentration of drug at time zero

t represents time

λ is the rate constant (either elimination or distribution/absorption).

From equation **4.6** the PK parameters for half-life (elimination or distribution/absorption), V$_d$ and Cl can be calculated. For half-life from equation **4.6** C$_0$ is considered to be 1 and C, 0.5; solving the equation yields:

$$t_{1/2} = \frac{ln2}{\lambda} \tag{4.7}$$

To calculate the volume of distribution it was assumed that all HI-6 administered was distributed instantaneously prior to any elimination. This was calculated by solving for the y-intercept or C$_0$ following administration of a quantity of HI-6 or the dose (D).

$$V_d = \frac{D}{C_0} \tag{4.8}$$

AUC represents the total amount of drug that entered the compartment and can be calculated for any time period using a trapezoidal calculation. For the studies discussed herein complete first order elimination was assumed and AUC was calculated from exponential terms as follows.

$$AUC_\infty = \Sigma \frac{C_n}{\lambda_n} \tag{4.9}$$

Where:

C_n represents the coefficient of each exponential term

λ_n represents the corresponding rate constant for each exponential term

In first order elimination *Cl* is a constant and serves as the basis for the infusion calculation (equation **4.1**) achieve a target plasma concentration. Clearance may be calculated from the area under the curve (equation **4.10**).

$$Cl = \frac{D}{AUC_\infty} \qquad (4.10)$$

Renal clearance was calculated from the total body clearance as follows:

$$Cl_r = \frac{Amount\ of\ HI-6\ in\ Urine}{AUC_\infty} \qquad (4.11)$$

The percent of HI-6 excreted in urine was calculated based upon the concentration of HI-6 determined in each urine sample over the course of the experimental period for each animal.

$$\%HI-6_u = \left(\frac{Amount\ of\ HI-6\ in\ Urine}{D}\right) x\ 100 \qquad (4.12)$$

Initial concentration, maximum concentration and time to maximum concentration were values determined directly from the logarithmic concentration verse time plots.

PK parameters were determined on an individual animal basis per group and then summarized as averages and standard deviations for reporting and comparison purposes.

4.5 Statistical Analysis

Reported averages and standard deviations were determined by InStat 3.10 (GraphPad Software, Inc., La Jolla, CA). Statistical analysis was performed on calculated PK parameters for each treatment group and comparisons between groups within species were determined using an un-paired t-test. The un-paired t-test (also known as the Student's t-test or two-sample t-test) assumes that the two groups being compared are independent of one another, the variable being compared approximates a Gaussian distribution and that there is equal variances in groups of data (homoscedasticity) (Motulsky, 2009; Petrie & Watson, 2006). The Kolmogorov and Smirnov test was used to determine if the data was sampled from a population that follows a Gaussian distribution and the F-test or Variance Ratio Test was used to confirm the

homoscedasticity of the data sets. Two-tailed P values were calculated and used to determine significance (P<0.05). Power values (value indicating the probability of a statistically significant effect) were determined using StatMate 2.00 (GraphPad Software, Inc., La Jolla, CA).

Physiological data and clinical chemistry data collected for all swine treatment groups were compared between groups using a one-way ANOVA with a Tukey post-test. Similarly PK parameters determined for guinea pigs and swine were compared to human data using a one-way ANOVA and Tukey-Kramer post-test. A one-way ANOVA compares the means of two or more groups assuming that the samples are independent and approximate a normal Gaussian distribution and indicates whether groups in the sample significantly differ. The Tukey-Kramer post-test was conducted following a significant ANOVA result and compares pairs of group means and indicates which groups in the sample tested were significantly different (Motulsky, 2009; Petrie & Watson, 2006).

5. Results

5.1 HPLC Method Validation

As previously reported by Bohnert, Vair and Mikler (2010) the HPLC method described and used for the quantification of HI-6 was fully validated under the principles of Good Laboratory Practices (GLP), and was found to pass all validation parameters when quantifying HI-6 (2Cl and DMS) in guinea pig plasma. Further method validation was conducted for the quantification of HI-6 in swine plasma and urine: validation results are presented in Table 5.1.

Table 5.1. HPLC method validation results for HI-6 2Cl and DMS swine (plasma and urine) and guinea pig plasma samples.

		Guinea Pig Plasma		Swine Plasma		Swine Urine	
		2Cl	DMS	2Cl	DMS	2Cl	DMS
Linearity (4-1000 µg/ml)	(R^2)	0.9991[4]	0.9995[4]	0.9978	0.9985	0.9972	0.9958
Accuracy	(%RE)	4[4]	5[4]	7	5	10	6
Precision	(%RE)	4.5[4]	1.5[4]	5	4	4	7
Selectivity		Pass[4]	Pass[4]	Pass	Pass	Pass	Pass
Lower Limit of detection (µg/ml)		1[4]	1[4]	1	1	1	1

All validation terms were defined in section 3.3.4. R^2: coefficient of determination; %RE: relative error.

Standard curves were found to be linear with the corresponding coefficients of determination to be greater than 0.99 (R^2) in all matrices and salts tested over the concentration range examined, indicating a close correlation between the peak area ratios of HI-6 and the internal standard 2PAM. Quantification of HI-6 2Cl and DMS was precise and accurate with percent errors of 10% or less in all cases tested. HI-6 2Cl and DMS were resolved at 6.33 ± 0.1 minutes in all matrices tested and 2PAM was resolved at 2.45 ± 0.1 minutes as shown in Figure 5.1 . The lower limit of detection of the method for all matrices was determined to be 1 µg/ml, while the lower limit of quantification was determined to be 4 µg/ml. The upper limit of quantification was determined to be 4 mg/ml in all matrices. All validation parameter limits were met indicating a robust and reliable method of quantifying HI-6 over a large concentration range in two separate species.

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[4] *Note.* The data presented under Guinea Pig Plasma for both HI-6 2Cl and HI-6 DMS are from "Development and validation of a sensitive HPLC method for the quantification of HI-6 in guinea pig plasma and evaluated in domestic swine", by S. Bohnert, C. Vair and J. Mikler, 2010, *Journal of Chromatography B, 878,* p.1412. Copyright 2010 by Elsevier Ltd. Adapted with permission.
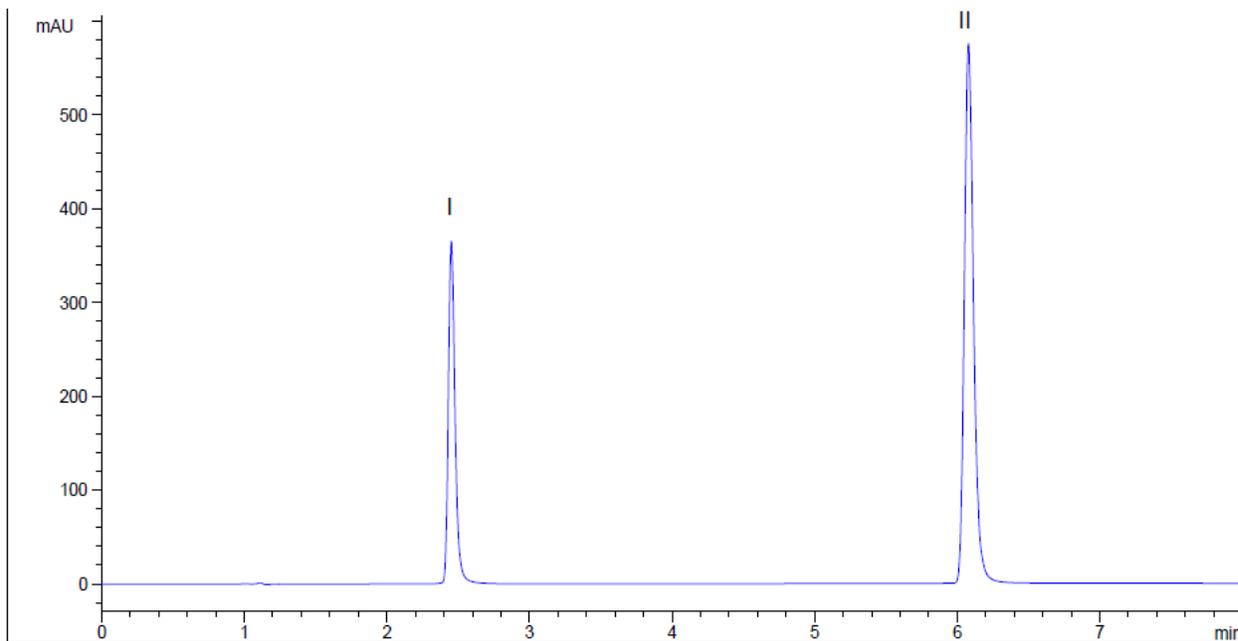
Figure 5.1. Representative chromatogram of 2PAM (I; 25 µg/ml) and HI-6 DMS (II; 30µg/ml) in guinea pig plasma. All plasma samples were separated using ion-pair chromatography on an Agilent 1100/1200 HPLC. The sample injection volume was 5 µL and samples were detected at 302 nm and a flow rate of 0.8 ml/min.

5.2 Dose Solution Characterization

Table 5.2 presents the concentrations, pH and osmolarities of HI-6 2Cl and DMS solutions administered to both guinea pigs and domestic swine.

Table 5.2 Concentration, pH and osmolarity of HI-6 2Cl and HI-6 DMS solutions administered to both guinea pigs and domestic swine.

HI-6	Concentration (µM)	pH	Osmolarity (mmol/kg)
2Cl (Treatment Group 1)	120	3.98	1642
DMS (Treatment Group 2)	120	2.93	2034
2Cl (Treatment Group A)	53	3.71	1116
DMS (Treatment Group B)	53	3.04	1286

5.3 Guinea Pig Experimental Results

The pharmacokinetics of HI-6 were determined, compared and evaluated in male Hartley guinea pigs as follows:

1. Case 1: Comparison of the pharmacokinetics of HI-6 2Cl and HI-6 DMS. Data found in section 5.3.2;

2. Case 2: Comparison of two different routes of administration (IM and IV) within each HI-6 salt. Data found in section 5.3.3;

3. Case 3: Evaluation of the effect of isoflurane anaesthetic on the pharmacokinetics of each HI-6 salt. Data found in section 5.3.4;

4. Case 4: Evaluation of the calculated pharmacokinetic parameters of HI-6 2Cl and DMS in guinea pigs to achieve and maintain a target plasma concentration of 100 µM over the course of an eight hour infusion. Data found in section 5.3.5; and

5. Case 5: Evaluation of the calculated pharmacokinetic parameters of HI-6 2Cl and DMS in guinea pigs to achieve and maintain a target plasma concentration of 100 µM over the course of an eight hour infusion when co-infused with atropine sulphate. Data found in section 5.3.6.

5.3.1 Guinea Pig Physiology

Anaesthetized guinea pigs were monitored on the day of HI-6 administration for changes in oxygen saturation, heart rate and respiration rate while under anaesthesia. Oxygen saturation rates for all animals fell to between 86 - 99% over the duration of the experimental period; no significant difference was observed for saturation rate between all guinea pig treatment groups. Heart rate values ranged from 196 – 245 beats per minute in anaesthetized animals and 240 – 360 beats per minute in non-anaesthetized guinea pigs. Respiration rates ranged from 20-38 breaths per minute while under anaesthesia and 40 – 80 breaths per minute for non-anaesthetized guinea pigs. A significant difference ($p < 0.05$) was noted for heart rate and respiration rate when comparing anaesthetized and non-anaesthetized guinea pigs.

Gross necropsies were completed for all guinea pigs at the end of their experimental period with no indication of abnormal pathology.

5.3.2 Comparison of HI-6 salts in Guinea Pigs

Case 1 compared the pharmacokinetics of HI-6 2Cl and HI-6 DMS administered in guinea pigs as follows:

1. Treatment Group 1 (non-anaesthetized guinea pigs administered 120 µmol/kg HI-6 2Cl IM) compared to Treatment Group 2 (non-anaesthetized guinea pigs administered 120 µmol/kg HI-6 DMS IM). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.2 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.3;
2. Treatment Group 3 (anaesthetized guinea pigs administered 120 µmol/kg HI-6 2Cl IM) compared to Treatment Group 4 (anaesthetized guinea pigs administered 120 µmol/kg HI-6 DMS IM). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.3 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.4; and
3. Treatment Group 5 (anaesthetized guinea pigs administered 110 µmol/kg HI-6 2Cl IV) compared to Treatment Group 6 (anaesthetized guinea pigs administered 110 µmol/kg

HI-6 DMS IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.4 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.5.
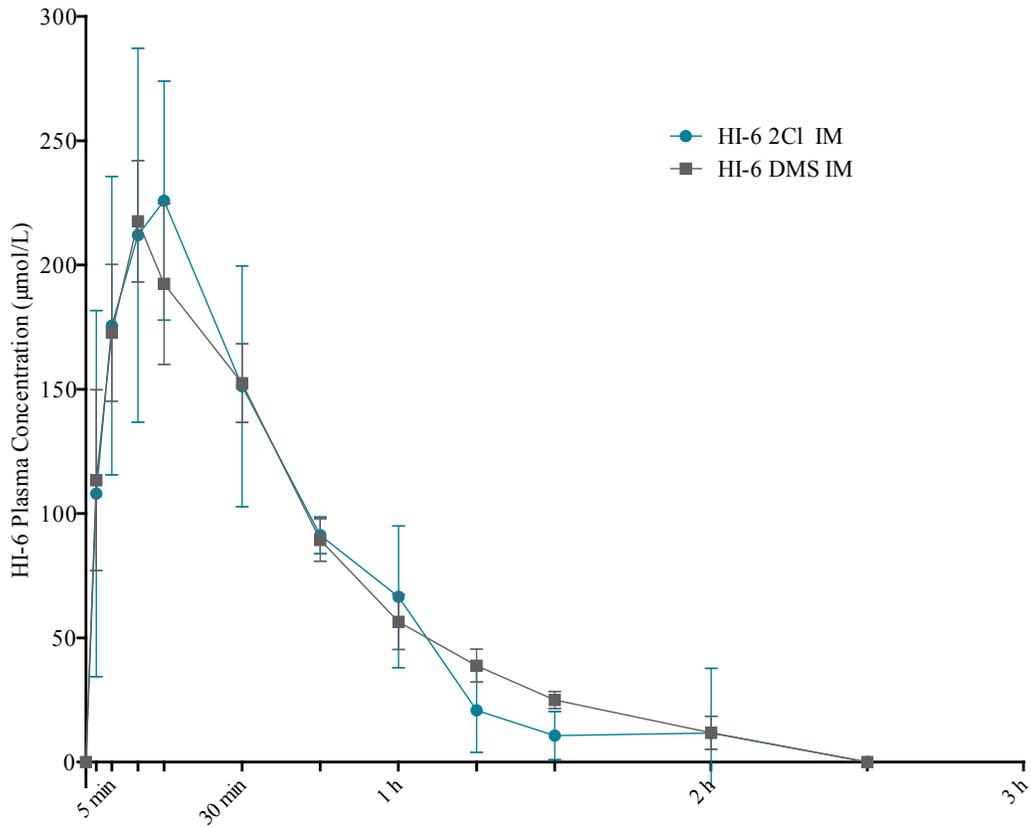
Figure 5.2 Mean (± SD) plasma HI-6 concentration-time profiles comparing equimolar (120 µmol/kg) doses of HI-6 2Cl (Treatment Group 1, $n = 8$) and HI-6 DMS (Treatment Group 2, $n = 5$) administered to non-anaesthetized guinea pigs IM.

Table 5.3 Pharmacokinetics of HI-6 2Cl (Treatment Group 1) and DMS (Treatment Group 2) administered IM at an equimolar dose (120 µmol/kg) to non-anaesthetized guinea pigs. Data are mean values ± SD. Vd volume of distribution; Cl total body clearance; AUC_∞ area under the curve; Cmax maximal concentration; Tmax time of maximal concentration; ns no significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl ($n = 8$)	HI-6 DMS ($n = 5$)	Significance
Elimination Half Life (min)	27.7 ± 11.9	25.9 ± 1.7	ns
V_d (ml/kg)	389.6 ± 74.3	440.3 ± 39.6	ns
Cl (ml/min/kg)	10.9 ± 3.4	11.8 ± 1.5	ns
AUC_∞ (µmol·min/L)	12288 ± 4774	10218 ± 1231	ns
C_{max} (µmol/L)	233.0 ± 56.5	225.9 ± 19.0	ns
T_{max} (min)	17.5 ± 8.0	12.0 ± 2.7	ns

No significant difference was found between the calculated PK parameters determined for awake (non-anaesthetized) guinea pigs administered equimolar doses of HI-6 2Cl and DMS, IM. These results suggest that the dichloride and dimethanesulfonate salts associated with the HI-6 moiety do not alter the pharmacokinetics of HI-6 in non-anaesthetized guinea pigs.
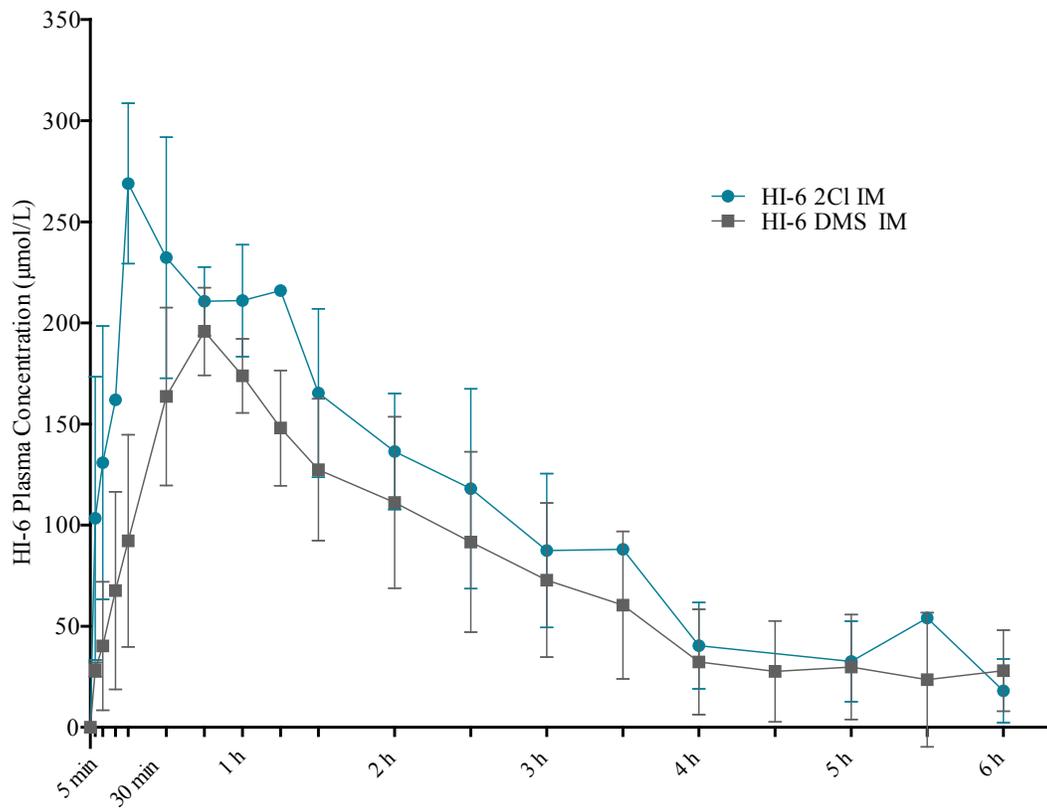
Figure 5.3 Mean (± SD) plasma HI-6 concentration-time profiles comparing equimolar (120 μmol/kg) doses of HI-6 2Cl (Treatment Group 3, $n = 6$) and HI-6 DMS (Treatment Group 4, $n = 5$) administered to anaesthetized guinea pigs IM.

Table 5.4 Pharmacokinetics of HI-6 2Cl (Treatment Group 3) and DMS (Treatment Group 4) administered IM at an equimolar dose (120 µmol/kg) to anaesthetized guinea pigs. Data are mean values ± SD. V_d volume of distribution; Cl total body clearance; AUC_∞ area under the curve; C_{max} maximal concentration; T_{max} time of maximal concentration; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl ($n = 6$)	HI-6 DMS ($n = 5$)	Significance
Elimination Half Life (min)	83.9 ± 34.7	97.3 ± 42.0	ns
V_d (ml/kg)	358.9 ± 99.0	498.8 ± 76.2	*
Cl (ml/min/kg)	3.2 ± 0.6	4.0 ± 1.5	ns
AUC_∞ (µmol·min/L)	39251 ± 7680	33410 ± 13686	ns
C_{max} (µmol/L)	259.9 ± 41.3	197.8 ± 28.1	*
T_{max} (min)	38.7 ± 15.6	48.0 ± 12.6	ns

A significant difference was observed for the V_d and C_{max} between isoflurane anaesthetized guinea pigs administered equimolar doses of either HI-6 2Cl or DMS, IM. V_d was greater in guinea pigs administered HI-6 DMS, IM. C_{max} was calculated to be greater in guinea pigs administered HI-6 2Cl, IM as can be seen in Figure 5.3.
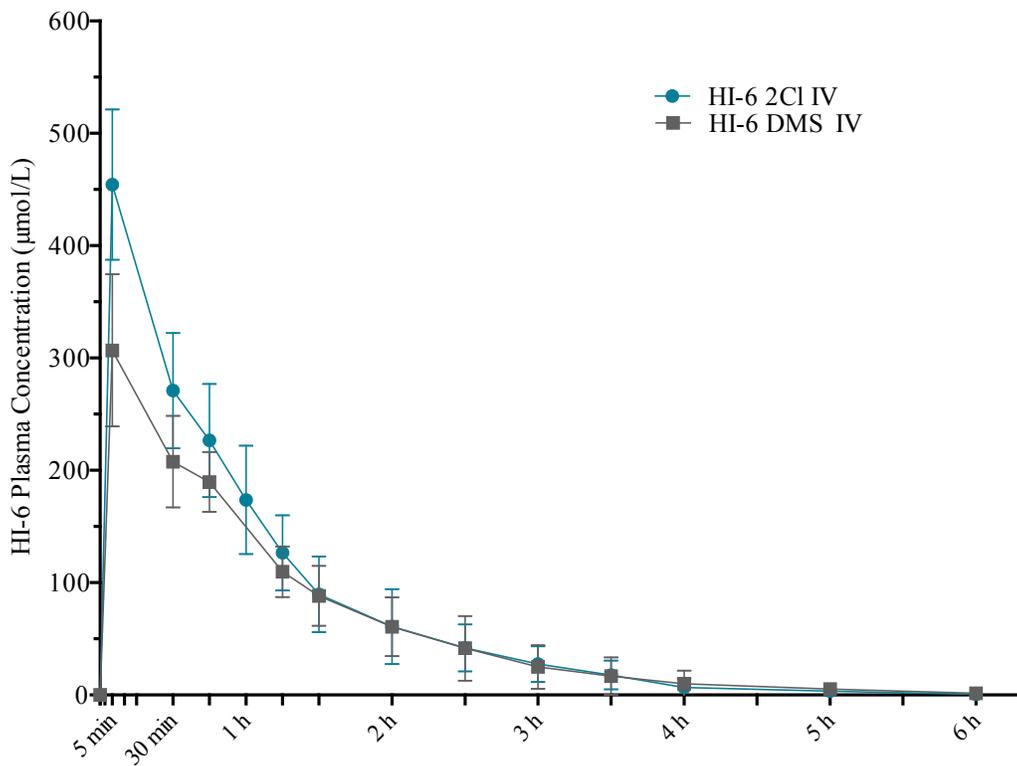
Figure 5.4 Mean (± SD) plasma HI-6 concentration-time profiles comparing equimolar (110 µmol/kg) doses of HI-6 2Cl (Treatment Group 5, *n* = 7) and HI-6 DMS (Treatment Group 6, *n* = 6) administered to anaesthetized guinea pigs IV.

Table 5.5 Pharmacokinetics of HI-6 2Cl (Treatment Group 5) and DMS (Treatment Group 6) administered IV at an equimolar dose (110 µmol/kg) to anaesthetized guinea pigs. Data are mean values ± SD. V_d volume of distribution; Cl total body clearance; AUC_∞ area under the curve; C_0 initial concentration; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl ($n = 7$)	HI-6 DMS ($n = 6$)	Significance
Elimination Half Life (min)	49.0 ± 7.7	44.8 ± 11.5	ns
V_d (ml/kg)	239.5 ± 42.1	307.8 ± 55.7	*
Cl (ml/min/kg)	3.5 ± 0.8	4.9 ± 1.1	*
AUC_∞ (µmol·min/L)	33616 ± 8841	23343 ± 5036	*
C_0 (µmol/L)	476.8 ± 108.7	367.1 ± 74.1	ns

V_d, Cl, AUC_∞ were all found to be significantly different when comparing equimolar doses of HI-6 2Cl and HI-6 DMS administered as an IV bolus to isoflurane anaesthetized guinea pigs. V_d and Cl were both greater in guinea pigs administered HI-6 DMS as reported in Table 5.5. As represented in Figure 5.4, AUC_∞ was calculated to be greater following HI-6 2Cl administration, IV.

5.3.3 Comparison of routes of administration of HI-6 in Guinea Pigs

Case 2 compared the pharmacokinetics of two different routes of administration of HI-6 2Cl and DMS in anaesthetized guinea pigs as follows:

1. Treatment Group 3 (anaesthetized guinea pigs administered 120 µmol/kg HI-6 2Cl IM) compared to Treatment Group 5 (anaesthetized guinea pigs administered 110 µmol/kg HI-6 2Cl IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.5 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.6; and

2. Treatment Group 4 (anaesthetized guinea pigs administered 120 µmol/kg HI-6 DMS IM) compared to Treatment Group 6 (anaesthetized guinea pigs administered 110 µmol/kg

HI-6 DMS IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.6 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.7.
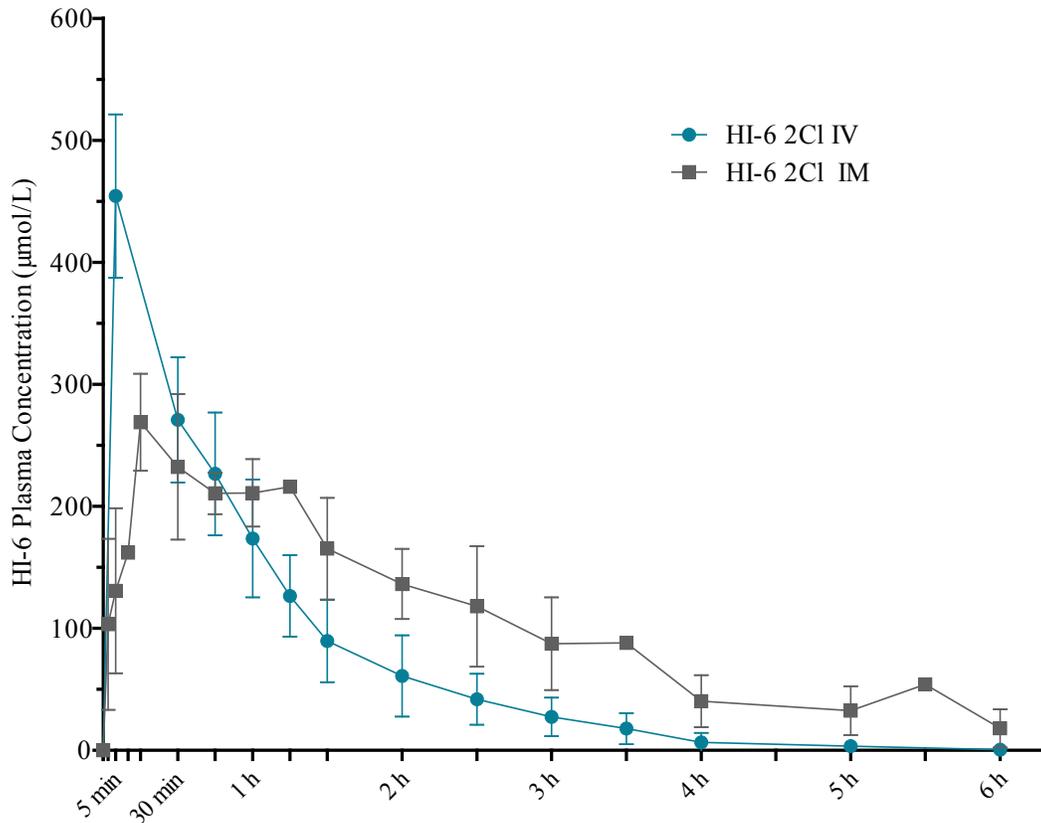
Figure 5.5 Mean (± SD) plasma HI-6 concentration-time profiles comparing HI-6 2Cl administered IV (Treatment Group 5, 110 µmol/kg, $n = 7$) and IM (Treatment Group 3, 120 µmol/kg, $n = 6$) in anaesthetized guinea pigs.

Table 5.6 Pharmacokinetics of HI-6 2Cl administered IV (Treatment Group 5, 110 µmol/kg) and IM (Treatment Group 3, 120 µmol/kg) to anaesthetized guinea pigs. Data are mean values ± SD. V_d volume of distribution; Cl total body clearance; AUC_∞ area under the curve; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	IV (n = 7)	IM (n = 6)	Significance
Elimination Half Life (min)	49.0 ± 7.7	83.9 ± 34.7	*
V_d (ml/kg)	239.5 ± 42.1	358.9 ± 99.0	*
Cl (ml/min/kg)	3.5 ± 0.8	3.2 ± 0.6	ns
AUC_∞ (µmol·min/L)	33616 ± 8841	39251 ± 7680	ns

A significant difference was found for both the elimination half-life and the V_d when comparing different routes of administration of HI-6 2Cl. It is important to note that the IV and IM doses administered were not equimolar (110 µg/kg, IV compared to 120 µg/kg, IM). The difference between doses administered between the two treatment groups was not considered to be significant and the calculated pharmacokinetics for each group were compared directly. Elimination half-life was longer and V_d was greater following HI-6 2Cl administration IM.
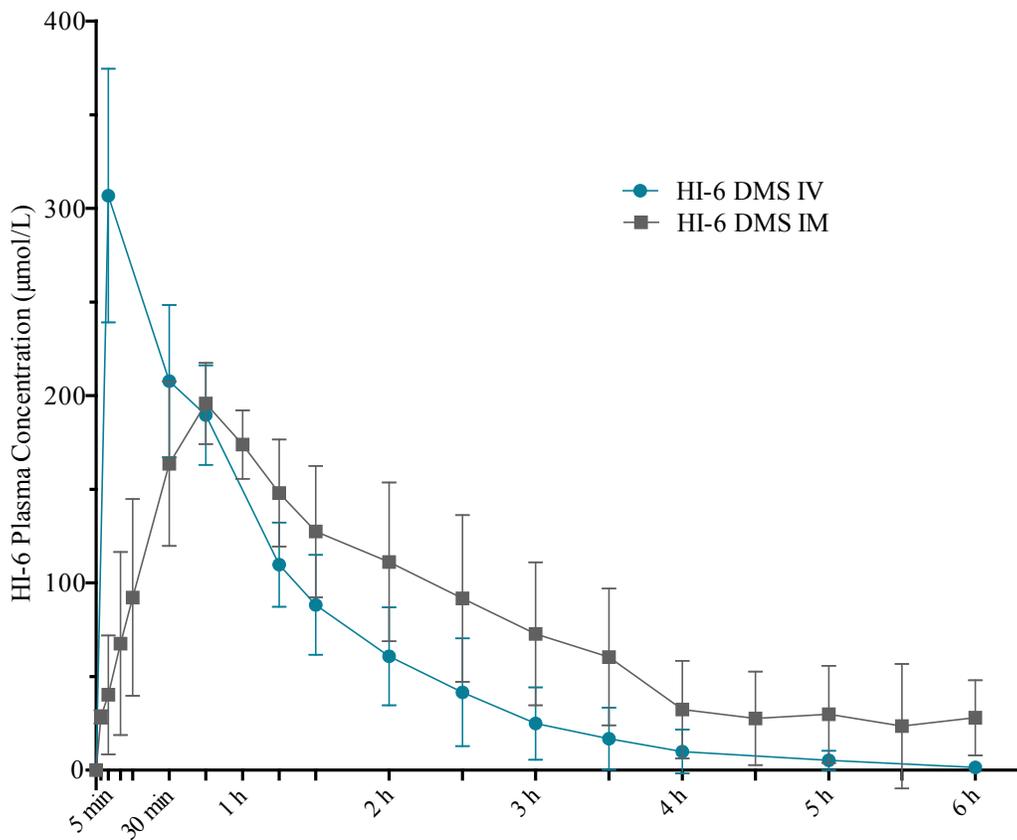
Figure 5.6 Mean (± SD) plasma HI-6 concentration-time profiles comparing HI-6 DMS administered IV (Treatment Group 6, 110 µmol/kg, *n* = 6) and IM (Treatment Group 4, 120 µmol/kg, *n* = 5) in anaesthetized guinea pigs.

Table 5.7 Pharmacokinetics of HI-6 DMS administered IV (Treatment Group 6, 110 µmol/kg) and IM (Treatment Group 4, 120 µmol/kg) to anaesthetized guinea pigs. Data are mean values ±SD. V_d volume of distribution; Cl total body clearance; AUC_∞ area under the curve; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	IV (*n* = 6)	IM (*n* = 5)	Significance
Elimination Half Life (min)	44.8 ± 11.5	97.3 ± 42.0	*
V_d (ml/kg)	307.8 ± 55.7	498.8 ± 76.2	*
Cl (ml/min/kg)	4.9 ± 1.1	4.0 ± 1.5	ns
AUC_∞ (µmol·min/L)	23343 ± 5036	33410 ± 13686	ns

Similar to the results comparing HI-6 2Cl administration either IM or IV, when HI-6 DMS was administered a significant difference was reported for both the elimination half-life and the V_d. It is important to note the dosing difference between the IV and IM groups as described earlier. The calculated elimination half-life was greater and V_d was larger when HI-6 DMS was administered IM compared to IV similar to HI-6 2Cl.

5.3.4 Comparison of effect of anaesthetic on HI-6 administration in Guinea Pigs

Case 3 compared the pharmacokinetics of HI-6 administered intramuscularly in non-anaesthetized and anaesthetized guinea pigs as follows:

1. Treatment Group 1 (non-anaesthetized guinea pigs administered 120 µmol/kg HI-6 2Cl IM) compared to Treatment Group 3 (anaesthetized guinea pigs administered 120 µmol/kg HI-6 2Cl IM). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.7 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.8; and
2. Treatment Group 2 (non-anaesthetized guinea pigs administered 120 µmol/kg HI-6 DMS IM) compared to Treatment Group 4 (anaesthetized guinea pigs administered 120 µmol/kg HI-6 DMS IM). Mean plasma HI-6 concentration-time profiles for both

treatment groups are shown in Figure 5.8 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.9
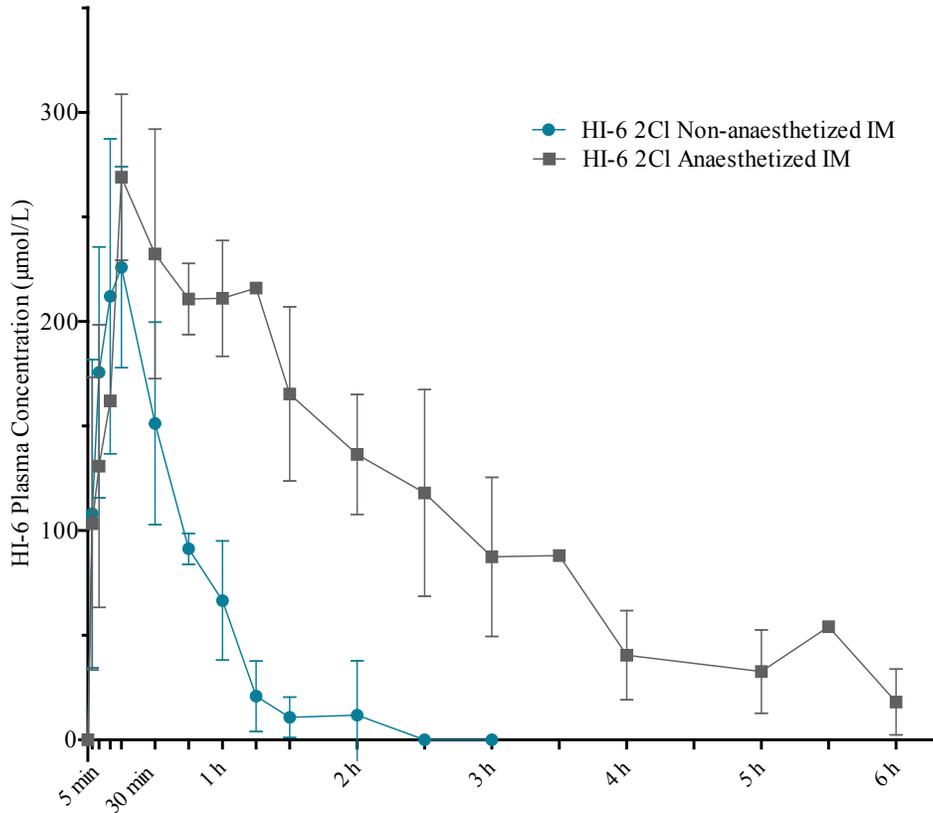

Figure 5.7 Mean (± SD) plasma HI-6 concentration-time profiles comparing equimolar (120 µmol/kg) doses of HI-6 2Cl administered IM to non-anaesthetized (Treatment Group 1, $n = 8$) and anaesthetized (Treatment Group 3, $n = 6$) guinea pigs.

Table 5.8 Pharmacokinetics of equimolar (120 µmol/kg) of HI-6 2Cl administered IM to non-anaesthetized (Treatment Group 1) and anaesthetized (Treatment Group 3) guinea pigs. Data are mean values ± SD. V_d volume of distribution; *Cl* total body clearance; AUC_∞ area under the curve; C_{max} maximal concentration; T_{max} time of maximal concentration; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	Non-anaesthetized ($n = 8$)	Anaesthetized ($n = 6$)	Significance
Elimination Half Life (min)	27.7 ± 11.9	83.9 ± 34.7	*
V_d (ml/kg)	389.6 ± 74.3	358.9 ± 99.0	ns
Cl (ml/min/kg)	10.9 ± 3.4	3.2 ± 0.6	*
AUC_∞ (µmol·min/L)	12288 ± 4774	39251 ± 7680	*
C_{max} (µmol/L)	233.0 ± 56.5	259.9 ± 41.3	ns
T_{max} (min)	17.5 ± 8.0	38.7 ± 15.6	*

Significantly different PK parameters were calculated for elimination half-life, *Cl,* AUC_∞ and T_{max} when comparing HI-6 2Cl administered IM, in anaesthetized and non-anaesthetized guinea pigs. Elmination half-life, AUC_∞ and T_{max} were greater in anaesthetized animals whereas *Cl* was reduced in anaesthetized guinea pigs. Finally the difference between AUC_∞ and T_{max} is best depicted in Figure 5.7, where it can be seen that the area under the curve is much great for the anaesthetized group.
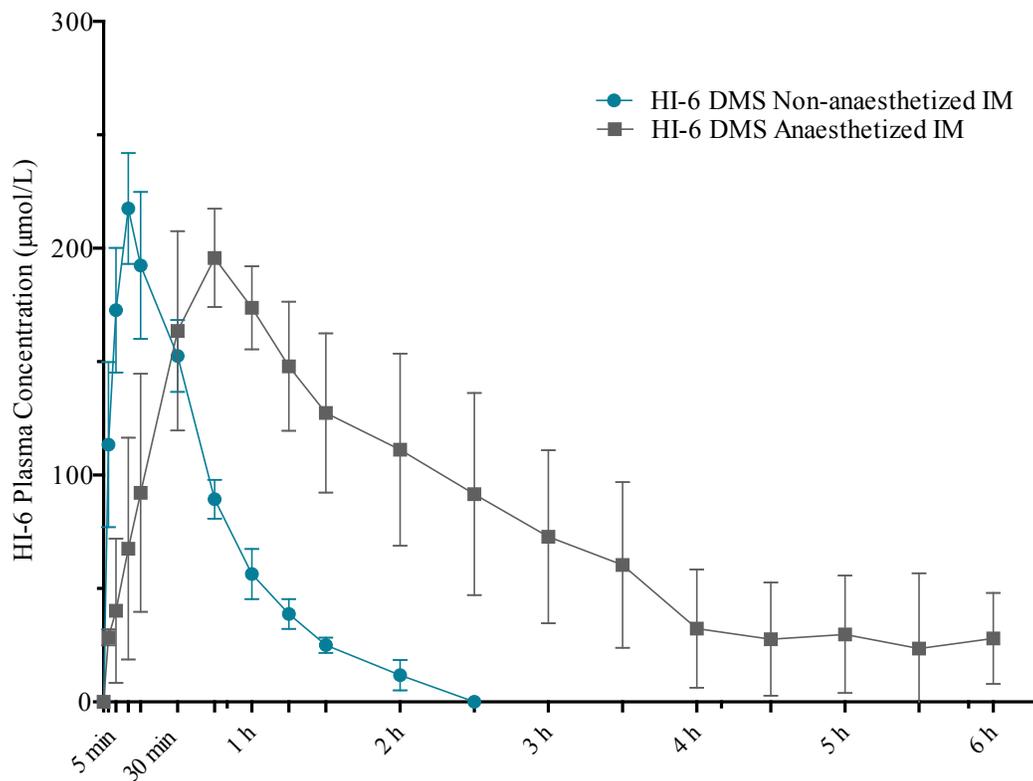
Figure 5.8 Mean (± SD) plasma HI-6 concentration-time profiles comparing equimolar (120 µmol/kg) doses of HI-6 DMS administered IM to non-anaesthetized (Treatment Group 2, *n* = 5) and anaesthetized (Treatment Group 4, *n* = 5) guinea pigs.

Table 5.9 Pharmacokinetics of equimolar (120 µmol/kg) of HI-6 DMS administered IM to non-anaesthetized (Treatment Group 2) and anaesthetized (Treatment Group 4) guinea pigs. Data are mean values ± SD. V_d volume of distribution; Cl total body clearance; AUC_∞ area under the curve; C_{max} maximal concentration; T_{max} time of maximal concentration; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	Non-anaesthetized ($n = 5$)	Anaesthetized ($n = 5$)	Significance
Elimination Half Life (min)	25.9 ± 1.7	97.3 ± 42.0	*
V_d (ml/kg)	440.3 ± 39.6	498.8 ± 76.2	ns
Cl (ml/min/kg)	11.8 ± 1.5	4.0 ± 1.5	*
AUC_∞ (µmol·min/L)	10218 ± 1231	33410 ± 13686	*
C_{max} (µmol/L)	225.9 ± 19.0	197.8 ± 28.1	ns
T_{max} (min)	12.0 ± 2.7	48.0 ± 12.6	*

Similar to HI-6 2Cl administration under anaesthesia, HI-6 DMS administration in anaesthetized guinea pigs resulted in significantly different elimination half-life, Cl, AUC_∞ and T_{max} values. The same trend was seen for HI-6 2Cl administration: elimination half-life, AUC_∞ and T_{max} values were greater in anaesthetized animals whereas Cl was reduced. The differences between groups for AUC_∞ and T_{max} are best represented in Figure 5.8.

5.3.5 Evaluation of Calculated PK Parameters when HI-6 is infused

Case 4 evaluated the calculated pharmacokinetic parameters determined from Treatment Group 5 (anaesthetized guinea pig administered 110 µmol/kg IV bolus of HI-6 2Cl) and Treatment Group 6 (anaesthetized guinea pig administered 110 µmol/kg IV bolus of HI-6 DMS) to calculate a constant infusion rate of HI-6 to reach and maintain a target plasma concentration of 100 µM HI-6 over the course of an eight hour infusion. Figure 5.9 presents mean plasma HI-6 concentration-time profiles for eight hour infusions of HI-6 2Cl (Treatment Group 7) or DMS (Treatment Group 8).
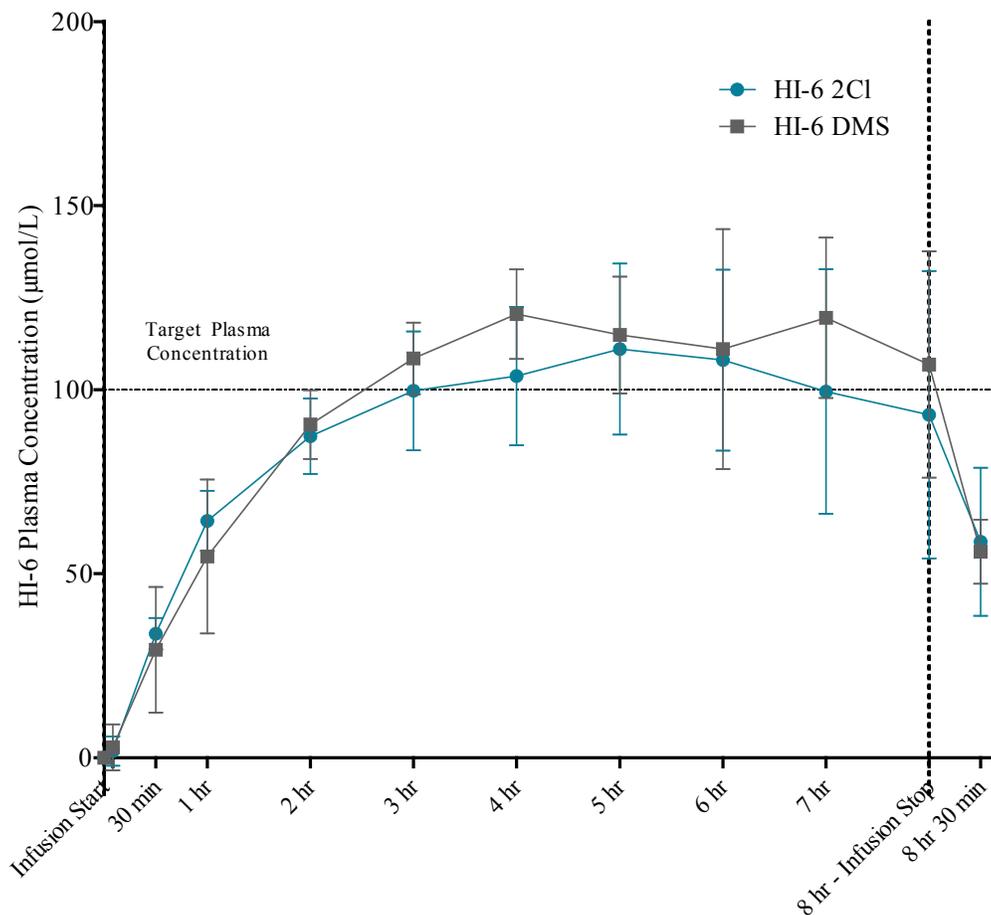
Figure 5.9 Mean (± SD) plasma HI-6 concentration-time profiles comparing calculated infusion rates of HI-6 2Cl (Treatment Group 7, *n* = 6) and HI-6 DMS (Treatment Group 8, *n* = 6) to reach and maintain a target plasma concentration of 100 μM HI-6 over the course of an eight hour infusion in anaesthetized guinea pigs. HI-6 2Cl infusion rate = 0.35 μmol/min·kg. HI-6 DMS infusion rate = 0.49 μmol/min·kg.

As shown in Figure 5.9 both HI-6 2Cl and HI-6 DMS infusions resulted in a target plasma concentration of 100 μM at approximately the three hour mark and this plasma concentration (steady state) was maintained until the infusion was stopped at eight hours at which point a rapid decline in plasma HI-6 concentrations was observed.

5.3.6 Evaluation of Calculated PK Parameters when HI-6 is co-infused with AS

Case 5 evaluated the calculated pharmacokinetic parameters of HI-6 (2Cl and DMS) in anaesthetized guinea pigs to achieve and maintain a target plasma concentration of 100 µM over the course of an eight hour infusion when co-infused with atropine sulphate. An HI-6 2Cl only infusion (Treatment Group 7, infusion rate = 0.35 µmol/min·kg) was directly compared to an equimolar infusion of HI-6 2Cl plus atropine sulphate (Treatment Group 9, atropine sulphate infusion rate = 4.4 µg/min·kg) as shown in Figure 5.10. The HI-6 DMS only infusion Treatment Group (8, infusion rate = 0.49 µmol/min·kg) was directly compared to an equimolar infusion of HI-6 DMS plus atropine sulphate (Treatment Group 10, atropine sulphate infusion rate = 4.4 µg/min·kg) as shown in Figure 5.11.
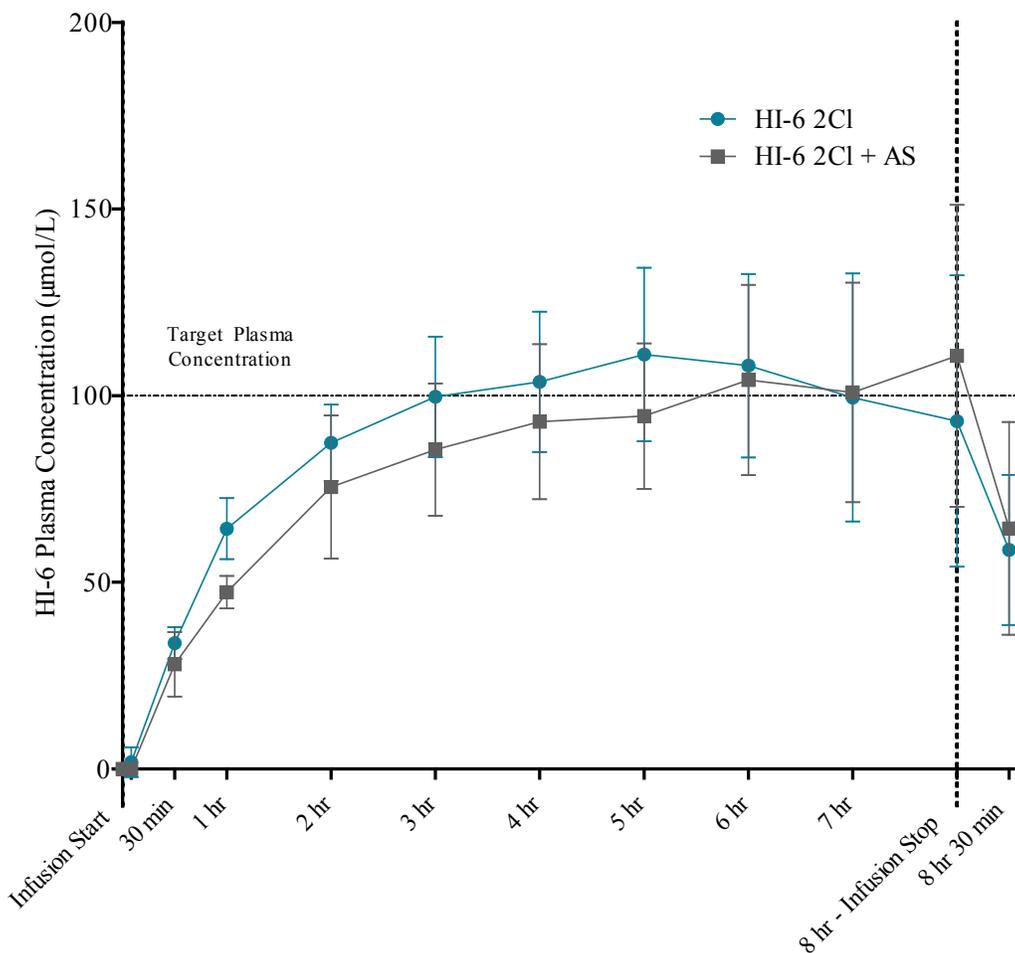
Figure 5.10 Mean (± SD) plasma HI-6 concentration-time profiles comparing calculated infusion rates of HI-6 2Cl (Treatment Group 7, *n* = 6) and HI-6 2Cl + AS (Treatment Group 9, *n* = 6) to reach and maintain a target plasma concentration of 100 μM HI-6 over the course of an eight hour infusion in anaesthetized guinea pigs. HI-6 2Cl infusion rate = 0.35 μmol/min·kg. Atropine sulphate infusion rate = 4.4 μg/min·kg.

Similar to HI-6 only infusions presented earlier, co-infusion of AS with HI-6 2Cl resulted in target concentration (100 μM) being reached around the three hour mark and was maintained over the eight hour experimental period. As shown in Figure 5.10 the error bars for both HI-6 2Cl infused alone and HI-6 co-infused with AS overlap at almost every time point with both

lines following a similar trend supporting that co-administration of AS does not significantly alter the pharmacokinetics of HI-6.
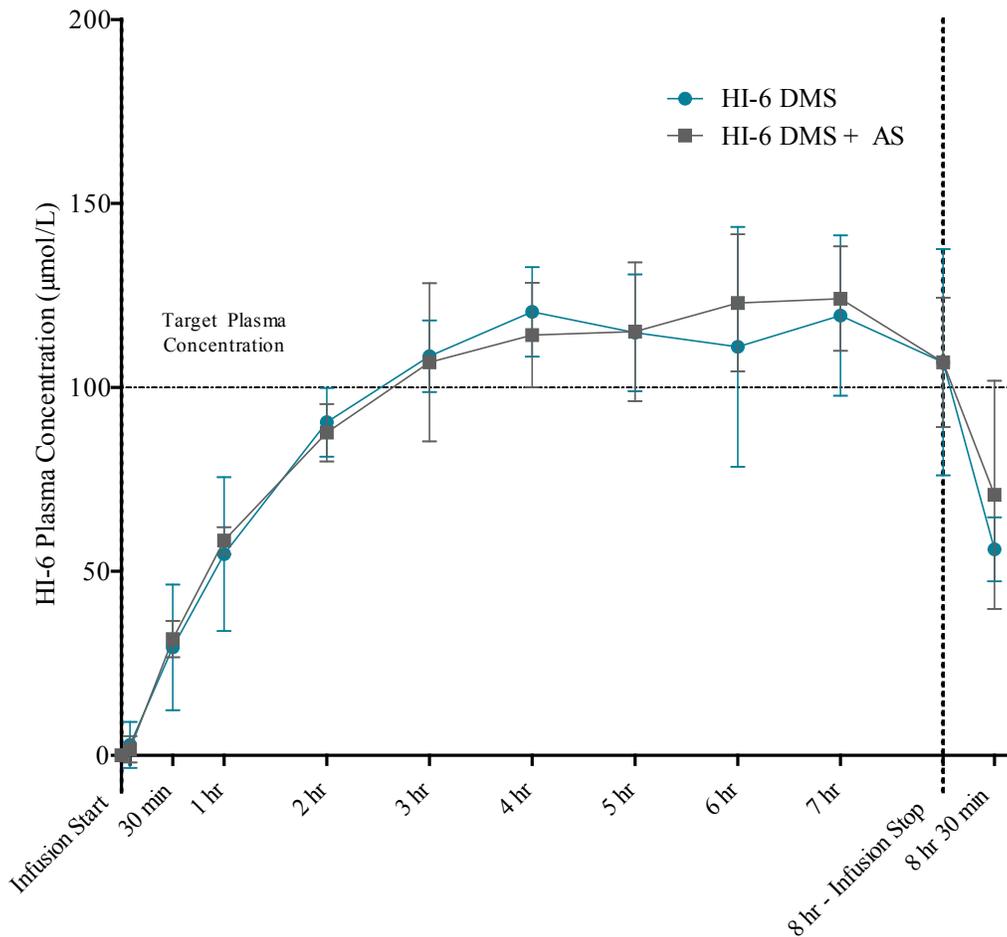
Figure 5.11 Mean (± SD) plasma HI-6 concentration-time profiles comparing calculated infusion rates of HI-6 DMS (Treatment Group 8, $n = 6$) and HI-6 DMS + AS (Treatment Group 10, $n = 5$) to reach and maintain a target plasma concentration of 100 μM HI-6 over the course of an eight hour infusion in anaesthetized guinea pigs. HI-6 DMS infusion rate = 0.49 μmol/min·kg. Atropine sulphate infusion rate = 4.4 μg/min·kg.

HI-6 DMS infused alone compared to HI-6 co-infused with AS did not result in any significant change to the time at which the target plasma concentration was reached or the shape of the plasma concentration curve over the experimental period as shown in Figure 5.11.

5.4 Domestic Swine Experimental Results

The pharmacokinetics of HI-6 were determined, compared and evaluated in anaesthetized domestic male castrated York-Landrace cross swine as follows:

1. Case 6: Comparison of the pharmacokinetics of HI-6 2Cl and HI-6 DMS. Data found in section 5.4.2;
2. Case 7: Comparison of two different routes of administration (IM and IV) within each HI-6 salt. Data found in section 5.4.3;
3. Case 8: Comparison of a low and high concentration of HI-6 (2Cl and DMS) administered intravenously. Data found in section 5.4.4;
4. Case 9: Determination of the effect of atropine co-administration on the pharmacokinetics of HI-6 (2Cl and DMS) when administered IM and IV. Data found in section 5.4.5; and
5. Case 10: Evaluation of the calculated pharmacokinetic parameters of HI-6 DMS in swine plasma to achieve and maintain a target plasma concentration of 100 µM over the course of an eight hour infusion. Data found in section 5.4.6.

5.4.1 Domestic Swine Physiology

Domestic swine Treatment Groups (A through J) were monitored throughout the course of the experimental period for changes in heart rate, mean arterial pressure, oxygen saturation and respiration rate. Figure 5.12 presents the normalized mean heart rate for Treatment Groups A through J over the six hour experimental period. No significant difference ($p < 0.05$) was observed for changes in heart rate between groups A through J; following SSA (stead state anaesthesia) the nominal heart rate was 121 beats per minute ± 13 beats per minute.

The normalized mean arterial pressure for Treatment Groups A through J over the course of the experimental period is presented in Figure 5.13. Following SSA the mean arterial pressure was 64 mmHg ± 7mmHg. No significant difference ($p < 0.05$) was observed for changes in mean arterial pressure between groups A through J.

Normalized mean oxygen saturation is presented in Figure 5.14 for Treatment Groups A through J. The nominal oxygen saturation was 96% ± 2% following SSA for the duration of the six hour experimental period. Larger variance in mean oxygen saturation is shown over short periods of time for Treatment Groups C and H. These variances can be directly attributed to improper sensor placement and this data was not included for statistical analysis between groups. No significant difference ($p < 0.05$) was observed between mean oxygen saturation levels of groups A through J.

Treatment Groups A through J normalized respiration rate data is presented in Figure 5.15 for the duration of the experimental period. The mean respiration rate following normalization based on SSA values was 40 breaths per minute ± 7 breaths per minute. No significant difference is reported between the mean respiration rates of Treatment Groups A through J.

Physiology data and clinical blood analysis levels for Treatment Group K (HI-6 DMS Infusion) is presented in section 5.4.6.

Blood gas levels including pH and bicarbonate (HCO_3^-), hemoglobin (Hgb) levels, electrolyte levels including sodium (Na), potassium (K), chloride (Cl), calcium (Ca), magnesium (Mg) and blood chemistry levels including phosphate (Phos), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), urea nitrogen (UREA) and creatine (CREA) were compared between Treatment Groups A through J. No significant difference ($p < 0.05$) was reported for any of the clinical blood sample analysis; mean and standard deviation values are presented for blood gas levels for Treatment Groups A through J in Table 5.10. Hemoglobin, electrolyte and blood chemistry mean levels for Treatment Groups A through J are presented in Table 5.11.

Complete necropsies were performed for all domestic swine at the end of the experimental period. Gross pathology did not indicate any abnormalities related to HI-6 administration.
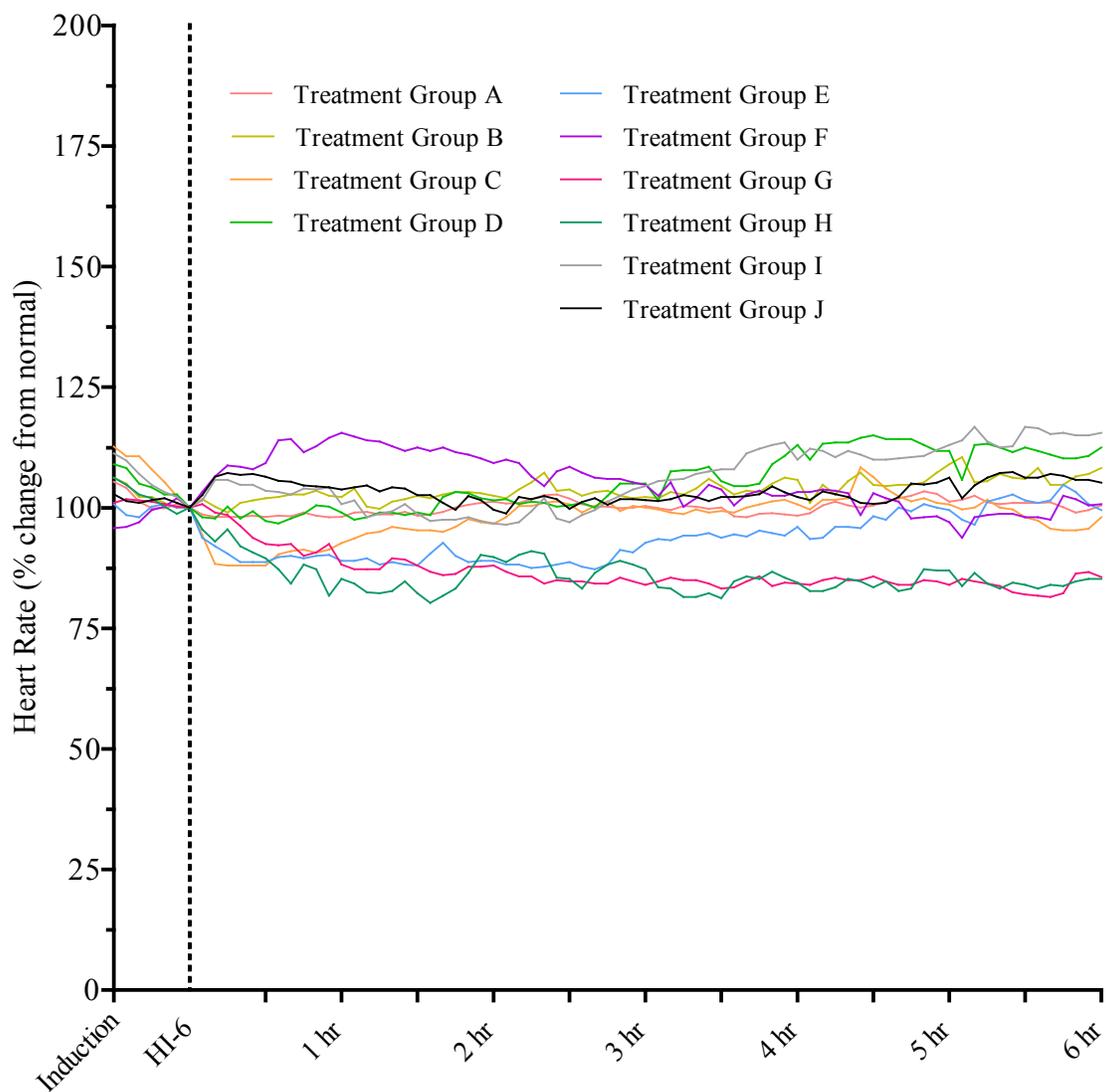
Figure 5.12 Normalized mean heart rate profile comparing Domestic Swine Treatment Groups A through J. Values were normalized to time zero: time at which steady state anaesthesia was reached. Treatment Group A: HI-6 2Cl (53 μmol/kg) IM; Treatment Group B: HI-6 DMS (53 μmol/kg IM); Treatment Group C: HI-6 2Cl (53 μmol/kg) + atropine sulphate (AS) (0.1 mg/kg) IM; Treatment Group D: HI-6 DMS (53 μmol/kg) + AS (0.1mg/kg) IM; Treatment Group E: HI-6 2Cl (26.8 μmol/kg) IV; Treatment Group F: HI-6 DMS (26.8 μmol/kg); Treatment Group G: 4008 μmol HI-6 2Cl IV; Treatment Group H: 4008 μmol HI-6 DMS IV; Treatment Group I: 4008 μmol HI-6 2Cl + 0.1 mg/kg AS IV; and Treatment Group J: 4008 μmol HI-6 DMS + 0.1 mg/kg AS IV.

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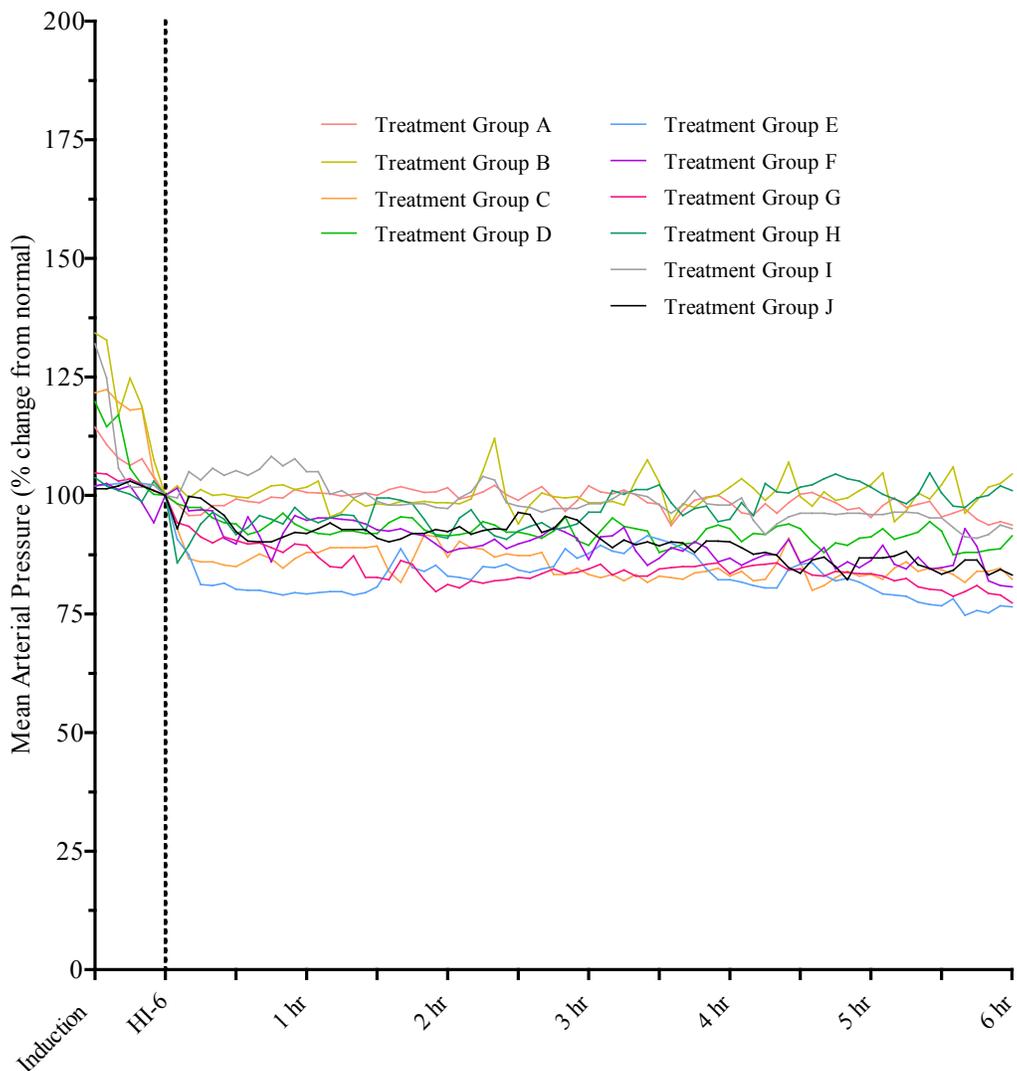
Figure 5.13 Normalized mean arterial pressure profile comparing Domestic Swine Treatment Groups A through J. Values were normalized to time zero: time at which steady state anaesthesia was reached. Treatment Group A: HI-6 2Cl (53 µmol/kg) IM; Treatment Group B: HI-6 DMS (53 µmol/kg IM); Treatment Group C: HI-6 2Cl (53 µmol/kg) + AS (0.1 mg/kg) IM; Treatment Group D: HI-6 DMS (53 µmol/kg) + AS (0.1mg/kg) IM; Treatment Group E: HI-6 2Cl (26.8 µmol/kg) IV; Treatment Group F: HI-6 DMS (26.8 µmol/kg); Treatment Group G: 4008 µmol HI-6 2Cl IV; Treatment Group H: 4008 µmol HI-6 DMS IV; Treatment Group I: 4008 µmol HI-6 2Cl + 0.1 mg/kg AS IV; and Treatment Group J: 4008 µmol HI-6 DMS + 0.1 mg/kg AS IV.

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Figure 5.14 Normalized oxygen saturation profile comparing Domestic Swine Treatment Groups A through J. Values were normalized to time zero: time at which steady state anaesthesia was reached. Treatment Group A: HI-6 2Cl (53 μmol/kg) IM; Treatment Group B: HI-6 DMS (53 μmol/kg IM); Treatment Group C: HI-6 2Cl (53 μmol/kg) + AS (0.1 mg/kg) IM; Treatment Group D: HI-6 DMS (53 μmol/kg) + AS (0.1mg/kg) IM; Treatment Group E: HI-6 2Cl (26.8 μmol/kg) IV; Treatment Group F: HI-6 DMS (26.8 μmol/kg); Treatment Group G: 4008 μmol HI-6 2Cl IV; Treatment Group H: 4008 μmol HI-6 DMS IV; Treatment Group I: 4008 μmol HI-6 2Cl + 0.1 mg/kg AS IV; and Treatment Group J: 4008 μmol HI-6 DMS + 0.1 mg/kg AS IV.

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Figure 5.15 Normalized respiration rate profile comparing Domestic Swine Treatment Groups A through J. Values were normalized to time zero: time at which steady state anaesthesia was reached. Treatment Group A: HI-6 2Cl (53 μmol/kg) IM; Treatment Group B: HI-6 DMS (53 μmol/kg IM); Treatment Group C: HI-6 2Cl (53 μmol/kg) + AS (0.1 mg/kg) IM; Treatment Group D: HI-6 DMS (53 μmol/kg) + AS (0.1mg/kg) IM; Treatment Group E: HI-6 2Cl (26.8 μmol/kg) IV; Treatment Group F: HI-6 DMS (26.8 μmol/kg); Treatment Group G: 4008 μmol HI-6 2Cl IV; Treatment Group H: 4008 μmol HI-6 DMS IV; Treatment Group I: 4008 μmol HI-6 2Cl + 0.1 mg/kg AS IV; and Treatment Group J: 4008 μmol HI-6 DMS + 0.1 mg/kg AS IV.

Table 5.10 Mean blood gas values for all domestic swine included in Treatment Groups A through J.

				Mean (±SD)				
	SSA	0.5 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
pH	7.40	7.38	7.39	7.42	7.41	7.41	7.40	7.40
	(0.05)	(0.07)	(0.06)	(0.06)	(0.05)	(0.05)	(0.05)	(0.06)
Bicarbonate (HCO_3^-)	29	28	29	29	30	29	30	29
(mmol/L)	(2.4)	(2.7)	(2.0)	(2.1)	(1.7)	(2.3)	(1.9)	(2.5)

Table 5.11 Mean hemoglobin, electrolyte and blood chemistry values for all domestic swine included in Treatment Groups A through J.

	Mean ± SD	
	SSA	6 hrs
Hemoglobin Levels		
Hemoglobin (Hgb) (g/dL)	10.3 ± 2.9	9.5 ± 1.9
	SSA	5 hrs
Electrolytes		
Sodium (Na) (mmol/L)	143 ± 8	143 ± 10
Potassium (K) (mmol/L)	4.5 ± 0.3	4.9 ± 0.7
Chloride (Cl) (mmol/L)	106 ± 2	107 ± 3
Calcium (Ca) (mmol/L)	2.3 ± 0.2	2.3 ± 0.2
Magnesium (Mg) (mmol/L)	0.85 ± 0.10	0.86 ± 0.08
Phosphate (Phos) (mmol/L)	2.9 ± 0.3	3.1 ± 0.4
Blood Chemistry		
Aspartate aminotransferase (AST) (u/L)	23.8 ± 5.8	25.7 ± 11.6
Gamma-glutamyltransferase (GGT) (u/L)	29.8 ± 10.5	28.6 ± 11.1
Urea nitrogen (UREA) (mmol/L)	3.13 ± 1.20	3.79 ± 0.94
Creatinine (CREA) (u/L)	81 ± 16	81 ± 20

5.4.2 Comparison of HI-6 salts in domestic swine

Case 6 compared the pharmacokinetics of the two salts of HI-6 (2Cl and DMS) administered in domestic swine as follows:

1. Treatment Group A (swine administered 53 μmol/kg HI-6 2Cl IM) compared to Treatment Group B (swine administered 53 μmol/kg HI-6 DMS IM). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.16 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.12;

2. Treatment Group C (swine administered 53 μmol/kg HI-6 2Cl + 0.1 mg/kg AS, IM) compared to Treatment Group D (swine administered 53 μmol/kg HI-6 DMS + 0.1mg/kg AS, IM. Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.17 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.13;

3. Treatment Group E (swine administered 26.8 μmol/kg HI-6 2Cl IV) compared to Treatment Group F (swine administered 26.8 μmol/kg HI-6 DMS IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are presented in Figure 5.18 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.14;

4. Treatment Group G (swine administered 4008 μmol of HI-6 2Cl IV) compared to Treatment Group H (swine administered 4008 μmol of HI-6 DMS IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are presented in Figure 5.19 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.15; and

5. Treatment Group I (swine administered 4008 μmol of HI-6 2Cl + 0.1mg/kg AS IV) compared to Treatment Group J (swine administered 4008 μmol of HI-6 DMS + 0.1mg/kg AS IV). Mean plasma HI-6 concentration-time profiles are presented in Figure 5.20 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.16.
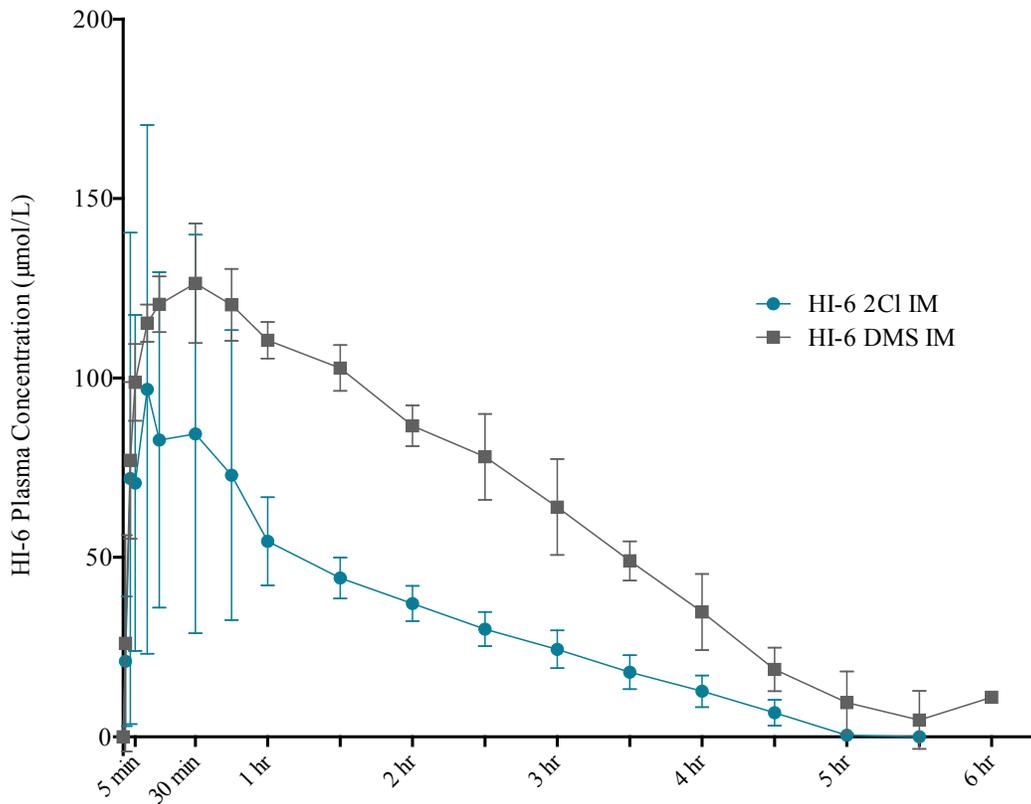
Figure 5.16 Mean (± SD) plasma HI-6 concentration-time profile comparing equimolar doses of HI-6 2Cl (Treatment Group A, *n* = 8) and HI-6 DMS (Treatment Group B, *n* = 4) administered to domestic swine IM.

Table 5.12 Pharmacokinetics of HI-6 2Cl (Treatment Group A) and DMS (Treatment Group B) administered IM at an equimolar dose (53 µmol/kg) to domestic swine. Data presented are mean values ± SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_{max} maximal concentration; T_{max} time of maximal concentration; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl ($n = 8$)	HI-6 DMS ($n = 4$)	Significance
Distribution/Absorption Half Life (min)	15.0 ± 15.4	26.5 ± 9.8	ns
Elimination Half Life (min)	58.2 ± 18.3	67.5 ± 6.1	ns
V_d (ml/kg)	414.8 ± 65.7	225.5 ± 30.4	*
Cl (ml/min/kg)	5.2 ± 1.2	2.3 ± 0.2	*
Cl_r (ml/min/kg)	3.4 ± 1.0	1.8 ± 0.1	ns
AUC_∞ (µmol·min/L)	10605 ± 2228	23156 ± 2181	*
C_{max} (µmol/L)	86.9 ± 29.3	131.7 ± 14.0	*
T_{max} (min)	28.9 ± 21.5	57.7 ± 13.4	*
HI-6 in Urine (%)	67.9 ± 11.7	75.8 ± 5.3	ns

A significant difference was observed for V_d, Cl, AUC_∞, C_{max} and T_{max} when equimolar doses of HI-6 2Cl and HI-6 DMS were administered IM in domestic anaesthetized swine. V_d and Cl were greater in swine dosed with HI-6 2Cl whereas AUC_∞, C_{max} and T_{max} were greater in swine treated with an IM injection of HI-6 DMS. The significant differences in AUC_∞, C_{max} and T_{max} can be seen in Figure 5.16.
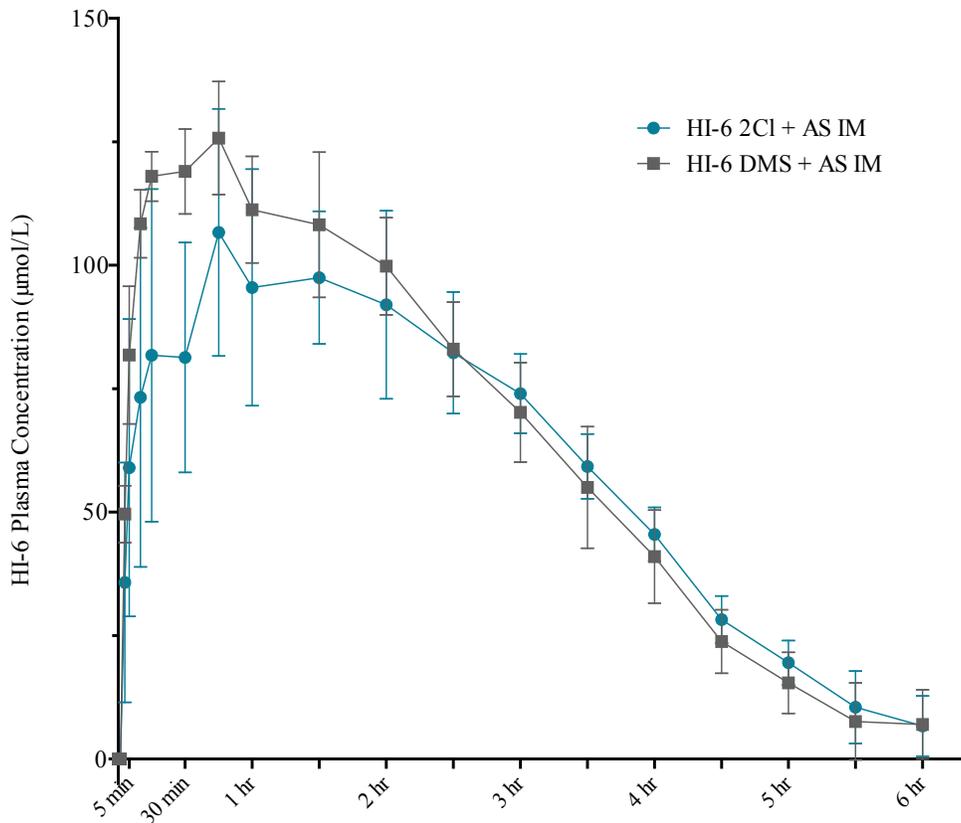
Figure 5.17 Mean (± SD) plasma HI-6 concentration-time profile comparing equimolar doses of HI-6 2Cl + AS (Treatment Group C, $n = 4$) and HI-6 DMS + AS (Treatment Group D, $n = 5$) administered to domestic swine IM.

Table 5.13 Pharmacokinetics of HI-6 2Cl + AS (Treatment Group C) and DMS + AS (Treatment Group D) administered IM at an equimolar dose (53 µmol/kg + 0.1mg/kg) to domestic swine. Data presented are mean values ± SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_{max} maximal concentration; T_{max} time of maximal concentration; nr not reported; nc not calculated; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl + AS (*n* = 4)	HI-6 DMS + AS (*n* = 5)	Significance
Distribution/Absorption Half Life (min)	35.8 ± 2.4	31.3 ± 3.2	ns
Elimination Half Life (min)	58.7 ± 7.3	58.6 ± 13.0	ns
V_d (ml/kg)	201.4 ± 23.6	184.4 ± 30.2	ns
Cl (ml/min/kg)	2.4 ± 0.5	2.2 ± 0.3	ns
Cl_r (ml/min/kg)	nr	1.8 ± 0.8	nc
AUC_∞ (µmol·min/L)	22609 ± 3782	24342 ± 3033	ns
C_{max} (µmol/L)	123.1 ± 17.2	140.9 ± 14.0	ns
T_{max} (min)	65.3 ± 3.9	60.0 ± 2.5	*
HI-6 in Urine (%)	nr	75.8 ± 5.3	nc

T_{max} was found to be significantly greater when HI-6 2Cl was co-administered with AS, IM when compared to equimolar HI-6 DMS and AS administration IM in domestic swine. The significance of any difference between both HI-6 salts when co-administered IM, could not be calculated for renal clearance or percent of HI-6 in urine as insufficient data was available for swine treated with HI-6 2Cl and AS.
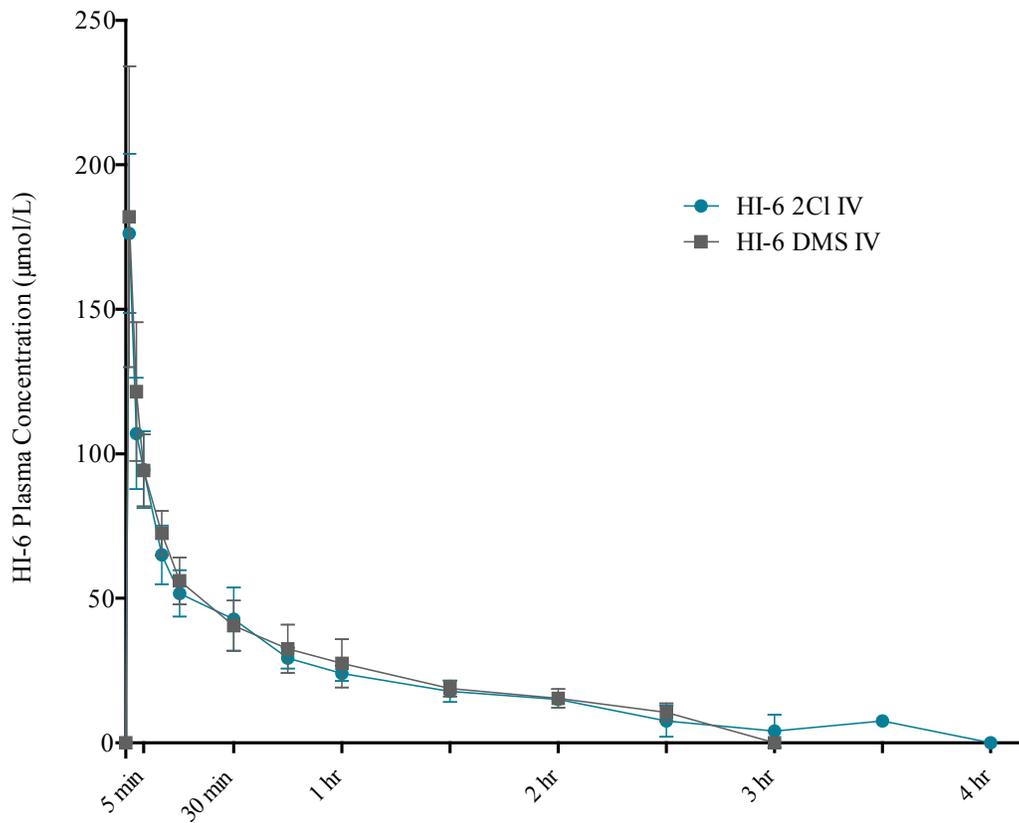
Figure 5.18 Mean (±SD) plasma HI-6 concentration-time profile comparing equimolar doses of HI-6 2Cl (Treatment Group E, *n* = 4) and HI-6 DMS (Treatment Group F, *n* = 4) administered to domestic swine IV.

Table 5.14 Pharmacokinetics of HI-6 2Cl (Treatment Group E) and DMS (Treatment Group F) administered IV at an equimolar dose (26.8 µmol/kg) to domestic swine. Data presented are mean values ± SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_0 initial concentration; ns no significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl ($n = 4$)	HI-6 DMS ($n = 4$)	Significance
Distribution Half Life (min)	4.4 ± 0.4	4.2 ± 0.6	ns
Elimination Half Life (min)	70.8 ± 11.2	62.5 ± 6.6	ns
V_d (ml/kg)	492.3 ± 37.6	428.4 ± 79.9	ns
Cl (ml/min/kg)	4.9 ± 1.0	4.8 ± 0.7	ns
Cl_r (ml/min/kg)	3.4 ± 0.6	3.0 ± 0.5	ns
AUC_∞ (µmol·min/L)	5603 ± 981	5709 ± 1003	ns
C_0 (µmol/L)	167.3 ± 26.6	179.8 ± 56.8	ns
HI-6 in Urine (%)	71.0 ± 14.9	65.5 ± 7.0	ns

No significant difference was reported for any of the calculated pharmacokinetic parameters when comparing IV administration of HI-6 2Cl and DMS at a low dose of 26.8µmol/kg. Both salts have a very similar plasma HI-6 profile as shown in Figure 5.18 with limited variation or deviation across the experimental period.
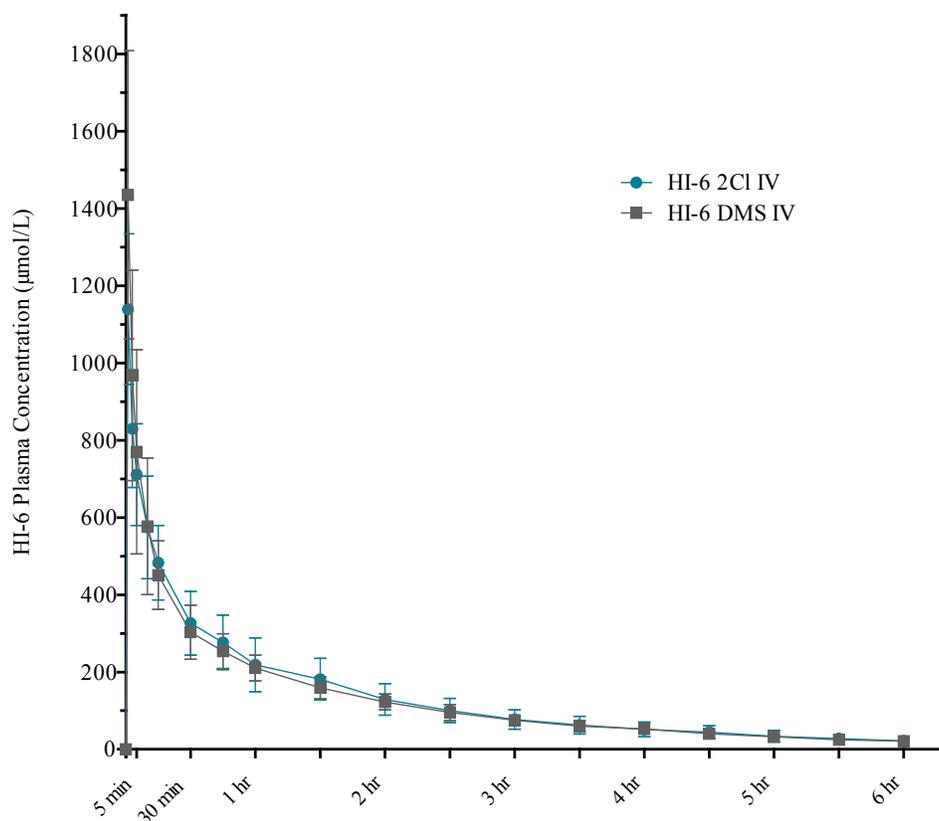
Figure 5.19 Mean (± SD) plasma HI-6 concentration-time profile comparing equimolar doses of HI-6 2Cl (Treatment Group G, *n* = 4) and HI-6 DMS (Treatment Group H, *n* = 4) administered to domestic swine IV.

Table 5.15 Pharmacokinetics of HI-6 2Cl (Treatment Group G) and DMS (Treatment Group H) administered IV at an equimolar dose (4008 µmol) to domestic swine. Data presented are mean values ± SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_0 initial concentration; ns no significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl (*n* = 4)	HI-6 DMS (*n* = 4)	Significance
Distribution Half Life (min)	6.5 ± 0.3	5.3 ± 1.2	ns
Elimination Half Life (min)	85.1 ± 5.9	86.3 ± 6.8	ns
V_d (ml/kg)	426.2 ± 72.2	453.7 ± 61.3	ns
Cl (ml/min/kg)	3.5 ± 0.8	3.7 ± 0.7	ns
Cl_r (ml/min/kg)	2.5 ± 0.5	2.9 ± 0.5	ns
AUC_∞ (µmol·min/L)	52441 ± 14362	50632 ± 10829	ns
C_0 (µmol/L)	1075.0 ± 182.3	1367.8 ± 431.1	ns
HI-6 in Urine (%)	72.9 ± 15.0	83.9 ± 9.3	ns

Similar to low dose IV administration of HI-6 no significant difference was reported for any of the calculated pharmacokinetic parameters when a high dose (4008µmol) of HI-6 2Cl or HI-6 DMS was administered IV in anaesthetized domestic swine.
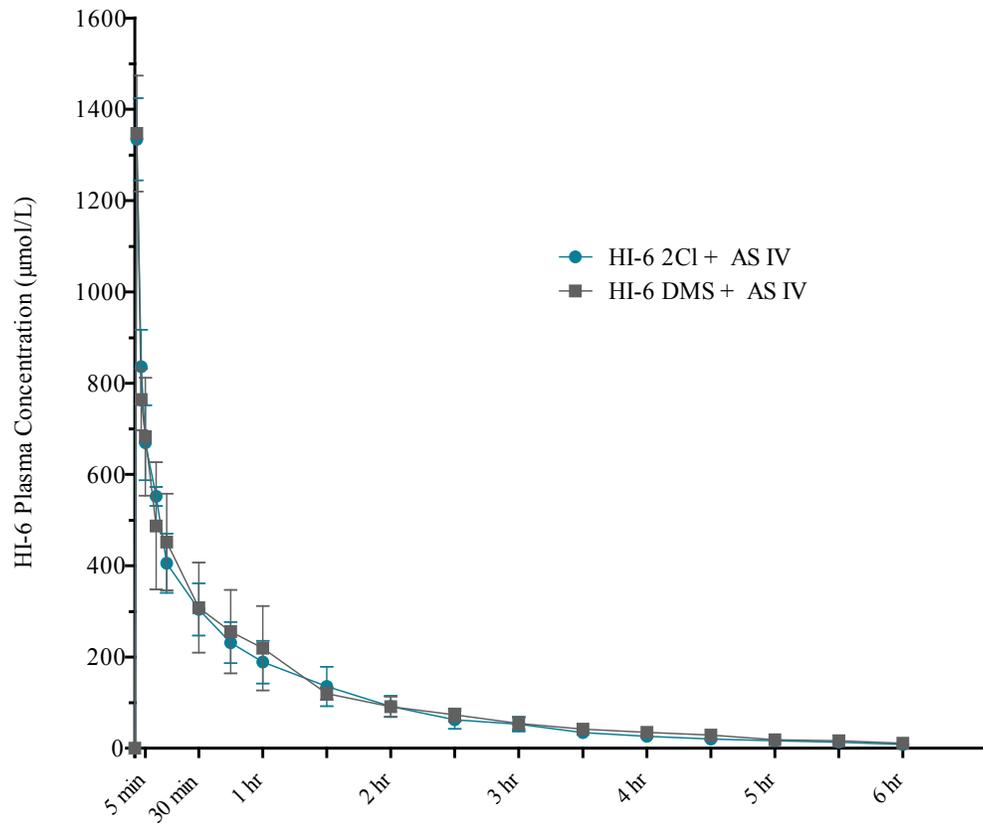
Figure 5.20 Mean (± SD) plasma HI-6 concentration-time profile comparing equimolar doses of HI-6 2Cl + AS (Treatment Group I, $n = 4$) and HI-6 DMS + AS (Treatment Group J, $n = 4$) administered to domestic swine IV.

Table 5.16 Pharmacokinetics of HI-6 2Cl + AS (Treatment Group I) and DMS + AS (Treatment Group J) administered IV at an equimolar dose (4008 µmol + 0.1mg/kg) to domestic swine. Data presented are mean values ± SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_0 initial concentration; ns no significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl + AS ($n = 4$)	HI-6 DMS + AS ($n = 4$)	Significance
Distribution Half Life (min)	5.0 ± 0.4	4.6 ± 1.3	ns
Elimination Half Life (min)	67.7 ± 3.0	75.7 ± 9.5	ns
V_d (ml/kg)	463.6 ± 50.1	440.2 ± 99.7	ns
Cl (ml/min/kg)	4.8 ± 0.6	4.0 ± 0.9	ns
Cl_r (ml/min/kg)	2.3 ± 1.0	2.8 ± 1.3	ns
AUC_∞ (µmol·min/L)	40434 ± 8004	44636 ± 9732	ns
C_0 (µmol/L)	1223.0 ± 85.2	1330.4 ± 265.4	ns
HI-6 in Urine (%)	49.5 ± 24.4	64.8 ± 23.1	ns

When AS was co-administered with HI-6 2Cl and HI-6 DMS, IV in anaesthetized domestic swine the pharmacokinetic profiles were found to be very similar with no significant differences in calculated pharmacokinetic parameters reported.

5.4.3 Comparison of routes of administration of HI-6 in domestic swine

Case 7 compared the pharmacokinetics of two different routes of administration of HI-6 (2Cl and DMS) in domestic swine as follows:

1. Treatment Group A (domestic swine administered 53 µmol/kg HI-6 2Cl IM) compared to Treatment Group E (domestic swine administered 26.8 µmol/kg HI-6 2Cl IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.21and their corresponding calculated pharmacokinetic parameters are presented in Table 5.17; and

2. Treatment Group B (domestic swine administered 53 µmol/kg HI-6 DMS IM) compared to Treatment Group F (domestic swine administered 26.8 µmol/kg HI-6 DMS IV). Mean

plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.22 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.18.
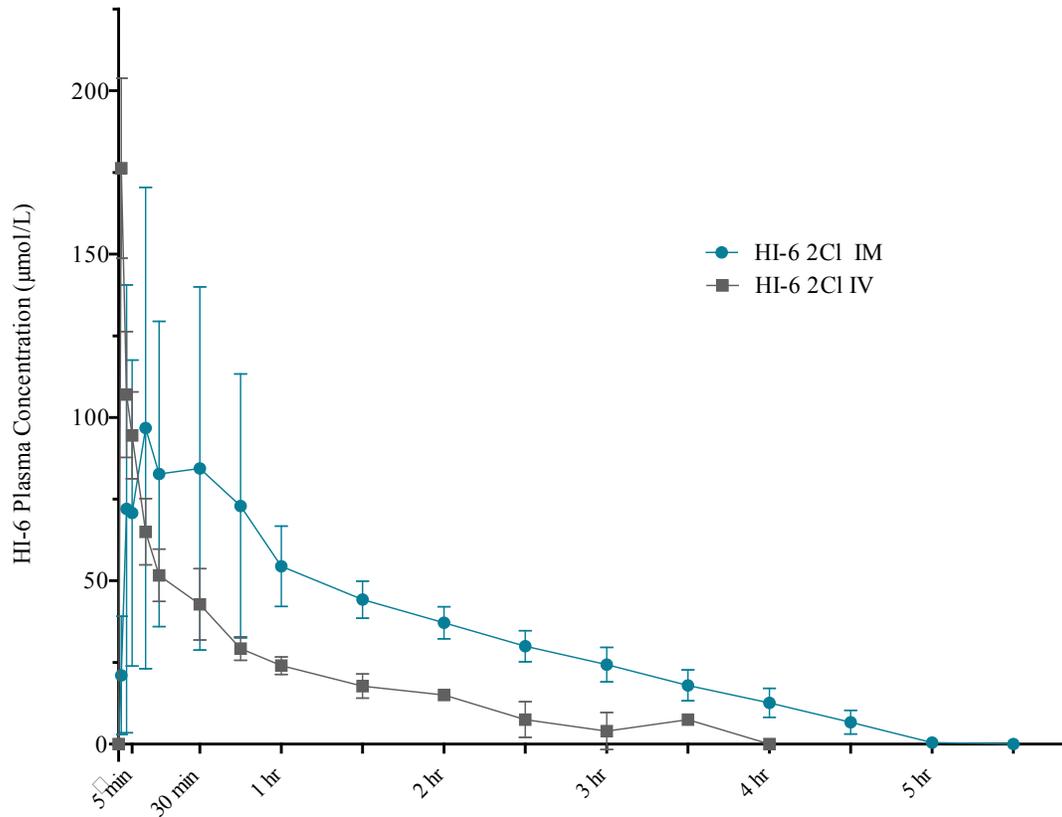
Figure 5.21 Mean (± SD) plasma HI-6 concentration-time profiles comparing HI-6 2Cl administered IM (Treatment Group A, 53 µmol/kg, *n* = 8) and IV (Treatment Group E, 26.8 µmol/kg, *n* = 4) in domestic swine.

Table 5.17 Pharmacokinetics of HI-6 2Cl administered IM (Treatment Group A, 53 µmol/kg) and IV (Treatment Group E, 26.8 µmol/kg) to domestic swine. Data are mean values ± SD. V_d volume of distribution; *Cl* total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	IM (*n* = 8)	IV (*n* = 4)	Significance
Distribution/Absorption Half Life (min)	15.0 ± 15.4	4.4 ± 0.4	ns
Elimination Half Life (min)	58.2 ± 18.3	70.8 ± 11.2	ns
V_d (ml/kg)	414.8 ± 65.7	492.3 ± 37.6	ns
Cl (ml/min/kg)	5.2 ± 1.2	4.9 ± 1.0	ns
Cl_r (ml/min/kg)	3.4 ± 1.0	3.4 ± 0.6	ns
AUC_∞ (µmol·min/L)	10605 ± 2228	5603 ± 981	*
HI-6 in Urine (%)	60.8 ± 21.6	71.0 ± 14.9	ns

Following IV and IM administration of HI-6 2Cl in anaesthetized domestic swine, AUC_∞ was the only pharmacokinetic parameter determined to be significantly different between the two groups. It is important to note that the doses of HI-6 2Cl administered were not similar. The IM dose administered (53 µmol/kg) was greater than the IV does (26.8 µmol/kg) administered which was reflected in the AUC_∞ calculated which was greater for domestic swine administered HI-6 2Cl, IM.
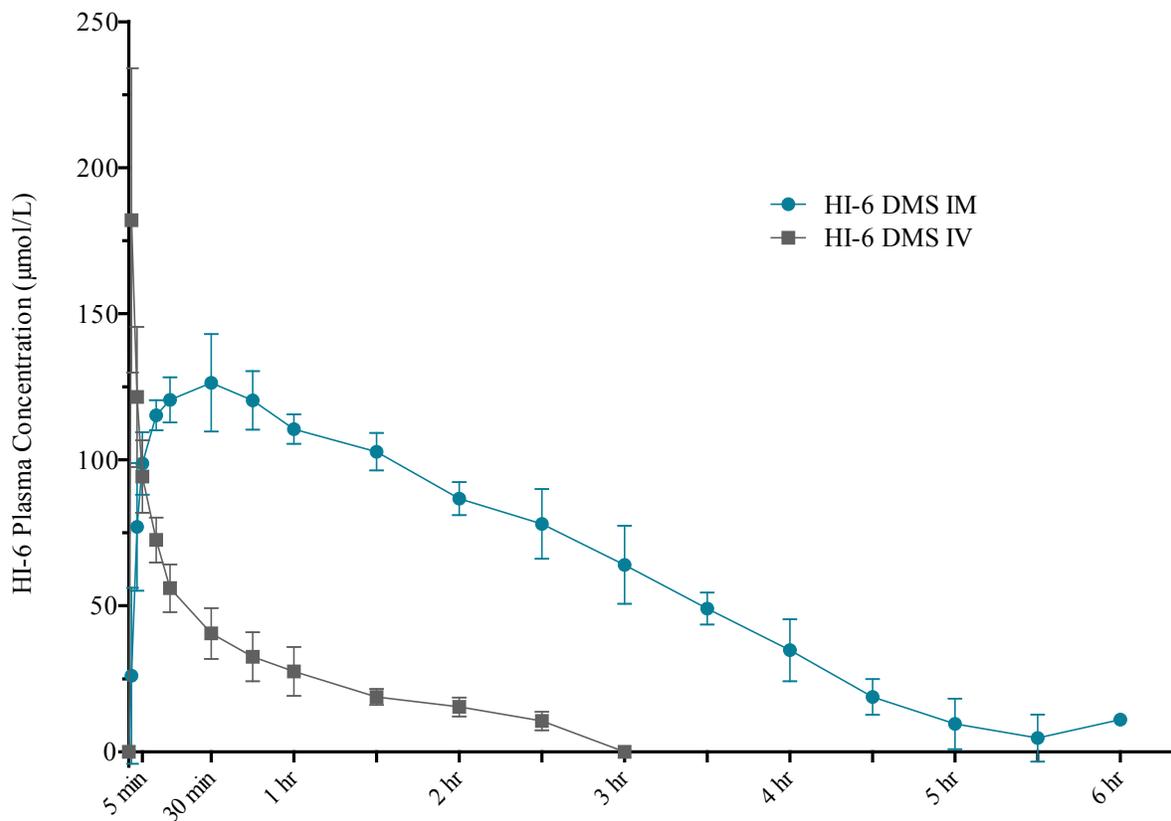
Figure 5.22 Mean (± SD) plasma HI-6 concentration-time profiles comparing HI-6 DMS administered IM (Treatment Group B, 53 µmol/kg, $n = 4$) and IV (Treatment Group F, 26.8 µmol/kg, $n = 4$) in domestic swine.

Table 5.18 Pharmacokinetics of HI-6 DMS administered IM (Treatment Group B, 53 µmol/kg) and IV (Treatment Group F, 26.8 µmol/kg) to domestic swine. Data are mean values ± SD. V_d volume of distribution; *Cl* total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	IM (*n* = 4)	IV (*n* = 4)	Significance
Distribution/Absorption Half Life (min)	26.5 ± 9.8	4.2 ± 0.6	*
Elimination Half Life (min)	62.5 ± 6.6	67.5 ± 6.1	ns
V_d (ml/kg)	225.5 ± 30.4	428.4 ± 79.9	*
Cl (ml/min/kg)	2.3 ± 0.2	4.8 ± 0.7	*
Cl_r (ml/min/kg)	1.8 ± 0.1	3.0 ± 0.5	ns
AUC_∞ (µmol·min/L)	23156 ± 2181	5709 ± 1003	*
HI-6 in Urine (%)	75.8 ± 5.3	65.5 ± 7.0	ns

The distribution/absorption half life, V_d, *Cl* and AUC_∞ calculated following either IM or IV administration of HI-6 DMS in anaesthetized domestic swine were found to be significantly different. The doses of HI-6 DMS administered IV and IM were not equimolar, with a larger dose being administered to the IM treated group. The distribution/absorption half life was longer and the AUC_∞ was greater in swine administered HI-6 DMS, IM. Additionally the V_d and *Cl* were greater following IV administration of HI-6 DMS in domestic swine.

5.4.4 Comparison of different HI-6 concentrations administered in domestic swine

Case 8 compared the pharmacokinetics of two different doses of HI-6 (2Cl and DMS) administered intravenously in domestic swine as follows:

1. Treatment Group E (domestic swine administered 26.8 µmol/kg HI-6 2Cl IV) compared to Treatment Group G (domestic swine administered 4008 µmol HI-6 2Cl IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.23 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.19; and

2. Treatment Group F (domestic swine administered 26.8 µmol/kg HI-6 DMS IV) compared to Treatment Group H (domestic swine administered 4008 µmol HI-6 DMS IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.24 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.20.
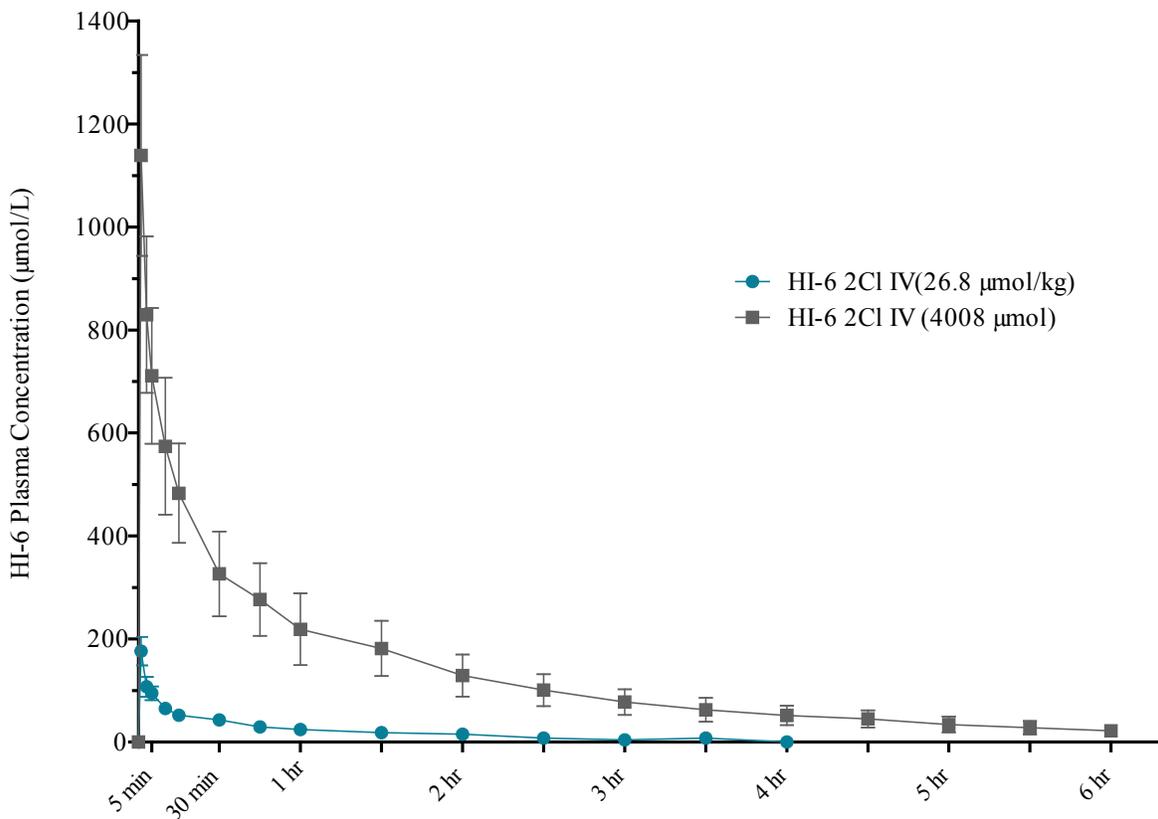
Figure 5.23 Mean (±SD) plasma HI-6 concentration-time profiles comparing a low (Treatment Group E, $n = 4$) and high (Treatment Group G, $n = 4$) dose of HI-6 2Cl administered IV in domestic swine.

Table 5.19 Pharmacokinetics of a low (Treatment Group E, 26.8 µmol/kg) and high (Treatment Group G, 4008 µmol) dose of HI-6 2Cl administered IV to domestic swine. Data are mean values ± SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_0 initial concentration; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	Low Dose (26.8 µmol/kg) (n = 4)	High Dose (4008 µmol) (n = 4)	Significance
Distribution Half Life (min)	4.4 ± 0.4	6.5 ± 0.3	*
Elimination Half Life (min)	70.8 ± 11.2	85.1 ± 5.9	ns
V_d (ml/kg)	492.3 ± 37.6	426.2 ± 72.2	ns
Cl (ml/min/kg)	4.9 ± 1.0	3.5 ± 0.8	ns
Cl_r (ml/min/kg)	3.4 ± 0.6	2.5 ± 0.5	ns
AUC_∞ (µmol·min/L)	5603 ± 981	52441 ± 14362	*
C_0 (µmol/L)	167.3 ± 26.6	1075.0 ± 182.3	*
HI-6 in Urine (%)	71.0 ± 14.9	72.9 ± 15.0	ns

Distribution half life, AUC_∞ and C_0 were significantly greater in domestic swine administered a high dose (4008 µmol) of HI-6 2Cl compared to a low dose (26.8 µmol/kg). The difference in AUC_∞ and C_0 are represented in Figure 5.23.
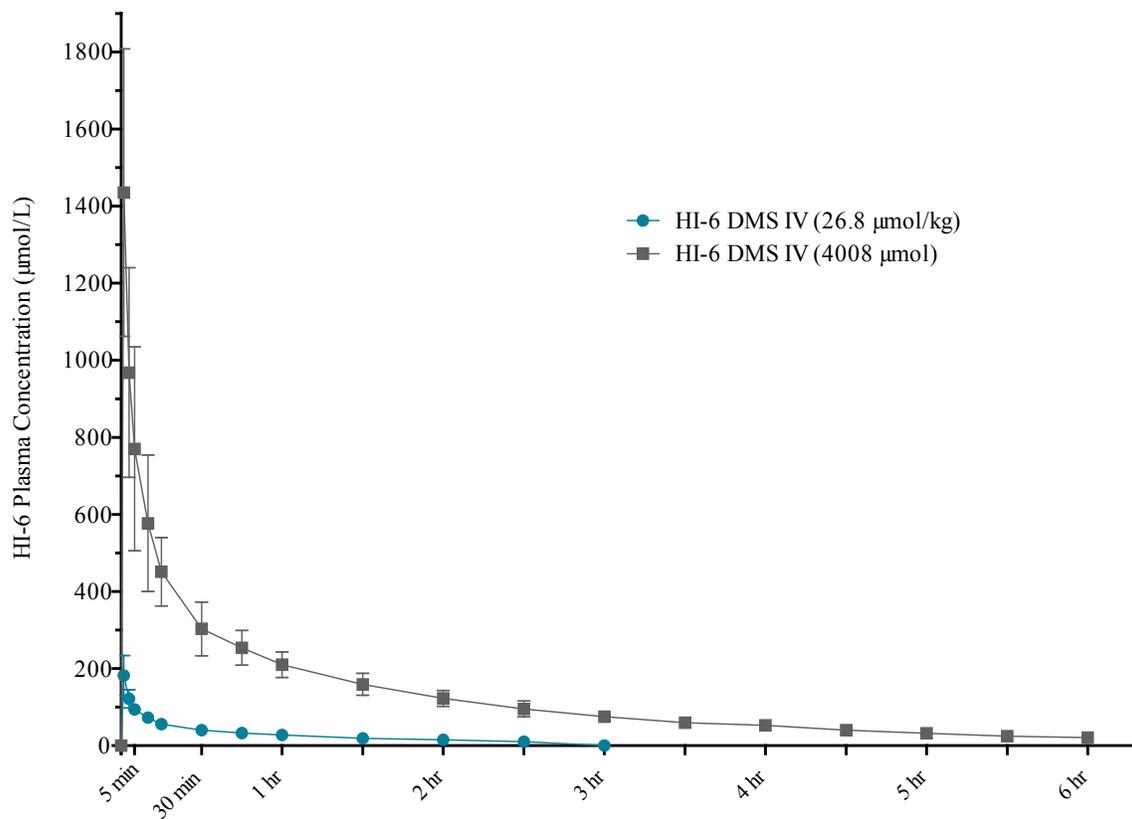
Figure 5.24   Mean (± SD) plasma HI-6 concentration-time profiles comparing a low (Treatment Group F, *n* = 4) and high (Treatment Group H, *n* = 4) dose of HI-6 DMS administered IV in domestic swine.

Table 5.20 Pharmacokinetics of a low (Treatment Group F, 26.8 µmol/kg) and high (Treatment Group H, 4008 µmol) dose of HI-6 DMS administered IV to domestic swine. Data are mean values ± SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_0 initial concentration; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	Low Dose (26.8 µmol/kg) ($n = 4$)	High Dose (4008 µmol) ($n = 4$)	Significance
Distribution Half Life (min)	4.2 ± 0.6	5.3 ± 1.2	ns
Elimination Half Life (min)	62.5 ± 6.6	86.3 ± 6.8	*
V_d (ml/kg)	428.4 ± 79.9	453.7 ± 61.3	ns
Cl (ml/min/kg)	4.8 ± 0.7	3.7 ± 0.7	ns
Cl_r (ml/min/kg)	3.0 ± 0.5	2.9 ± 0.5	ns
AUC_∞ (µmol·min/L)	5709 ± 1003	50632 ± 10829	*
C_0 (µmol/L)	179.8 ± 56.8	1367.8 ± 431.1	*
HI-6 in Urine (%)	65.5 ± 7.0	83.9 ± 9.3	ns

Elimination half life, AUC_∞ and C_0 were found to be significantly different following IV administration of a high dose (4008 µmol) of HI-6 DMS when compared to a lower dose (26.8 µmol/kg) of HI-6 DMS in domestic swine. The differences between AUC_∞ and C_0 when directly comparing a high and low dose of HI-6 DMS administered IV is best represented by Figure 5.24.

5.4.5 Determination of effect of atropine sulphate co-administration

Case 9 determined the effect of atropine sulphate co-administration with HI-6 (2Cl or DMS) on the pharmacokinetics of HI-6 when administered IV or IM as follows:

1. Treatment Group A (domestic swine administered 53 µmol/kg HI-6 2Cl IM) compared to Treatment Group C (domestic swine administered 53 µmol/kg HI-6 2Cl + 0.1mg/kg AS, IM). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.25 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.21;

2. Treatment Group G (domestic swine administered 4008 μmol HI-6 2Cl IV) compared to Treatment Group I (domestic swine administered 4008 μmol HI-6 2Cl + 0.1mg/kg AS, IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.26 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.22;

3. Treatment Group B (domestic swine administered 53 μmol/kg HI-6 DMS IM) compared to Treatment Group D (domestic swine administered 53 μmol/kg HI-6 DMS + 0.1mg/kg AS, IM). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.27 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.23; and

4. Treatment Group H (domestic swine administered 4008 μmol HI-6 DMS IV) compared to Treatment Group J (domestic swine administered 4008 μmol HI-6 DMS + 0.1mg/kg AS, IV). Mean plasma HI-6 concentration-time profiles for both treatment groups are shown in Figure 5.28 and their corresponding calculated pharmacokinetic parameters are presented in Table 5.24.
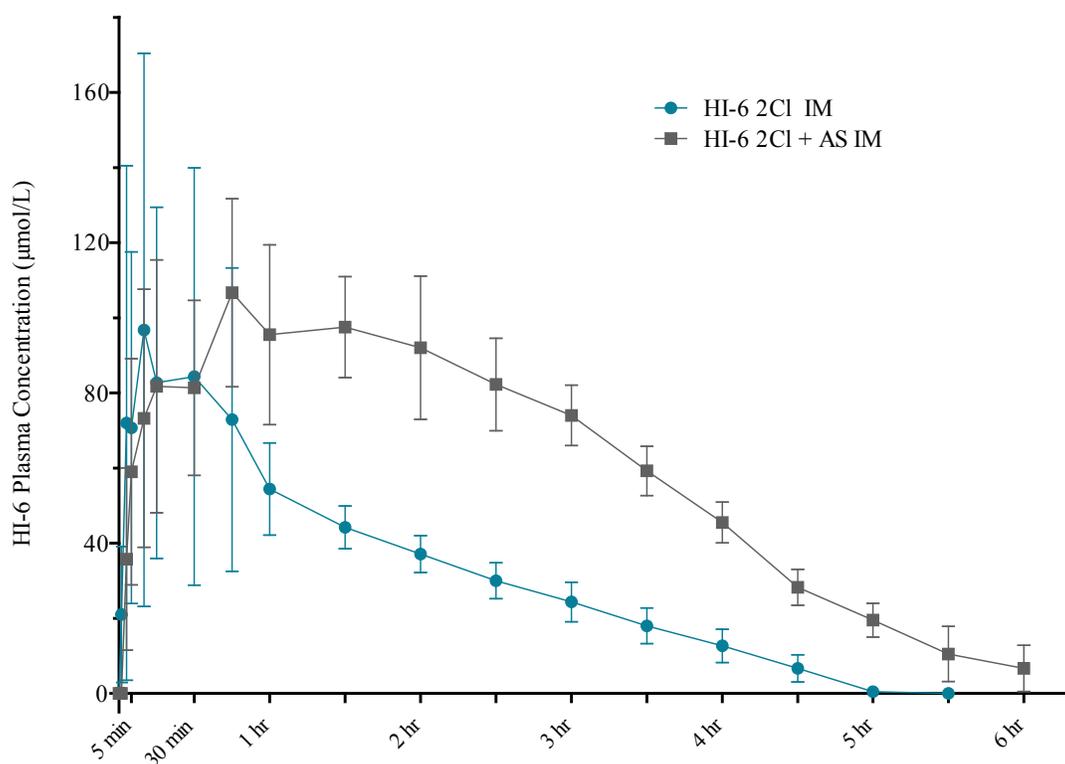
Figure 5.25 Mean (± SD) plasma HI-6 concentration-time profiles comparing equimolar doses of HI-6 2Cl when administered alone (Treatment Group A, $n = 8$) or with 0.1 mg/kg atropine sulphate (Treatment Group C, $n = 4$) IM in domestic swine.

Table 5.21 Pharmacokinetics of equimolar doses of HI-6 2Cl (53 µmol/kg) administered alone (Treatment Group A) or concomitantly with 0.1 mg/kg atropine sulphate (Treatment Group C) IM in domestic swine. Data are mean values $\pm$ SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_{max} maximal concentration; T_{max} time to maximal concentration; nr not reported; nc not calculated; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl ($n = 8$)	HI-6 2Cl + AS ($n = 4$)	Significance
Distribution/Absorption Half Life (min)	15.0 ± 15.4	35.8 ± 2.4	*
Elimination Half Life (min)	58.2 ± 18.3	58.7 ± 7.3	ns
V_d (ml/kg)	414.7 ± 65.7	201.4 ± 23.6	*
Cl (ml/min/kg)	5.2 ± 1.2	2.4 ± 0.5	*
Cl_r (ml/min/kg)	3.4 ± 1.0	nr	nc
AUC_∞ (µmol·min/L)	10605 ± 2228	22609 ± 3782	*
C_{max} (µmol/L)	86.9 ± 29.3	123.1 ± 17.2	ns
T_{max} (min)	28.9 ± 21.5	65.3 ± 3.9	*
HI-6 in Urine (%)	67.9 ± 11.7	nr	nc

When comparing the pharmacokinetics of HI-6 2Cl and HI-6 2Cl co-administered with AS, IM significant differences were reported for the distribution/absorption half life V_d, Cl, AUC_∞ and T_{max}. V_d and Cl were greater following HI-6 2Cl administration IM in domestic swine, where as distribution/absorption half life, AUC_∞ and T_{max} were greater when HI-6 2Cl was co-administered with AS. Values for Cl_r and percent of HI-6 in urine were not reported for swine treated with HI-6 2Cl and AS due to insufficient data and as such the significance of these parameters compared to the administration of HI-6 2Cl alone could not be determined.
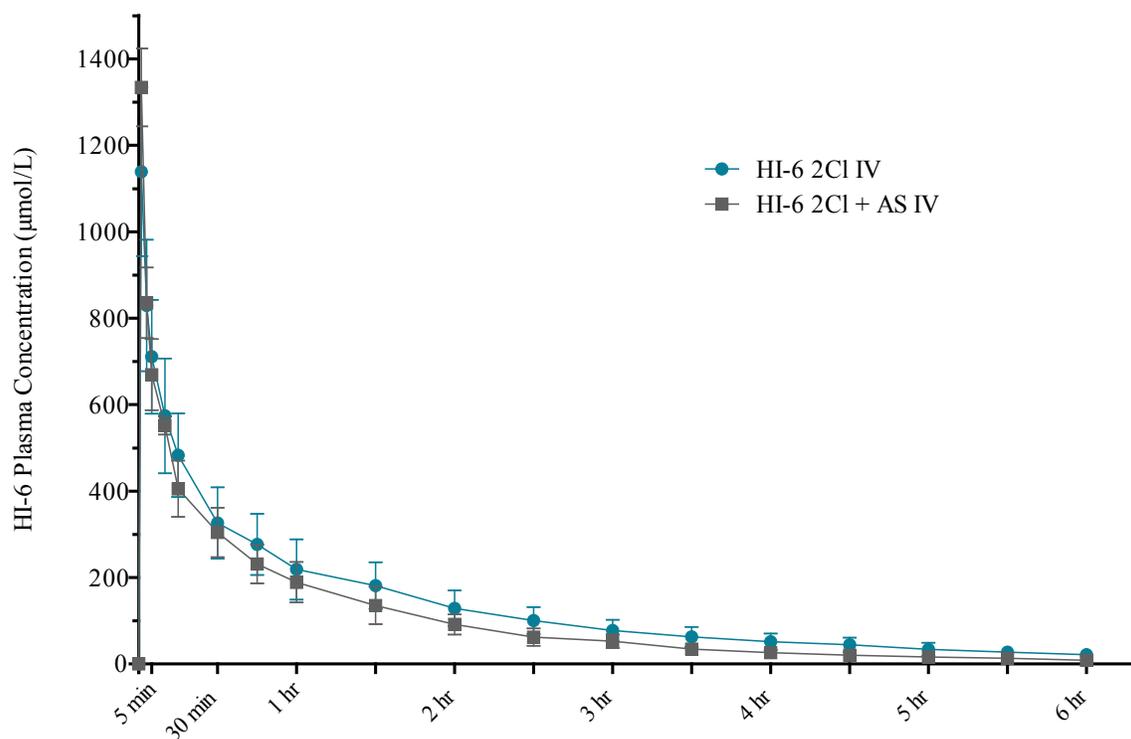
Figure 5.26 Mean (± SD) plasma HI-6 concentration-time profiles comparing equimolar doses of HI-6 2Cl when administered alone (Treatment Group G, $n = 4$) or with 0.1 mg/kg atropine sulphate (Treatment Group I, $n = 4$) IV in domestic swine.

Table 5.22 Pharmacokinetics of equimolar doses of HI-6 2Cl (26.8 µmol/kg) administered alone (Treatment Group G) or concomitantly with 0.1 mg/kg atropine sulphate (Treatment Group I) IV in domestic swine. Data are mean values ± SD. V_d volume of distribution; *Cl* total body clearance; *Cl$_r$* renal clearance; AUC$_\infty$ area under the curve; C$_0$ initial concentration; ns no significant difference; * significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 2Cl (*n* = 4)	HI-6 2Cl + AS (*n* = 4)	Significance
Distribution/Absorption Half Life (min)	6.5 ± 0.3	5.0 ± 0.4	*
Elimination Half Life (min)	85.1 ± 5.9	67.7 ± 3.0	*
V$_d$ (ml/kg)	426.2 ± 72.2	463.6 ± 50.1	ns
Cl (ml/min/kg)	3.5 ± 0.8	4.8 ± 0.6	*
Cl$_r$ (ml/min/kg)	2.5 ± 0.5	2.8 ± 0.2	ns
AUC$_\infty$ (µmol·min/L)	52441 ± 14362	40434 ± 8004	ns
C$_0$ (µmol/L)	1075.0 ± 182.3	1223.0 ± 85.2	ns
HI-6 in Urine (%)	72.9 ± 15.0	49.5 ± 24.4	ns

Following equimolar IV administration of either HI-6 2Cl or HI-6 2Cl and AS in domestic swine a significant difference was reported when distribution/absorption half life, elimination half life and *Cl* were calculated. Both the elimination half life and the distribution/absorption half life were greater when HI-6 2Cl was administered alone. Total body clearance was greater when HI-6 2Cl was co-administered with AS. The pharmacokinetic parameters that were found to be significantly different had very small standard deviations as shown in Table 5.22 and supported in Figure 5.26 by the very minimal differences of the plasma profiles of both treatment groups.
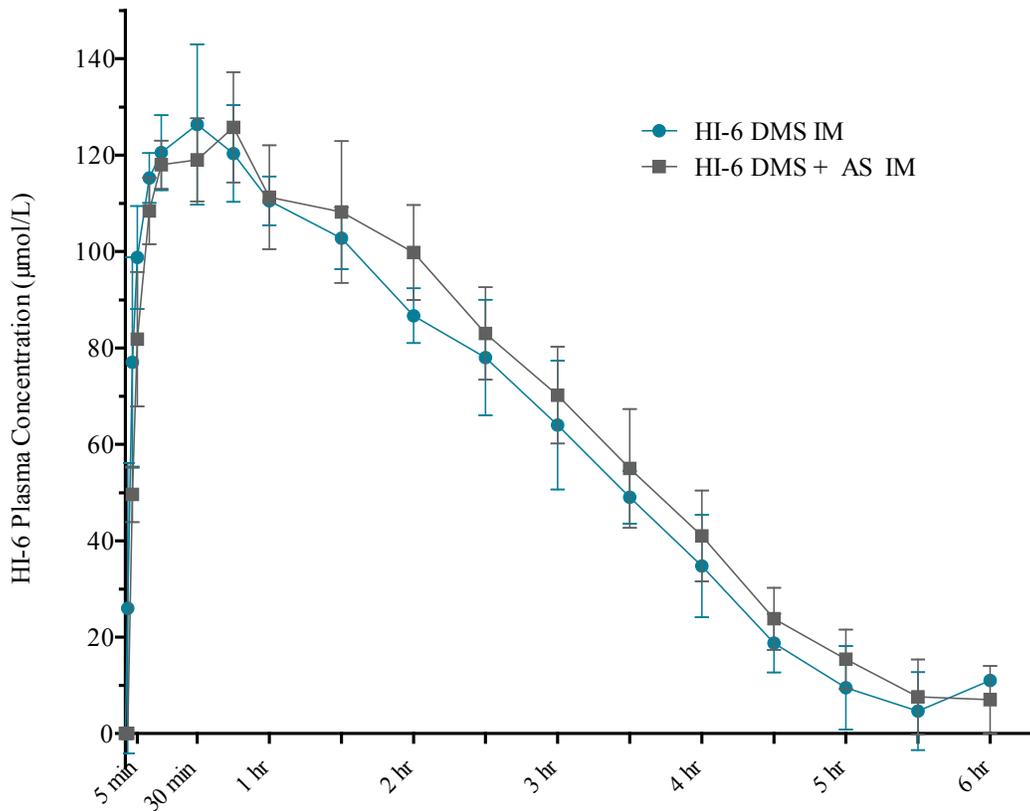
Figure 5.27 Mean (± SD) plasma HI-6 concentration-time profiles comparing equimolar doses of HI-6 DMS when administered alone (Treatment Group B, *n* = 4) or with 0.1 mg/kg atropine sulphate (Treatment Group D, *n* = 5) IM in domestic swine.

Table 5.23 Pharmacokinetics of equimolar doses of HI-6 DMS (53 µmol/kg) administered alone (Treatment Group B) or concomitantly with 0.1 mg/kg atropine sulphate (Treatment Group D) IM in domestic swine. Data are mean values ± SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_{max} maximal concentration; T_{max} time to maximal concentration; ns no significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 DMS ($n = 4$)	HI-6 DMS + AS ($n = 5$)	Significance
Distribution/Absorption Half Life (min)	26.8 ± 9.8	31.3 ± 3.2	ns
Elimination Half Life (min)	67.5 ± 6.1	58.6 ± 13.0	ns
V_d (ml/kg)	225.5 ± 30.4	184.4 ± 30.2	ns
Cl (ml/min/kg)	2.3 ± 0.2	2.2 ± 0.3	ns
Cl_r (ml/min/kg)	1.8 ± 0.1	1.8 ± 0.8	ns
AUC_∞ (µmol·min/L)	23156 ± 2181	24342 ± 1356	ns
C_{max} (µmol/L)	131.7 ± 14.0	140.9 ± 14.0	ns
T_{max} (min)	57.7 ± 13.4	60.0 ± 2.5	ns
HI-6 in Urine (%)	75.8 ± 5.3	66.3 ± 16.2	ns

No significant difference was found when examining all of the calculated pharmacokinetic parameters following IM administration of HI-6 DMS and HI-6 DMS and AS in domestic swine. The very similar plasma HI-6 profiles reflective of similar pharmacokinetic parameters are presented in Figure 5.27.
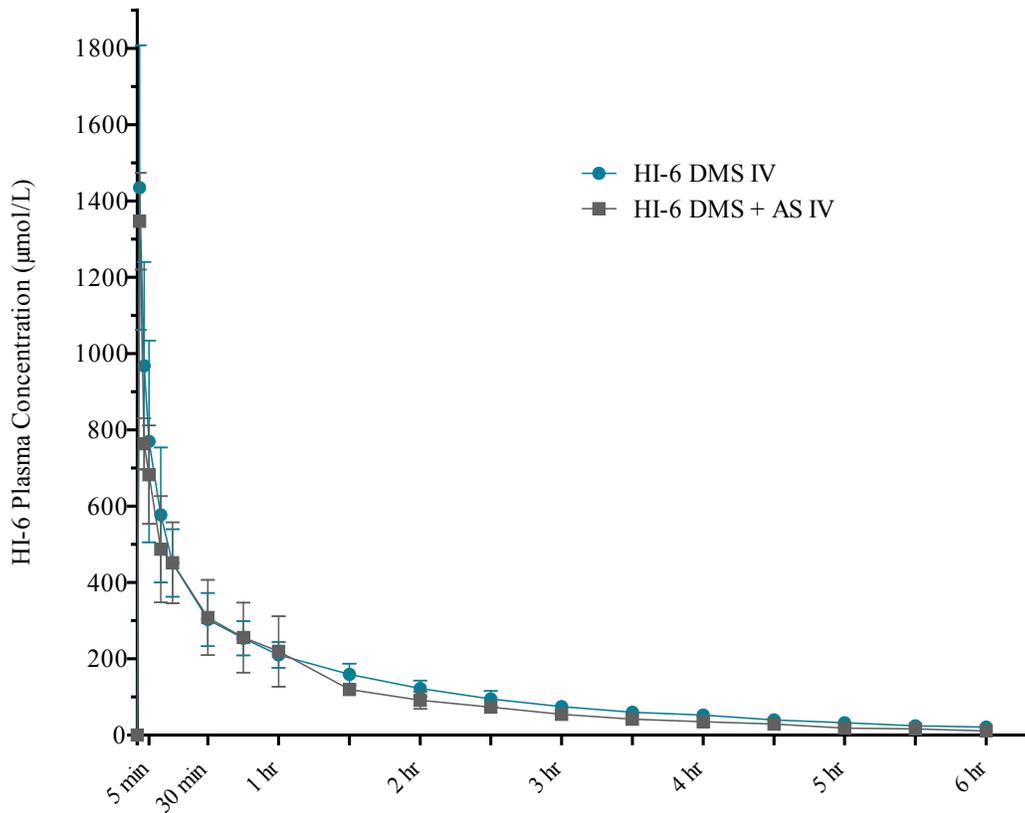
Figure 5.28 Mean (± SD) plasma HI-6 concentration-time profiles comparing equimolar doses of HI-6 2Cl when administered alone (Treatment Group H, *n* = 4) or with 0.1 mg/kg atropine sulphate (Treatment Group J, *n* = 4) IV in domestic swine.

Table 5.24 Pharmacokinetics of equimolar doses of HI-6 DMS (26.8 µmol/kg) administered alone (Treatment Group H) or concomitantly with 0.1 mg/kg atropine sulphate (Treatment Group J) IV in domestic swine. Data are mean values ± SD. V_d volume of distribution; Cl total body clearance; Cl_r renal clearance; AUC_∞ area under the curve; C_0 initial concentration; ns no significant difference at $p < 0.05$ (unpaired t-test).

PK Parameter	HI-6 DMS (*n* = 4)	HI-6 DMS + AS (*n* = 4)	Significance
Distribution Half Life (min)	5.3 ± 1.2	4.6 ± 1.3	ns
Elimination Half Life (min)	86.3 ± 6.8	75.7 ± 9.5	ns
V_d (ml/kg)	453.7 ± 61.3	440.2 ± 99.7	ns
Cl (ml/min/kg)	3.7 ± 0.7	4.0 ± 0.9	ns
Cl_r (ml/min/kg)	2.9 ± 0.5	2.8 ± 1.3	ns
AUC_∞ (µmol·min/L)	50632 ± 10829	44636 ± 9732	ns
C_0 (µmol/L)	1367.8 ± 431.1	1330.4 ± 265.4	ns
HI-6 in Urine (%)	83.9 ± 9.3	64.8 ± 23.1	ns

Similar to IM administration of both HI-6 DMS and HI-6 DMS and AS no significant difference was calculated following IV administration of both of these treatments. Figure 5.28 best represents how similar the pharmacokinetics of HI-6 DMS and HI-6 DMS co-administered with AS are. In particular over several periods of the experimental period presented it is very difficult to discern to two plasma HI-6 concentration profiles from one another.

5.4.6 Evaluation of Calculated PK Parameters when HI-6 is infused

Case 10 evaluated the calculated pharmacokinetic parameters determined for Treatment Group F (domestic swine administered 26.8 µmol/kg IV bolus of HI-6 DMS) used to calculate a constant infusion rate of HI-6 to reach and maintain a target plasma concentration of 100 µM HI-6 over the course of an eight hour infusion.

Physiology data (heart rate, mean arterial pressure, oxygen saturation and respiration rate) was collected for Treatment Group K (infusion of HI-6 DMS) in the same manner as it was collected for Treatment Groups A through J. Figure 5.29 presents the normalized mean heart rate for

Treatment Groups K over the eight hour experimental period. No significant difference ($p <$ 0.05) was observed for changes in heart rate within the group or when compared to Treatment Groups A through J; following SSA (stead state anaesthesia) the nominal heart rate was 115 beats per minute ± 11 beats per minute.

The normalized mean arterial pressure for Treatment Group K over the course of the experimental period is presented in Figure 5.30. Following SSA the mean arterial pressure was 59 mmHg ± 5mmHg. No significant difference ($p < 0.05$) was observed for changes in mean arterial pressure within the group or when compared to Treatment Groups A through J.

Normalized mean oxygen saturation is presented in Figure 5.31 for Treatment Group K. The nominal oxygen saturation was 93% ± 2% following SSA for the duration of the six hour experimental period. Larger variance in mean oxygen saturation is shown over short periods of time: these variances can be directly attributed to improper sensor placement and values returned to mean levels following adjustment of the sensor placement. No significant difference ($p <$ 0.05) was observed between mean oxygen saturation levels within the group or when compared to Treatment groups A through J.

Treatment Group K normalized respiration rate data is presented in Figure 5.32 for the duration of the experimental period. The mean respiration rate following normalization based on SSA values was 40 breaths per minute ± 2 breaths per minute. No significant difference is reported between the mean respiration rates of the domestic swine included in Treatment Group K or when comparing the group to Treatment Groups A through J.
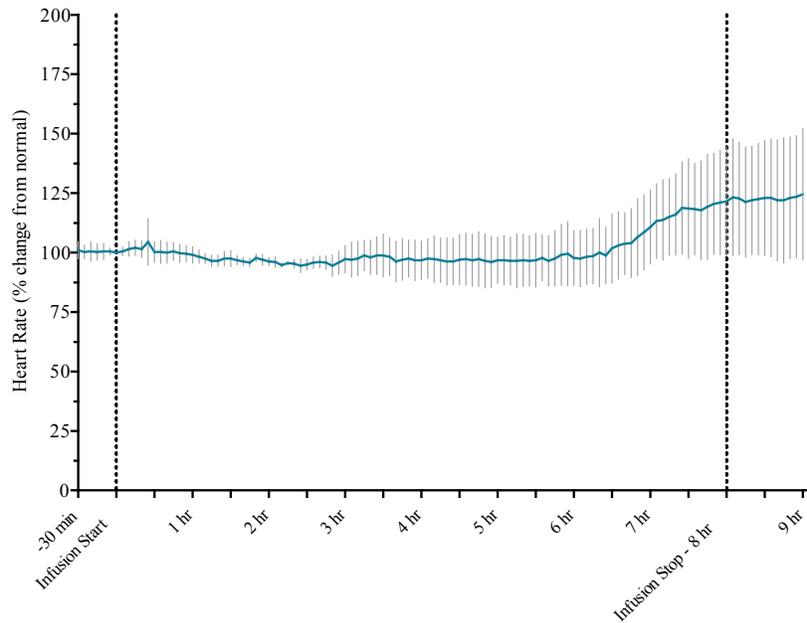
Figure 5.29 Normalized mean heart rate profile for Treatment Group K (HI-6 DMS infusion, $n = 4$). The bars represent standard deviation.
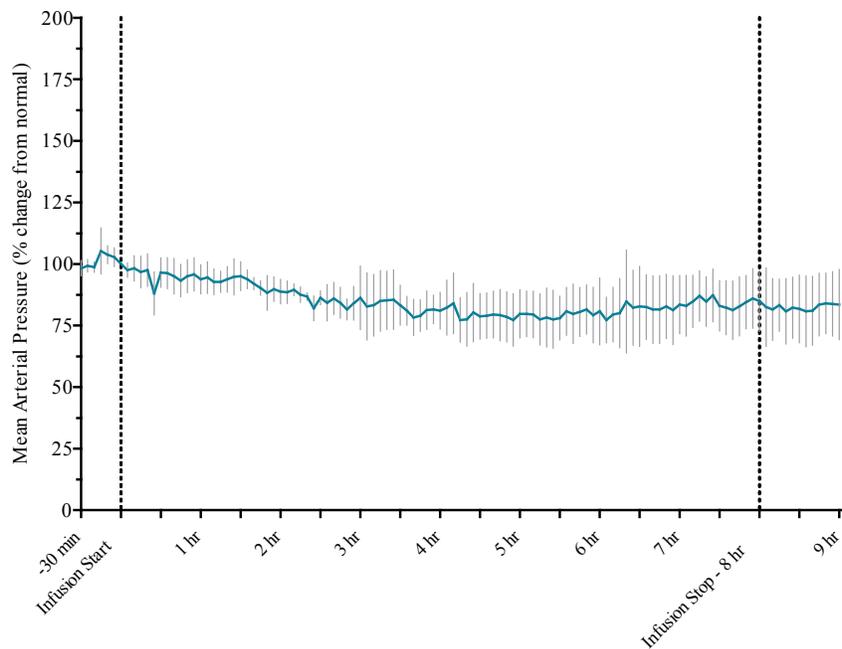
Figure 5.30 Normalized mean arterial pressure profile for Treatment Group K (HI-6 DMS infusion, $n = 4$). The bars represent standard deviation.
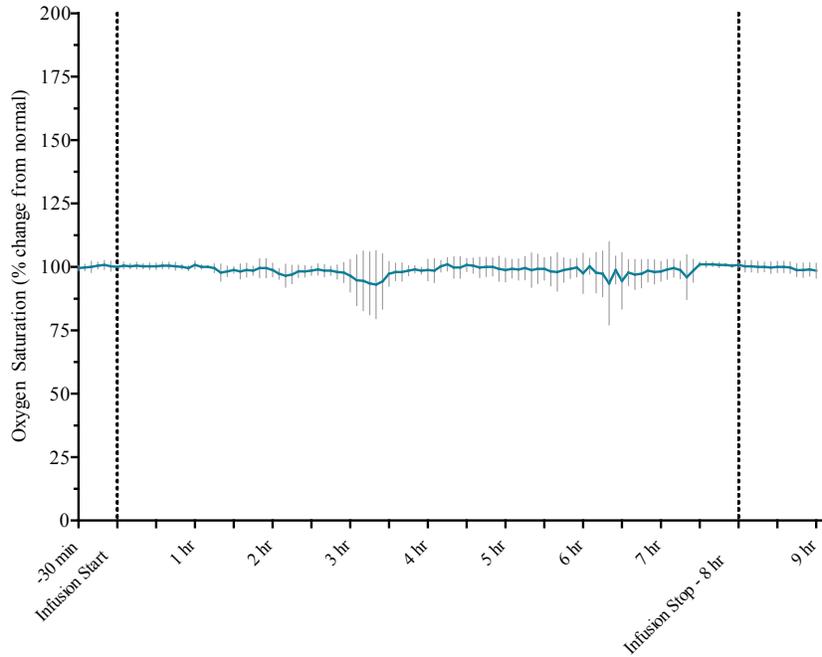
Figure 5.31 Normalized oxygen saturation profile for Treatment Group K (HI-6 DMS infusion, $n = 4$). The bars represent standard deviation.
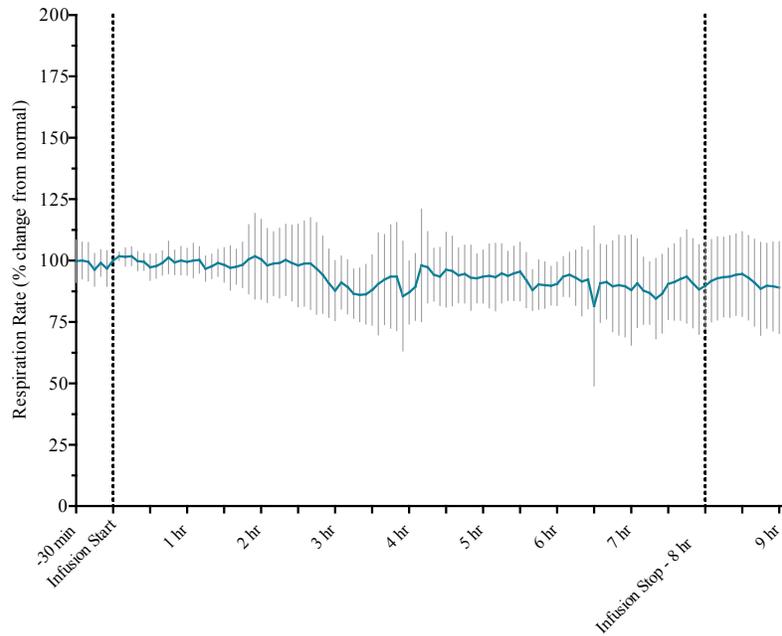
Figure 5.32 Normalized respiration rate profile for Treatment Group K (HI-6 DMS infusion, $n = 4$). The bars represent standard deviation.

Blood gas levels including pH and bicarbonate (HCO$_3^-$), hemoglobin (Hgb) levels, electrolyte levels including sodium (Na), potassium (K), chloride (Cl), calcium (Ca), magnesium (Mg) and blood chemistry levels including phosphate (Phos), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), urea nitrogen (UREA) and creatine (CREA) are reported in Table 5.25 and Table 5.26. No significant difference ($p < 0.05$) was reported for any of the clinical blood sample analysis between Group K and any other domestic swine group (Treatment Groups A through J).

Table 5.25 Mean blood gas values for Treatment Group K (HI-6 DMS infusion, $n = 4$).

	Mean ± SD	
	pH	**Bicarbonate (HCO$_3^-$) (mmol/L)**
SSA	7.39 ± 0.02	30.7 ± 2.9
0.5 hr	7.40 ± 0.03	31.9 ± 1.4
1 hr	7.41 ± 0.03	30.9 ± 1.9
2 hr	7.41 ± 0.01	30.5 ± 1.8
3 hr	7.41 ± 0.05	30.4 ± 0.9
4 hr	7.40 ± 0.04	30.8 ± 1.0
5 hr	7.41 ± 0.05	28.6 ± 1.2
5 hr 40 min	7.41 ± 0.05	30.6 ± 1.1
6 hr 20 min	7.39 ± 0.09	30.5 ± 1.4
7 hr	7.39 ± 0.07	30.7 ± 1.2
7 hr 40 min	7.39 ± 0.07	28.5 ± 1.2
8 hr 15 min	7.38 ± 0.06	28.9 ± 1.2
8 hr 45 min	7.36 ± 0.04	29.7 ± 2.2

Table 5.26 Mean hemoglobin, electrolyte and blood chemistry values for Treatment Group K (HI-6 DMS infusion, $n = 4$).

		Mean ± SD	
	SSA	4 hrs	8 hrs
Hemoglobin Levels			
Hemoglobin (Hgb) (g/dL)	7.9 ± 0.7	8.2 ± 0.5	8.6 ± 0.9
Electrolyte Levels			
Sodium (Na) (mmol/L)	139 ± 3	137 ± 2	137 ± 1
Potassium (K) (mmol/L)	4.2 ± 0.7	4.5 ± 0.3	4.5 ± 0.3
Chloride (Cl) (mmol/L)	106 ± 6	103 ± 2	103 ± 2
Calcium (Ca) (mmol/L)	8.0 ± 1.5	8.6 ± 0.5	8.2 ± 0.3
Magnesium (Mg) (mmol/L)	1.9 ± 0.4	2.0 ± 0.1	2.1 ± 0.1
Phosphate (Phos) (mmol/L)	9.8 ± 2.0	10.2 ± 0.4	11.1 ± 0.7
Blood Chemistry			
Aspartate aminotransferase (AST) (u/L)	22.8 ± 2.5	23.8 ± 2.6	36.0 ± 5.7
Gamma-glutamyltransferase (GGT) (u/L)	26.8 ± 6.7	28.3 ± 6.3	29.3 ± 6.2
Urea nitrogen (UREA) (mmol/L)	7.9 ± 2.5	9.5 ± 1.2	15.2 ± 1.6
Creatinine (CREA) (u/L)	95.0 ± 29.2	90.6 ± 29.2	72.9 ± 11.1

 Complete necropsies were performed for all domestic swine included in Treatment Group K at the end of the experimental period. Gross pathology did not indicate any abnormalities related to HI-6 administration.

Figure 5.33 presents mean plasma HI-6 concentration-time profiles for eight hour infusions of HI-6 DMS (Treatment Group K) and Figure 5.34 presents the urinary excretion profile of HI-6 DMS over the course of the continuous eight hour infusion.
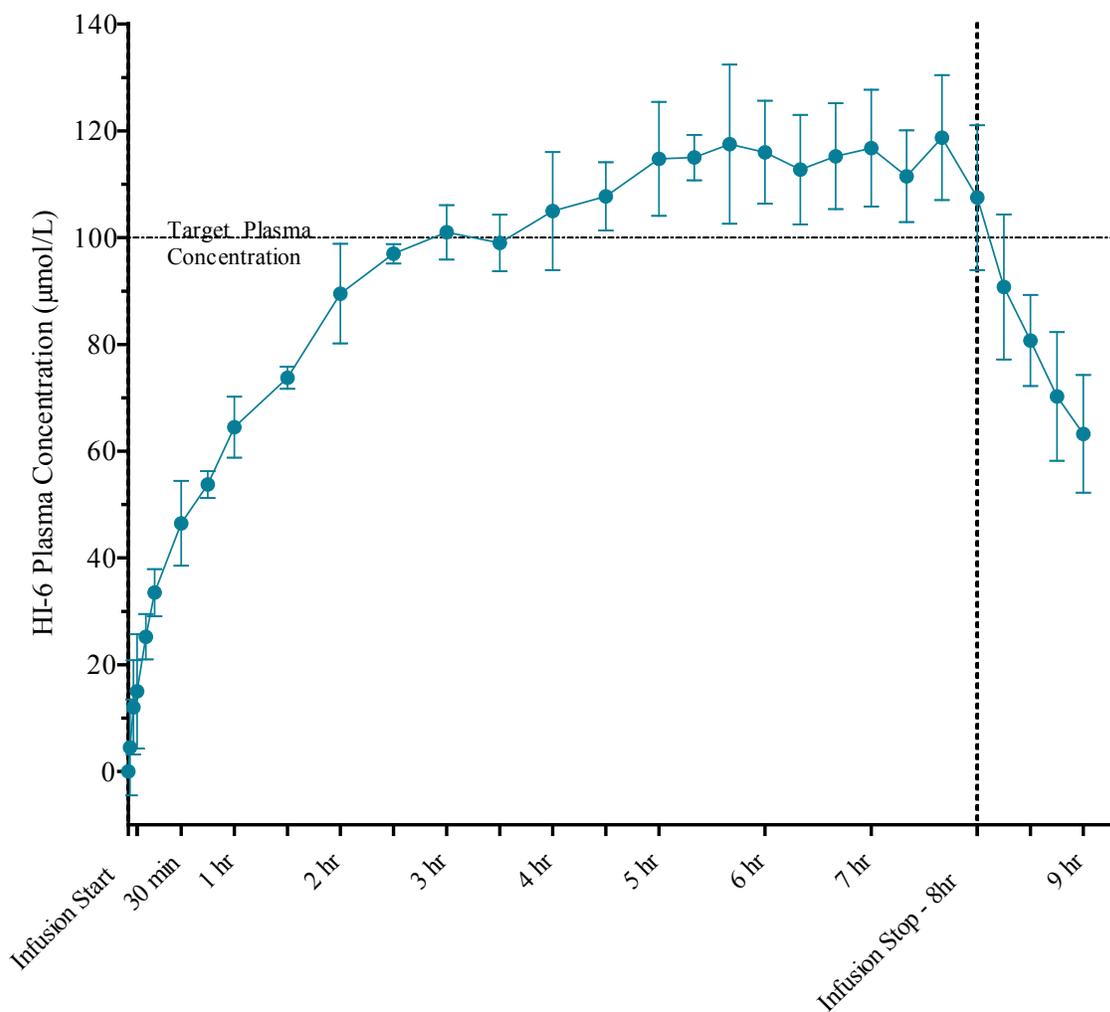
Figure 5.33 Mean (± SD) plasma HI-6 concentration-time profile when HI-6 DMS is infused (Treatment Group K, *n* = 4) at a constant rate of 0.48 µmol/min·kg for eight hours in domestic swine.

As shown in Figure 5.33 a target plasma concentration of 100 µM of HI-6 DMS was reached around the three hour mark and steady state was maintained for the duration of the infusion (8 hours). Once the infusion was stopped a rapid decline in plasma HI-6 concentration was observed.
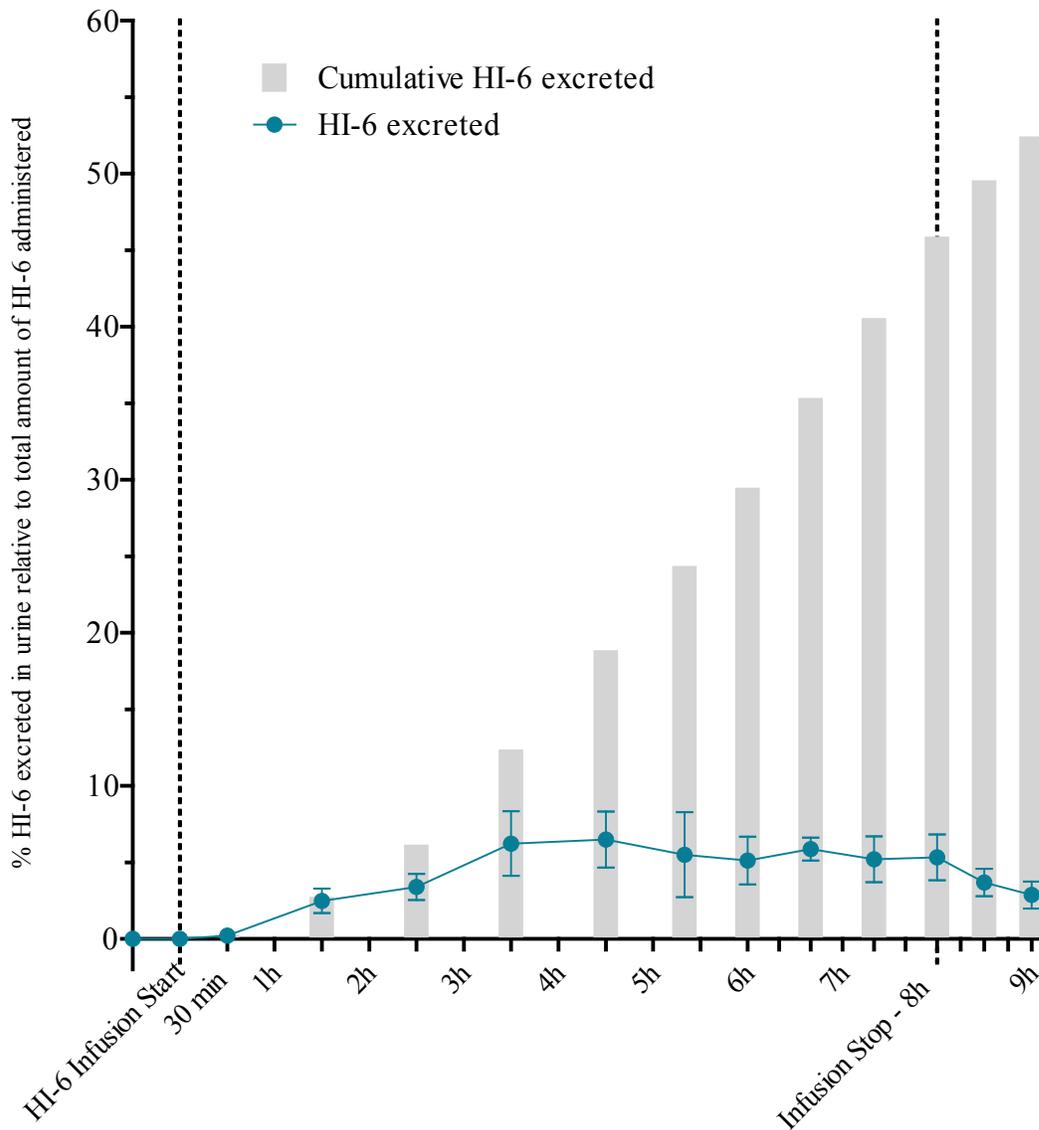
Figure 5.34 Percent of HI-6 DMS excreted in urine over the course of a continuous eight hour infusion of HI-6 DMS (Treatment Group K, $n = 4$). The bars represent the cumulative percent of HI-6 excreted in the urine up to that point, whereas the line graph represents the percent of HI-6 excreted ($\pm$ SD) for that specific time point.

The percent of HI-6 excreted in urine was examined over the course of the infusion of HI-6 DMS as shown in Figure 5.34: similar to the infusion graph (Figure 5.33) the percentage of HI-6

excreted (represented by line graph) reaches a steady state level around three and a half hours and this level is maintained until the infusion was stopped at which point the percentage of HI-6 DMS excreted in urine decreased as did the plasma concentration of HI-6 DMS. Over the course of the nine hour total experimental period an average of 52% of the total amount of HI-6 administered over the course of the eight hour infusion period was excreted in the urine.

6. Discussion

The threat of nerve agent poisoning has existed for several decades and continues to be a concern in parts of the world. HI-6, an oxime first developed in Germany in the 1960's, has been shown to be a very effective therapeutic in animal studies, and with low toxicity in man. Although HI-6 is accessible to the Canadian Armed Forces under a Special Access Provision for use, there is a requirement for licensure of an HI-6 product that can be used in extended care situations. However, limited data is available on the pharmacokinetics of both HI-6 2Cl and DMS when administered in different animal species, by different routes, in the presence or absence of anaesthesia or with or without atropine co-administration. In support of further licensure of HI-6 additional pharmacokinetic studies needed to be conducted in two animal models to allow for the evaluation of HI-6 administered under various conditions and ultimately as an infusion product.

6.1 Comparison HI-6 2Cl and HI-6 DMS Salts

A large body of work evaluating HI-6 2Cl is available. However, there is very limited data available on the DMS formulation. Previously, two studies examining the pharmacokinetics of both HI-6 2Cl and DMS following IM administration reported no significant difference between the two salts (Karasova, et al., 2013; Lundy, et al., 2005). The known solubility differences between HI-6 2Cl and DMS are well documented (Karasova, et al., 2013; Lundy, et al., 2005; Thiermann, Seidl, et al., 1996). Although HI-6 2Cl and DMS are both present as ionized forms when in solution and have similar pKa's of approximately 7.3 (Eyer, Hagedorn, & Ladstetter, 1988), the pH of HI-6 in solution (2.93 – 3.98) and osmolarity (917 – 2034 mmol/kg) vary

slightly between salts at the same concentration and within the same salt over different concentrations as reported in Table 5.2. These differences in pH and osmolarity at varying concentrations could potentially lead to differences in pharmacokinetic parameters directly related to absorption and distribution following an IM injection. It is unlikely that these physiochemical differences will affect the pharmacokinetics of either salt when administered IV as the absorption phase is almost non-existent as 100% of the drug administered is immediately available in the bloodstream. The pharmacokinetics of HI-6 2Cl and DMS were evaluated and compared following equimolar administration as either an IM injection or an IV bolus in guinea pigs and swine under various conditions.

6.1.1 Guinea Pig Studies (Comparison of salts)

Previous work of Karasova et. al (2013) showed that HI-6 2Cl and HI-6 DMS had similar pharmacokinetics following an IM injection. This was found to be true when equimolar doses of both HI-6 2Cl and HI-6 DMS were administered to non-anaesthetized guinea pigs IM.

Conversely when equimolar doses of HI-6 2Cl and HI-6 DMS were administered to anaesthetized guinea pigs either IM or IV the volume of distribution of HI-6 DMS was significantly greater than that of HI-6 2Cl. The significant difference between V_d suggests that under anaesthesia HI-6 2Cl and HI-6 DMS do not follow the same absorption/distribution pattern as in non-anaesthetized animals, where no significant difference was reported. The observed differences in V_d as reported in Table 5.4 and Table 5.5 are most likely due to the depressant action of anaesthesia and the associated decrease in renal clearance, decreased blood flow and muscle movement. Differences in V_d could be attributed to dehydration as a result of anaesthetic when comparing non-anaesthetized models (potentially dehydrated) to anaesthetized models (appropriately hydrated with access to water as desired) no significant differences were reported suggesting hydration associated with anaesthesia did not affect the volume of distribution.

The reported significantly higher C_{max} values when HI-6 2Cl was administered IM in anaesthetized guinea pigs most likely suggests an error or variance in dose administration. Dose confirmation analysis determined that all dosing solutions were equimolar within $\pm 5\%$

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indicating that an error in dose solution preparation could not account for the differences reported in maximal concentration.

Both Cl and AUC_∞ were found to be significantly different following equimolar IV administration of HI-6 2Cl or HI-6 DMS in anaesthetized guinea pigs. Similar to differences in V_d, the reported Cl differences (Table 5.5) suggest some sort of effect related to the HI-6 counter ions and their associated movement throughout the model. HI-6 2Cl solutions administered were determined to have an average pH of 3.79 and an osmolarity of 917 mmol/kg. Conversely DMS solutions had an average pH of 3.04 and an osmolarity of 1011 mmol/kg. The difference in pH between salts may contribute to the difference noted in Cl. However, this would need to be confirmed through analysis of urine samples which could not be collected from guinea pigs. Based upon previously reported results the differences observed are most likely due to a differential effect of anaesthesia on an unknown component of the absorption/distribution kinetics.

6.1.2 Swine Studies (Comparison of Salts)

A significant difference was observed for V_d, Cl, AUC_∞, C_{max} and T_{max} when comparing equimolar doses of HI-6 2Cl and DMS administered IM in swine as reported in Figure 5.16 and Table 5.12. Similar to the results reported for guinea pigs, significant differences in V_d, Cl and AUC_∞ suggest a counter ion effect that alters the distribution pattern of HI-6 as well as its clearance. In contrast to the guinea pigs, swine were administered replacement fluids over the course of their experimental period ruling out any changes in V_d to be due to hydration levels of the model. Similar to guinea pigs large differences were noted between HI-6 2Cl and DMS for their respective pH values (3.71 compared to 3.04). A difference in the renal clearance as postulated earlier in the guinea pig studies (supporting a difference in clearance due to a counter ion effect) was examined. No significant difference was reported between renal clearance of HI-6 2Cl and DMS and the percentage of HI-6 excreted urine over the course of the experimental period was also not significantly different suggesting that renal clearance was not affected by either counter ion. Unfortunately the effect of anaesthesia cannot be ruled out for the swine studies at this time and as postulated earlier for the guinea pig studies, the differences in the salts

could be due to differences related to anaesthesia rather than counter ion, suggesting that studies conducted with both salts in non-anaesthetized swine may show very similar pharmacokinetic profiles for both salts as reported previously in swine studies by Karasova et al.(2013), and Lundy et al. (2005).

A significant difference in C_{max} suggests that different doses of HI-6 were administered. However doses were confirmed to be equimolar. This difference could potentially be attributed to slight differences in IM administration such as the force or location of administration leading to different absorption/distribution kinetics, a theory that is supported by the fact that T_{max} was also found to be significantly different following IM administration of both HI-6 salts either alone or in the presence of AS. Although all IM injections were administered in the same muscle group the force of administration and depth of needle insertion were not closely monitored and as such minor variations in these, in addition to individual differences in animal tissue composition could account for the variations reported. F.R. Sidell, Markis, Groff & Kaminskis (1974) reported that when greater forces is applied for an IM injection an increased rate of absorption is noted because the solution is "sprayed" or distributed through a larger tissue mass instead of being deposited at one site as is seen when an IM injection is administered at a slower rate with less force. The reported absorption/distribution half life was not found to be significantly different indicating that both salts follow a similar absorption/distribution pattern as has been reported earlier and also seen in non-anaesthetized guinea pigs.

No significant differences were reported between the pharmacokinetics of HI-6 2Cl and HI-6 DMS when administered intravenously at varying equimolar doses in the presence or absence of AS. This suggests that under IV administration conditions the HI-6 counter ions do not alter the PK of HI-6.

6.1.3 Summary of Comparison of HI-6 2Cl and HI-6 DMS salts

Following HI-6 IM administration in anaesthetized guinea pigs or IM administration in swine, a significant difference was noted for V_d, Cl and AUC_∞ between the two salts. These pharmacokinetic differences suggest that the dichloride and dimethanesulfonate counter ions

affect the absorption/distribution kinetics of HI-6 differently when administered IM in anaesthetized animals. This difference could be due to the slight difference in pH and osmolarity of the solutions, both of which are physiochemical properties that are known to affect the absorption and distribution of a compound. Differences more likely are due to an effect of anaesthetic effect that is discussed later in section 6.3.

IV administration of both HI-6 2Cl and HI-6 DMS in swine did not result in a significant difference between PK parameters as was reported previously by Lundy, et al. (2005).

6.2 Comparison of Routes of Administration

Previous studies compared the pharmacokinetics of HI-6 2Cl and HI-6 DMS when administered either as an IV bolus or an IM injection (Baggot, et al., 1993; Lukey, et al., 1989; Nyberg, et al., 1995). Differences were reported in the calculated pharmacokinetic values for HI-6 2Cl and DMS following an IM injection. Intramuscular injections result in the initial deposition of HI-6 near the site of injection in the muscle tissue, from where the HI-6 is transported via passive diffusion and absorbed into the bloodstream for distribution throughout the body. IM injections can lead to tissue damage at the site of injection depending on the size of needle used and the force applied during injection; factors that may alter the absorption and distribution kinetics slightly on a case by case basis. In general, following an IM injection of HI-6 there is a distinct absorption phase until the maximal concentration is reached (anywhere from 30 to 60 minutes following injection depending on the species being examined), followed by a distribution phase and finally an elimination phase. Intravenous injections result in 100% of the HI-6 administered being available immediately following injection, leading to undetectable absorption and distribution phases and only a very apparent elimination phase. The slopes of the elimination phases following either an IM injection or an IV bolus were expected to be very similar. However in contrast, the absorption and distribution parameters were expected to be significantly different due to the large difference in these phases as a result of the administration method. Previous studies have indicated that virtually 100% of HI-6 administered IM is absorbed (Lukey, et al., 1989; Simons & Briggs, 1983, 1985) suggesting that there is no difference in the

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bioavailability or absorption of HI-6 when administered IV or IM and that any differences in pharmacokinetics would not be due to bioavailability of HI-6.

Historically much of the efficacy data collected for HI-6 has been obtained from studies using the dichloride salt. In order for regulatory agencies to accept that both HI-6 2Cl and DMS are similar it is necessary to conduct studies that examine the pharmacokinetic of both salts when administered either IV or IM. The pharmacokinetics of HI-6 (2Cl and DMS) were compared when administered either as an IM injection or IV bolus in guinea pigs and swine.

6.2.1 Guinea Pig Studies (Comparison of routes of administration)

It is important to note that the IM and IV doses administered to anaesthetized guinea pigs were not equimolar. The difference in dose administered did not prove to have a significant effect on any of the PK parameters calculated as the difference between doses on a per animal weight basis was less than 10 %.

A significant difference was found for both the elimination half-life and the V_d when comparing IM and IV administration for both HI-6 salts in guinea pigs. The elimination half-life is greater for both salts when administered IM as shown in Tables 5.5 and 5.6. The difference in elimination half-life between routes of administration could be attributed to the manner in which the elimination half-life was calculated. Elimination half-life was determined by calculating the exponential rate constant of the HI-6 plasma concentration-time curve from the point of maximal concentration until the end of the experimental period. It is possible that due to the limited data points available following injection (due to sampling restrictions of using a small animal model such as the guinea pig), that the point selected and designated as the start of the elimination phase (C_{max} for IM subjects) may have still been part of the absorption/distribution phase. When looking at Figures 5.5 and 5.6 from the two hour sample forward, the slopes of the plasma HI-6 concentrations for both IM and IV administration follow a similar pattern (are parallel) indicating that from two hours until the end of the experimental period the elimination rates are not significantly different (are of a similar rate), supporting the theory that absorption/distribution phases continue for longer than is apparent and a different time point

should have been selected as the first point for calculating the elimination half-life. This could be further assessed by using a more sophisticated pharmacokinetic software for analysis. Alternatively further studies could be conducted with increased blood sample collection for the first hour following HI-6 administration.

The V_d was greater for both salts when administered IM as shown in Tables 5.5 and 5.6. When HI-6 is administered IV all of the HI-6 is initially distributed through the circulatory system, whereas IM administration results in initial localized distribution into muscle tissue surrounding the site of injection and then further distribution. Additionally, IM administration may result in the creation of a localized depot of HI-6 as a result of the force of injection into the muscle resulting in sustained and delayed release over time, leading to errors in the calculation of V_d.

6.2.2 Swine Studies (Comparison of routes of administration)

It is important to note that the IM and IV doses administered to anaesthetized domestic swine were not equimolar.

A significant difference was observed for the AUC_∞ when comparing the administration of HI-6 2Cl either IM or IV as reported in Figure 5.21 and Table 5.17. This difference in AUC_∞ was expected as significantly different doses of HI-6 were administered and the calculation of AUC_∞ is dose dependent.

V_d, *Cl* and AUC_∞ were all found to be significantly different when comparing the administration of HI-6 DMS as either an IM injection or and IV bolus as presented in Figure 5.22 and Table 5.18. Differences in V_d are due not only to the difference in dose administered by also due to the delay in the absorption of HI-6 as a result of potential variations in IM injections as described earlier. Finally the significant difference reported between *Cl* as also reported for guinea pigs is most likely due to differences in absorption distribution patterns that could not be detected with the pharmacokinetic modeling software used.

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6.2.3 Summary of comparison of routes of administration

As postulated and supported by previous studies there was no significant difference between the calculated elimination pharmacokinetic parameters of HI-6 2Cl or DMS following either IM or IV administration. Although not discernable due to the limited number of samples collected when using the guinea pig model, it is reasonable to expect to see a difference in the distribution/absorption parameters when comparing IM and IV administration as was seen in the swine model.

In general IM and IV injections of both HI-6 2Cl and DMS resulted in similar calculated pharmacokinetic parameters indicating equal bioavailability of HI-6 independent of route of administration. It is important to note that we can expect HI-6 pharmacokinetics to be similar between routes of administration. All of the currently available human pharmacokinetic data has been determined based upon the IM administration of either HI-6 2Cl or DMS. In order to provide extended titrated therapy against nerve agent poisoning an intravenous formulation of HI-6 DMS is being examined. We now know based upon the results of this work, that results previously published on the pharmacokinetics of HI-6 in man following an IM injections can easily and accurately be translated to IV dosing and ultimately a sustained intravenous infusion of HI-6 in the event that extended therapy is required. The differences reported earlier in section 6.1 (comparison of salts of HI-6) were not observed when comparing routes of administration supporting the hypothesis that there is a difference in the pharmacokinetic between salts.

6.3 Comparison of Effect of Anaesthetic

The previous section demonstrated that the pharmacokinetics of HI-6 are similar when administered IM or IV, supporting that HI-6 is readily bioavailable and thus effective via either route of administration. This allows for the immediate treatment of OP poisoning in the field with an auto-injector (IM injection) followed by additional IV therapy that can be titrated to the casualty's needs in a more clinical setting, without the concern of HI-6 behaving differently under different routes of administration. A large percentage of the pharmacokinetic data available for evaluation has been conducted in anaesthetized animals whereas all of the human

pharmacokinetic data has been collected from conscious volunteers. It is important to evaluate and understand any potential changes anaesthetic may have on the pharmacokinetics of HI-6 when translating pharmacokinetic data from an anaesthetized animal model to humans.

Previous studies that have examined an anaesthetic effect in monkeys (Clement, et al., 1990) and dogs (Klimmek & Eyer, 1986) reported a significantly faster absorption rate, decreased maximal concentration, longer elimination half-life and decreased renal clearance in anaesthetized animals compared to conscious ones. It is expected that a significant difference will be seen for clearance, elimination half life and distribution/absorption half life when comparing anaesthetized and non-anaesthetized guinea pigs based upon the results of previous studies.

The pharmacokinetics of HI-6 2Cl and DMS were compared when administered in conscious guinea pigs and guinea pigs anaesthetized with isoflurane. Swine studies were not conducted due to the complexity of working with non-anaesthetized swine.

6.3.1 Guinea Pig Studies (Comparison of effect of anaesthetic)

A significant difference was observed for elimination half-life, Cl, AUC_∞ and T_{max} when comparing non-anaesthetized and anaesthetized animals following an IM injection of either HI-6 2Cl or HI-6 DMS. The elimination half-life was longer for both HI-6 salts when administered to anaesthetized animals as shown in Tables 5.7 and 5.8. Under anaesthesia cardiac output is decreased, leading to decreased blood flow and circulation, in addition to a reduced metabolic rate and thus a decrease in both elimination half-life and clearance values. These results are similar to those reported for studies conducted in dogs (Klimmek & Eyer, 1986) and monkeys (Clement, et al., 1990) as described in the Introduction. Although AUC_∞ is dose dependent, it is not unreasonable to note a significant difference when comparing anaesthetized and non-anaesthetized groups after examining their HI-6 plasma concentration profiles in Figures 5.7 and 5.8 due to the large differences in glomerular filtration and thus renal clearance of HI-6 between anaesthetized and non-anaesthetized animals. As presented in the figures the distribution/absorption phases and elimination phases for anaesthetized animals are much greater, leading to a decreased clearance rate most likely due to decreased renal clearance while

under anaesthesia. However, this could not be confirmed as urine samples were not collected for guinea pig studies.   Previous studies have shown that HI-6 is eliminated by tubular secretion (Shek, et al., 1976) and that the renal clearance of HI-6 resembles the glomerular filtration rate (Clement, et al., 1995). Under anaesthesia the glomerular filtration rate may be reduced. The delayed absorption/distribution phase leads to a delay in the time to maximal concentration as can be seen in both Figure 5.7 and Figure 5.8.

6.4 Comparison of Different HI-6 Concentrations

As reported in section 6.3 administration of HI-6 in an anaesthetized model compared to a non-anaesthetized animal results in different calculated pharmacokinetics of HI-6. In particular the clearance and elimination half life were significantly different suggesting that anaesthetic directly changes how HI-6 is removed from the body. If anaesthetic can affect the handling of HI-6 it is not unreasonable to postulate that with increasing dose administration HI-6 concentrations approach a point at which absorption, distribution, metabolism and elimination processes may be saturated, leading to changes in the calculated pharmacokinetic parameters. Previously conducted ascending dose studies (Clement, et al., 1995; Lundy, et al., 2005; Maksimović, 1979) reported no significant difference between pharmacokinetic parameters between ascending doses. However, there were trends of time to maximal concentration and absorption half-life increasing as doses were increased whereas, elimination half-life and clearance decreased as doses were increased. These trends suggest a shift in the calculated pharmacokinetics directly linked to increasing dose, that may lead to significant differences if even higher concentrations or a constant infusion of HI-6 is administered. It is important to understand if the absorption, distribution, metabolism and/or elimination (ADME) of HI-6 is altered following increasing dose administration as discussed in section 6.4.1 or over the course of an intravenous infusion as discussed in section 6.6. The pharmacokinetics of HI-6 2Cl and DMS were compared when different doses of HI-6 were administered in domestic swine.

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6.4.1 Swine Studies (Comparison of different HI-6 concentrations)

A significant difference was observed for distribution/absorption half-life, AUC_∞ and C_0 when comparing two different doses of HI-6 2Cl administered IV as presented in Figure 5.23 and Table 5.19. The significant difference in AUC_∞ and C_0 were to be expected as both of these parameters are dose dependent. The difference in distribution/absorption half-life may be due to an osmolarity or pH difference between the two solutions that becomes significant at higher concentrations and starts to affect the absorption/distribution of HI-6 even if administered IV. In other words the highest concentration of HI-6 2Cl evaluated may be reaching a saturation limit for absorption/distribution and thus the difference in calculated parameters. HI-6 has a low lipophilic character (Karasova, et al., 2013) and is charged in solution. Distribution of HI-6 is dependent upon passive diffusion (Herkert, et al., 2012) across membranes and as such is subject to the varying structure of membranes (in particular its lipid and protein composition). Changes in the pH and osmolarity of a dosing solution as a result of concentration differences may affect the absorption and distribution of HI-6 across membranes and into the bloodstream, although this should not be a factor following IV administration.

A significant difference was observed for elimination half-life, AUC_∞ and C_0 when comparing two different doses of HI-6 DMS administered IV as presented in Figure 5.24 and Table 5.20. Similar to HI-6 2Cl the significant difference in AUC_∞ and C_0 were expected and are reasonable as both of these parameters are dose dependent. The difference in elimination half-life could possibly be due to saturation of either hepatic or renal clearance although the values for total body clearance and renal clearance reported were both similar to one another with no change in urine output noted in either. A second possibility is that at high concentrations of HI-6 DMS there is a shift away from first order kinetics. However, the more likely explanation is inaccurate calculation of the elimination half-life due to an unrecognizable extended distribution/absorption phase similar to that seen for HI-6 2Cl as a result of large osmolarity or pH differences between the two different doses administered. A shift away from first-order elimination kinetics toward zero-order kinetics would indicate that the elimination pathway affected by increasing concentrations of HI-6. Zero order kinetics results in a set amount of drug in this case HI-6, being eliminated per unit of time.

6.5 Determination of effect of AS Co-Administration

Atropine sulphate is a competitive antagonist for muscarinic acetylcholine receptors and is administered when organophosphate poisoning is suspected, to directly inhibit acetylcholine binding to muscarinic receptors. Oximes such as HI-6 cannot directly inhibit acetylcholine binding but rather mitigate OP poisoning through their action on acetylcholinesterase which in turn breaks down excess acetylcholine reducing cholinergic stimulation. Atropine acts on cholinergic receptors to reduce meiosis, salivation, lacrimation, diarrhea and bronchorrea. Co-administration of HI-6 and AS is standard protocol for the Canadian Armed Forces in the form of an auto-injector. In an extended care situation where continued treatment is required, atropine will continue to be administered to the casualty in addition to an oxime. It is important to understand if co-administration of HI-6 and AS results in any changes to the pharmacokinetics of HI-6, thus potentially reducing the efficacy of the combination.

As demonstrated previously, HI-6 pharmacokinetics are altered with increasing dose and when administered to animals under anaesthesia, suggesting that HI-6 ADME is affected and in turn, is handled differently. It is important to understand if AS changes the handling of HI-6 and if so, how it changes the calculated pharmacokinetics. Previous studies examining both HI-6 and AS reported no significant difference in calculated pharmacokinetic parameters (Thiermann, Radtke, Spöhrer, Klimmek, & Eyer, 1996b). However, an increased absorption of HI-6 was reported (Jovanović, et al., 1992). Based upon the previously published studies of AS and HI-6 (Clement, et al., 1990; Thiermann, Radtke, et al., 1996a), a change in the pharmacokinetics of HI-6 is not expected when they are co-administered. The pharmacokinetics of HI-6 2Cl and DMS were compared when administered alone or co-administered with AS.

6.5.1 Guinea Pig Studies (Determination of effect of AS co-administration)

The calculated pharmacokinetic parameters of HI-6 (2Cl and DMS) in anaesthetized guinea pigs were evaluated to achieve and maintain a target plasma concentration of 100 µM over the course of an eight hour infusion when co-infused with atropine sulphate. An HI-6 2Cl only infusion (infusion rate = 0.35 µmol/min·kg) was directly compared to an equimolar infusion of HI-6 2Cl

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plus atropine sulphate (HI-6 infusion rate = 0.35 µmol/min·kg, atropine sulphate infusion rate = 4.4 µg/min·kg) as shown in Figure 5.10. An HI-6 DMS only infusion (infusion rate = 0.49 µmol/min·kg) was directly compared to an equimolar infusion of HI-6 DMS plus atropine sulphate (atropine sulphate infusion rate = 4.4 µg/min·kg) as shown in Figure 5.11.

As shown in Figures 5.10 and 5.11 there was not a significant difference between the HI-6 plasma concentration profile when comparing an HI-6 only infusion or an infusion of HI-6 plus AS for both HI-6 salts. AS does not appear to affect the pharmacokinetics of HI-6 2Cl or HI-6 DMS when co-infused over a period of eight hours in guinea pigs. Although a direct comparison of pharmacokinetic parameters between HI-6 administration alone and HI-6 co-administered with AS was not examined in the guinea pig model based upon the infusion results it appears that AS does not significantly alter the PK parameters of either HI-6 salt in guinea pigs. Future studies should directly examine the effect of atropine co-administration with HI-6 in guinea pigs.

Glycopyrrolate (an anticholinergic similar to atropine) was administered for all anaesthetized guinea pig studies to aid in the reduction of salivary, tracheobronchial and pharyngeal secretions generated while anaesthetized with an inhaled anaesthetic. If an anticholinergic is not administered to aid in secretion reduction often rodents may die due to respiratory complications while under anaesthesia. In the case of the experiments discussed within this thesis premature death due to anaesthetic complications would have led to very limited data collection and incomplete profiles of HI-6. The administration of an anticholinergic prior to and over the course of anaesthesia in rodents is common (Clemons & Seeman, 2011); in particular glycopyrrolate is often selected over atropine due to its longer period of action and the fact that it does not cross the blood brain barrier (Fassi & Rosenberg, 1979). The total amount of glycopyrrolate administered over the experimental period per animal was approximately 0.04 mg whereas the total amount of atropine sulphate administered per animal was approximately 0.240 mg. Following glycopyrrolate administration no observable changes in heart rate were observed only the most sensitive anti-muscarinic effect of reduction of secretions (Helller Brown & Laiken, 2011) (the desired effect) was observed. The effect and dose of glycopyrrolate was considered to be negligible compared to that of atropine administered and for the experiments presented in this thesis not considered a confounding variable. Additional studies should be carried to examine the effect of glycopyrrolate administration in nonanaesthetized animals with

and without atropine administration in addition to further studies examining AS co-administration IM or IV in anaesthetized guinea pigs.

6.5.2 Swine Studies (Determination of effect of AS co-administration)

A significant difference was observed for the distribution/absorption half-life, V_d, Cl, AUC_∞ and T_{max} (as presented in Figure 5.25 and Table 5.21) when comparing the pharmacokinetics of HI-6 2Cl administered in the presence or absence of AS. The distribution/absorption half-life is greater when HI-6 2Cl is co-administered with AS, IM suggesting that AS delays the distribution of HI-6 possibly due to decreased blood flow and increases the length of time until T_{max} is reached. Additionally the reduced V_d reported following AS co-administration suggests that AS restricts the distribution of HI-6.

Atropine sulphate (a competitive muscarinic receptor antagonist) administered at low doses typically results in slight bradycardia, dryness of the mouth and inhibition of sweating (Helller Brown & Laiken, 2011). Increasing doses of atropine sulphate result in increased heart rate, dry mouth, dilated pupils, hallucinations, delirium, blurred vision and what is commonly referred to as atropine flush, a dilation of cutaneous blood vessels (Kolka & Stephenson, 1987). In the absence of an antagonist following acetylcholine release a vasodilation of vascular beds can be seen as a result of acetylcholine's action on M_3 receptors in smooth muscle tissue resulting in increased nitric oxide production (Furchgott & Zawadzki, 1980). This vasodilation is not seen when smooth muscle endothelium is damaged (Vallance & Chan, 2001). In the event of a cholinergic crisis (the result of nerve agent poisoning) initial vasodilation of the smooth muscle tissue would be observed due to increased nitric oxide levels as a result of muscarinic receptor binding. However, following treatment with low to moderate doses of AS (competitive antagonist of acetylcholine) peripheral vasodilation is rapidly counteracted. The studies presented within this thesis were completed in healthy models; as such normal levels of circulating acetylcholine would have been present unlike those found in the event of NA intoxication. In the absence of an agonist, AS (competitive antagonist) would not result in any change to the vascular tone through muscarinic receptors as antagonists do not confer any activity on a receptor but rather only block the activity of the agonist. Additionally, in the event

that high (approaching toxic doses) of AS are administered in healthy patients a systemic flush is observed. This systemic flush may be due to a direct action of AS on α-adrenergic blockade of the receptor and not a result of its anticholinergic properties as cholinergic fibers play a relatively minor role in smooth muscle vascular tone. Chang & Hang (1995) demonstrated that AS has a strong affinity for α-adrenergic receptors at higher concentrations. More recently Kwan, Zhang, Kwan & Sakai (2003), have shown that the atropine flush (prominent vasodilation) is mediated through the activation of voltage dependent potassium channels resulting in increased potassium concentrations and inhibition of smooth muscle contraction (Guyton & Hall, 2006). Data presented shows, co-administration of AS and HI-6 resulted in a decreased absorption rate of the latter. It is likely that at the doses of AS employed in the current study and a resultant decrease in local blood flow within the muscle tissue at the site of injection or blunt shunting of blood to cutaneous sites delays the absorption and distribution of HI-6.

The significant difference in *Cl* suggests that AS may affect renal or hepatic clearance in this case reducing the clearance. The significant differences reported when comparing HI-6 2Cl administration in the presence or absence of AS, IM to domestic swine are most likely a result of the large variance of Treatment Group A and not a true effect of AS.

A significant difference was observed for distribution/absorption half-life, elimination half-life and *Cl* when evaluating the effect of atropine co-administration with HI-6 2Cl, IV (results presented in Figure 5.26 and Table 5.22. The distribution/absorption half-life of HI-6 2Cl was reduced when HI-6 2Cl was co-administered with AS IV suggesting that AS may increase the distribution of HI-6 possibly due to the local vasodilator properties of AS, as was proposed for HI-6 2Cl, AS co-administration IM. Additionally the clearance rate was greater when AS was co-administered following an IV bolus. Renal clearance, however, was not significantly altered suggesting the renal clearance is not altered by the co-administration of AS and some other mechanism of clearance is affected. The elimination half-life was significantly reduced following AS co-administration. However upon examination of Figure 5.26 the slopes are very similar and the difference reported could be due to very small standard deviation reported as shown in Table 5.22. Upon evaluation of the power statistics for these two groups, completing further animal studies leading to increased group size and statistical power the reported significant differences seen between groups would no longer exist.

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Finally co-administration of HI-6 DMS and AS following either an IM injection or an IV bolus resulted in very similar pharmacokinetic profiles when compared to HI-6 DMS administration alone. These results indicate that AS co-administration does not alter the pharmacokinetics of HI-6 DMS.

6.5.3 Summary of determination of effect of AS co-administration

Although significant differences between pharmacokinetic parameters were found between HI-6 2Cl administration alone compared to HI-6 2Cl co-administration with AS in swine these variances can be attributed to a large variance of plasma concentrations across all time points present within the HI-6 2Cl, IM group that cannot be explained. Additional studies should be carried out in both guinea pigs and swine examining the co-administration of HI-6 2Cl and AS to confirm that no significant difference in fact does exist, or provide an explanation for why a difference may exist. In both guinea pigs and swine co-administration of HI-6 DMS and AS resulted in very similar pharmacokinetics indicating that AS does not alter the pharmacokinetics of HI-6 DMS.

6.6 Evaluation of Calculated PK Parameters when HI-6 is Infused

The pharmacokinetic parameters calculated in both guinea pigs and swine were used to determine an infusion rate of HI-6 2Cl or HI-6 DMS to attain a target plasma concentration of 100 μM. The calculated infusion rate was evaluated for its accuracy in attaining and maintaining a plasma HI-6 concentration of 100 μM over the course of an eight hour period.

6.6.1 Guinea Pig Studies (Evaluation of calculated PK parameters)

As shown in Figure 5.9 both HI-6 2Cl and HI-6 DMS infusions resulted in a target plasma concentration of 100 μM at approximately the 3 hour mark and this plasma concentration (steady state) was maintained until the infusion was stopped at 8 hours at which point a rapid decline in plasma HI-6 concentrations can be seen. The rapid decline in plasma concentration reported at 8.5 hours and the lack of accumulation of HI-6 over the course of the infusion period indicates

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that an infusion of HI-6 does not lead to an accumulation of HI-6 within the guinea pig model. Furthermore, clearance, elimination and volume of distribution are not altered when HI-6 is continually infused. HI-6 2Cl was infused at a rate of 0.35 µmol/min·kg while HI-6 DMS was infused at a rate of 0.49 µmol/min·kg: the difference in infusion rates reflects the reported differences in pharmacokinetics parameters when comparing salts of HI-6 as discussed earlier for anaesthetized guinea pigs.

6.6.2 Swine Studies (Evaluation of calculated PK Parameters)

The calculated pharmacokinetic parameters determined for domestic swine administered an IV bolus of HI-6 DMS were used to calculate a constant infusion rate of HI-6 to reach and maintain a target plasma concentration of 100 µM HI-6 over the course of an eight hour infusion.

As shown in Figure 5.33 a target plasma concentration of 100 µM of HI-6 DMS was reached around the 3 hour mark and stead state was maintained for the duration of the infusion (8 hours). Similar to the guinea pig infusions a rapid decline in plasma concentration was reported once the HI-6 infusion was stopped and HI-6 plasma levels did not appear to rise significantly once steady state was achieved. The percent of HI-6 excreted in urine was examined over the course of the infusion as shown in Figure 5.34: similar to the infusion graph the percentage of HI-6 excreted reaches a steady state level around 3.5 hours and this level is maintained until the infusion was stopped at which point the percentage of HI-6 excreted in urine decreased as did the plasma concentration of HI-6. Over the course of the nine hour experimental period an average of 52 % of the total amount of HI-6 administered over the course of the eight hour infusion period was excreted in the urine. HI-6 DMS was infused at a rate of 0.48 µmol/min·kg.

6.6.3 Summary of evaluation of calculated PK parameters

The pharmacokinetic parameters determine in the IV bolus studies for both guinea pigs and swine were accurate: target plasma concentrations of 100 µM were attained and maintained in both models based upon the infusion rates calculated. The infusion rate for HI-6 DMS in guinea

pigs (0.49 µmol/min·kg) and swine (0.48 µmol/min·kg) were very similar suggesting that the pharmacokinetics of HI-6 DMS are very similar between the two species.

6.7 Comparison of Calculated PK Parameters with Human Data

The pharmacokinetic parameters determined for guinea pigs in Treatment Groups 5 and 6 and swine in Treatment Groups E and F were compared to previously published pharmacokinetic data determined in human clinical trials as shown in Table 6.1 and Table 6.2.

Table 6.1 Comparison of Elimination Half-life (min) following HI-6 2Cl and HI-6 DMS administration in guinea pigs (IV), swine (IV) and humans (IM). Guinea pigs were administered 110 µmol/kg of HI-6 2Cl or HI-6 DMS in 0.2 ml of saline as an IV bolus. Swine were administered 26.8 µmoll/kg of HI-6 2Cl or HI-6 DMS in 3 ml of saline as an IV bolus. Humans were administered various doses (62.5, 125, 250 or 500 mg) of HI-6 2Cl IM (Clement, et al., 1995) or 636 mg of HI-6 DMS, IM using a syringe and 22G 1" needle (Morelli & Jensen, 2010).

	Guinea Pig	Swine	Human
HI-6 2Cl[5]	49.0 ± 2.9*	70.8 ± 5.6	64.2 ± 13.2[a]
HI-6 DMS	44.8 ± 4.7	62.5 ± 3.3	76.2 ± 9.0[b]

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[5] *Note.* [a]Values from "The Acetylcholinesterase Oxime Reactivator HI-6 in Man: Pharmacokinetics and Tolerability in Combination with Atropine," by Clement, et al., 1995, Biopharmaceutics and Drug Disposition, 16(5), p.415-425. [b]Values from "Phase I, Open-Label, Fed, Single-dose, Pharmacokinetic, Safety and Tolerance Evaluation of Intramuscularly Administered HI-6 Dimethanesulfonate," by Morelli, G. & Jensen, M.L., 2010, Directorate Health Services Operations, AA81840. *Significant difference reported $p < 0.05$ (one-way anova) when comparing the elimination half-life of HI-6 2Cl in guinea pigs compared to swine and humans. Significant difference reported across all species when comparing the elimination half-life of HI-6 DMS.

The reported and calculated elimination half-life values presented in Table 6.1 are significantly different from one another as would be expected between species with the exception of the values presented for HI-6 2Cl in swine and humans. The differences between species supports a possible species variance. However, further research needs to be conducted with larger group sizes and equimolar doses per body weight administered across all species to provide for a less biased comparison. Species differences are most likely due to physiological differences that directly affect the absorption, distribution, metabolism and elimination (ADME) of HI-6.

Table 6.2 Comparison of calculated clearance (ml/kg) values following HI-6 and HI-6 DMS administration in guinea pigs (IV), swine (IV) and humans (IM). Guinea pigs were administered 110 μmol/kg of HI-6 2Cl or HI-6 DMS in 0.2 ml of saline as an IV bolus. Swine were administered 26.8 μmoll/kg of HI-6 2Cl or HI-6 DMS in 3 ml of saline as an IV bolus. Humans were administered various doses (62.5, 125, 250 or 500 mg) of HI-6 2Cl IM (Clement, et al., 1995) or 636 mg of HI-6 DMS, IM using a syringe and 22G 1" needle (Morelli & Jensen, 2010).

	Guinea Pig	Swine	Human
HI-6 2Cl[6]	3.5 ± 0.3	4.9 ± 0.5*	3.6 ± 0.2[a]
HI-6 DMS	4.9 ± 0.7	4.8 ± 0.4	4.8 ± 0.2[b]

The clearance data presented in Table 6.2 is very similar across all species within a salt with the exception of HI-6 administered IV in swine, indicating that the clearance of HI-6 is not species

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[6] *Note.* [a]Values from "The Acetylcholinesterase Oxime Reactivator HI-6 in Man: Pharmacokinetics and Tolerability in Combination with Atropine," by Clement, et al., 1995, Biopharmaceutics and Drug Disposition, 16(5), p.415-425. [b]Values from "Phase I, Open-Label, Fed, Single-dose, Pharmacokinetic, Safety and Tolerance Evaluation of Intramuscularly Administered HI-6 Dimethanesulfonate," by Morelli, G. & Jensen, M.L., 2010, Directorate Health Services Operations, AA81840. * Significant difference reported $p < 0.05$ (one-way anova) when comparing swine clearance values following HI-6 2Cl administration with guinea pig and human values.

dependent and can easily be translated from an animal model to humans. Currently clearance values are often used in conjunction with an algorithm (Food, 2002; Mahmood, Green, & Fisher, 2003) for scaling between animal model and humans for determination of dosing in human clinical trials with success. The use of an algorithm rather than a straight body weight/dose scaling method is preferred as algorithms take into account physiological factors that are different between species and as such alter ADME differently between species. Algorithms take into consideration factors such as oxygen utilization, blood volume, basal metabolism, renal function and many other biological processes (Reagan-Shaw, Nihal, & Ahmad, 2008), allowing for more specialized scaling calculations between species that are more meaningful than straight body weigh equivalences or body surface normalizations.

7. Conclusion

In all studies comparing HI-6 2Cl andHI-6 DMS, a significant difference was reported for absorption/distribution parameters. Absorption/distribution of HI-6 DMS was delayed compared to HI-6 2Cl both out of the muscle (following an IM injection) and into the tissue (following an IV bolus). In general IM and IV injections of both HI-6 2Cl and DMS resulted in similar calculated pharmacokinetic parameters indicating equal bioavailability of HI-6 independent of route of administration. This is important to note, as all currently available human pharmacokinetic data has been determined based upon IM injections. Based upon the results of this work this data can now be easily and accurately translated into IV formulation dosing for humans. As expected, a significant difference was reported when comparing administration of HI-6 in anaesthetized and non-anaesthetized models. Similar to previous ascending dose studies, differences were reported for absorption/distribution kinetics when comparing a low and high dose of HI-6. Co-administration of HI-6 with atropine sulphate did not have a significant effect on the pharmacokinetics of HI-6. This is important to know as atropine sulphate is always co-administered with HI-6 in the auto-injector formulation. The calculated pharmacokinetic parameters for both HI-6 2Cl and HI-6 DMS were accurate for the determination of an infusion rate to reach and maintain a target plasma concentration of 100 μM in both guinea pigs and swine. Additionally the infusion rates calculated for HI-6 DMS were very similar for both

guinea pigs and swine indicating that a species difference does not exist. This suggests that both the guinea pig and swine are good models to be used not only for pharmacokinetic studies of HI-6 DMS, but may also be for efficacy studies whose results could be translated for human use in the event of nerve agent poisoning requiring sustained treatment with a licensed HI-6 DMS product. It is important to keep in mind however that there are very large differences in the *in vitro* reactivation of inhibited human, swine and guinea pig AChE by HI-6. The percent of HI-6 eliminated in urine was very similar to the reported human values supporting the similarity across species for the pharmacokinetics of HI-6. The pharmacokinetic parameters determined for HI-6 2Cl and HI-6 DMS in swine and guinea pigs are similar to the reported human clinical trial data. In particular the clearance values, which are often used for scaling between animal models and humans were very similar. Based upon the results of these studies the guinea pig and swine models are useful models not only for the determination of the pharmacokinetics of HI-6 2Cl and HI-6 DMS under various conditions but will continue to be useful models for any future efficacy experiments required.

8. Future Considerations

There is considerable on going threat of the use of chemical warfare agents for both military and civilian populations. The oxime HI-6 has been shown to be an effective treatment against several different organophosphate nerve agents. This thesis provided a comprehensive look at the pharmacokinetics of both HI-6 2Cl and HI-6 DMS, however there are still large areas of research regarding HI-6 that should be examined.

Further studies examining the distribution the HI-6 throughout body tissues will be important to determine if there is any way to encourage HI-6 entry into the CNS by crossing the blood brain barrier and allow for maximal treatment in a relatively short period of time.

Future studies should examine the efficacy of HI-6 against exposure of different nerve agents. In particular studies should be undertaken to evaluate scenarios where following intoxication and initial auto-injector treatment of NA poisoning an infusion of HI-6 is administered for sustained treatment and models are evaluated following treatment with behavioral tests to determine any

cognitive/behavioral deficit. Building on these efficacy studies it may be critical to examine the effect of some form of trauma and blood loss as would be seen in a combat environment and how these physiological changes resulting in an already health compromised state effect the pharmacokinetics and efficacy of HI-6. Similarly research should be undertaken to understand the role that other medications such as those used for pain management (i.e. morphine) may play in the tolerability, pharmacokinetics and efficacy of HI-6.

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