The Impact of MK-467 on Certain Pharmacokinetic and Pharmacodynamic Properties of Selected Drugs Affecting the Central Nervous System in Dogs

Rachel C. Bennett

ACADEMIC DISSERTATION

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To my family
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ABSTRACT

Background and rationale

$\alpha_2$-adrenoceptor agonists, such as medetomidine, are commonly used in veterinary medicine for the sedation and premedication of animals. Their use is associated with a range of undesirable pharmacodynamic effects most notably vasoconstriction, bradycardia (Pyndop and Verstegen 1998), hyperglycaemia (Hsu and Hummel 1981) and diuresis (Thurmon et al. 1978). MK-467 is a peripheral $\alpha_2$-adrenoceptor antagonist (Clineschmidt et al. 1988), which ameliorates the aforementioned side effects of (dex-)medetomidine whilst maintaining the sedative effects (Enouri et al. 2006; Honkavaara et al. 2008; Restitutti et al. 2012; Rolfe et al. 2012). Therefore MK-467 may offer some important clinical benefits, although some questions remain concerning its pharmacodynamic and pharmacokinetic interaction with other anaesthetic drugs.

The main objectives of the following studies were to: determine the protein-binding fraction of MK-467, to assess the possible role of MK-467 as a P-glycoprotein substrate in vitro; and to evaluate the impact of MK-467 on the disposition of medetomidine in vivo. During in vivo studies, the impact of MK-467 on the centrally mediated effects: sedation and antinociception and peripherally mediated cardiovascular effects of medetomidine were assessed simultaneously.

The protein-binding characteristics of MK-467 were investigated using the technique of equilibrium dialysis. Drug concentrations were measured using the technique of liquid chromatography and tandem mass spectrometry. Protein-binding fraction of MK-467 was approximately 70% and it was unaltered by the presence of medetomidine.

Transcellular drug movement was determined using Madin-Darby Canine Kidney cells - wild type (WT-MDCKII) and cells transfected with the human gene encoding P-glycoprotein (MDR1-MDCKII). In addition to MK-467, acepromazine, a putative P-glycoprotein substrate, and dexmedetomidine were also investigated. Based on measured drug concentrations, apparent permeability of the cells was calculated and used to determine the role of active transport in the transcellular movement of the selected drugs. Passive movement of MK-467 was undetectable. Therefore, efflux ratios for MK-467 were not determined. However, movement in the basolateral to apical direction occurred in both cell lines. The identity of the possible transporter remains unclear. Transport ratios for acepromazine were 1.17:1.0 and 1.51:1.0 for WT-MDCKII and MDR1-MDCKII transfected cells, respectively. Currently acepromazine cannot be defined as a P-glycoprotein substrate based on these results. Whilst, dexmedetomidine transport ratios were 0.98:1.0 and 1.15:1.0 for WT-MDCKII and MDR1-MDCKII respectively. It does not appear to be a substrate for an active transport mechanism.

In vivo drug concentration data underwent non-compartmental analysis. MK-467 increased the volume of distribution and clearance of dexmedetomidine and levomedetomidine, whilst area under the curve and elimination half-life were significantly decreased when compared with medetomidine alone. Medetomidine significantly decreased the clearance
of alfaxalone during co-administration, whilst the additional administration of MK-467 counteracted the effect of medetomidine on alfaxalone clearance.

The quality and duration of sedation were assessed using a composite sedation score, whilst hypnosis was evaluated by measurement of bispectral index or analysis of the electroencephalogram. Antinociception was determined by the measurement of limb withdrawal times and head lift times following the application of a nociceptive stimulus applied to the nailbed of a hind limb digit. Measured haemodynamic variables included arterial blood pressure, heart rate, cardiac output and systemic vascular resistance. Ventilatory effects of medetomidine and co-administered MK-467 were also assessed by the analysis of arterial and venous blood gas samples taken during these studies. The co-administration of MK-467 did not alter the initial quality of sedation but reduced the duration of sedation produced by medetomidine. MK-467 significantly diminished the antinociceptive action of medetomidine. MK-467 ameliorated the cardiovascular, haemodynamic and ventilatory effects of medetomidine prior to and during general anaesthesia.

In conclusion, MK-467 is moderately protein bound and it is unlikely to be subject to drug-drug interactions in vivo. MK-467 shows little passive movement in MDCKII cells but may undergo active cellular efflux. It is unclear whether MK-467 is suitable for use in animals carrying the P-glycoprotein mutation. The addition of MK-467 alters the disposition of co-administered drugs resulting in lower plasma drug concentrations. The reduction in some pharmacodynamic effects may be attributed to the alteration in pharmacokinetics caused by the peripheral α₂-adrenoceptor antagonist.
LIST OF ORIGINAL PUBLICATIONS

Study I

Study II
RC Bennett, M Palviainen, M Peltoniemi, L Vuorilehto, M Scheinin, MR Raekallio, OM Vainio. The role of active transport in the transcellular movement of the peripheral α2-adrenoceptor antagonist, MK-467: an in vitro pilot study. Accepted for publication in the Canadian Journal of Veterinary Research.

Study III

Study IV

Study V
K Salla, RC Bennett, F Restitutti, J Junnila, M Raekallio M, O Vainio (2014). A comparison in dogs of medetomidine, with or without MK-467, and the combination acepromazine-butorphanol as premedication prior to anaesthesia induced by propofol and maintained with isoflurane. Veterinary Anaesthesia and Analgesia 41, 163-73.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette transporters</td>
</tr>
<tr>
<td>AGP</td>
<td>α1-acid glycoprotein</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic nervous system</td>
</tr>
<tr>
<td>AP-BL</td>
<td>Apical to basolateral</td>
</tr>
<tr>
<td>ASA</td>
<td>American Society of Anesthesiologists</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt;</td>
<td>Area under the concentration time curve from zero to infinity</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;last&lt;/sub&gt;</td>
<td>Area under the curve to the last sampling point</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-60&lt;/sub&gt;</td>
<td>Area under the curve between time zero and 60 minutes</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood–brain barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast cancer resistance protein</td>
</tr>
<tr>
<td>BSCB</td>
<td>Blood–spinal cord barrier</td>
</tr>
<tr>
<td>BIS</td>
<td>Bispectral index</td>
</tr>
<tr>
<td>BL-AP</td>
<td>Basolateral to apical</td>
</tr>
<tr>
<td>CI</td>
<td>Cardiac index</td>
</tr>
<tr>
<td>Cl&lt;sub&gt;ss&lt;/sub&gt;</td>
<td>Clearance at steady state</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum measured plasma concentration</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>CRI</td>
<td>Constant rate infusion</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450 enzyme system (e.g. CYP2A6)</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyograph</td>
</tr>
<tr>
<td>f&lt;sub&gt;R&lt;/sub&gt;</td>
<td>Respiratory frequency</td>
</tr>
<tr>
<td>f&lt;sub&gt;u&lt;/sub&gt;</td>
<td>Unbound fraction</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>HLT</td>
<td>Head lift time</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
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<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography &amp; tandem mass spectrometry</td>
</tr>
<tr>
<td>LWT</td>
<td>Limb withdrawal time</td>
</tr>
<tr>
<td>MAC</td>
<td>Minimum alveolar concentration</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial blood pressure</td>
</tr>
<tr>
<td>MDCKII</td>
<td>Madin–Darby canine kidney II epithelial cells</td>
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</tbody>
</table>
MDR1  Multi-drug resistance gene 1
MMK  Medetomidine and MK-467 treatment
MRPs  Multidrug resistance-associated proteins
MRP1  Multidrug Resistance-associated protein 1
MRP2  Multidrug Resistance-associated protein 2
mRNA  Messenger ribonucleic acid
NCA  Non-compartmental analysis
PaCO₂  Arterial carbon dioxide partial pressure
PaO₂  Arterial oxygen partial pressure
P_app  Apparent permeability
PBS  Phosphate buffered saline
P-gp  P-glycoprotein
PPB  Plasma protein-binding
Q-Q  Quantile-quantile
SD  Standard deviation
SEF  Spectral edge frequencies
SVR  Systemic vascular resistance
SVRI  Systemic vascular resistance index
T  Time from drug administration
T₁/₂  Elimination half-life
T10 etc.  Ten minutes after drug injection
TEER  Transepithelial electrical resistance
Vₖdes  Steady state volume of distribution
Vₐ  Apparent volume of distribution
V_max/Kₘ  Ratio of maximum velocity of transformation and affinity of an enzyme for a given substance
WT  Wild type
1. INTRODUCTION

The importance of the autonomic nervous system (ANS) cannot be overemphasized. Its maintenance and action is vital to life and to homeostasis. In broad terms, it controls cardiovascular and visceral functions, which by definition are not under conscious control. The range of functions performed by the autonomic system may best be illustrated or described by the eponymous ‘Ondine’s curse’ derived from Greek mythology. The myth relates to the water nymph (Greek: νόμφη) – Ondine – who sadly had an unfaithful mortal lover. He swore to her that his “every waking breath would be a testimony of [his] love.” On witnessing his adultery, she cursed him such that if he should fall asleep, he would forget to breathe. Eventually, from sheer exhaustion, he succumbed to sleep, his breathing stopped and he duly died.

Due to the many physiological processes mediated by this part of the nervous system, it is perhaps unsurprising that drugs acting on the ANS have wide-ranging pharmacodynamic effects, some of which are both desirable and beneficial, whilst others are unwanted. The opportunity to use a drug that may ameliorate the problematic effects of another drug whilst maintaining the latter’s desired effects has great potential in clinical veterinary medicine.

Alpha₂-adrenoceptor agonists have been used in veterinary medicine for nearly fifty years (Rosenberger et al. 1968; Clarke & Hall 1969; Müller et al. 1969; Kerr et al. 1972). They are known to have profound sedative and analgesic effects, both of which are mediated within the central nervous system (CNS). However, their clinical use has been restricted by the other pharmacodynamic effects associated with their administration, which for the most part are mediated via a peripheral site of action. The common peripheral effects include hypertension due to their action at α₂-adrenoceptors on vascular smooth muscle. Binding at this site leads to vasoconstriction and an increase in peripheral vascular resistance (Pyndpend & Verstegen 1998). Alpha₂-adrenoceptor agonists may precipitate cardiac arrhythmias (Muir et al. 1975). They cause hyperglycaemia by preventing the release of insulin from the pancreas (Hsu and Hummel 1981), and this also contributes to diuresis (Thurmon et al. 1978), alongside their inhibitory effect on the action of vasopressin within the tubules of the kidney (Gellai 1990).

MK-467 is a peripherally acting α₁-adrenoceptor antagonist (i.e. it has not been demonstrated to cross the blood-brain barrier (BBB) in rats or marmosets (Clineschmidt et al. 1988)), which in previous studies has been shown to ameliorate the side effects of medetomidine (Enouri et al. 2006; Honkavaara et al. 2008; Restitutti et al. 2012; Rolfe et al. 2012). Its use therefore offers potential clinical benefits and could widen the indications for the administration of medetomidine in small animals.

This thesis focuses on the interaction between certain pharmacokinetic parameters and pharmacodynamic effects of medetomidine and MK-467, encompassing both in vitro and in vivo studies.
2. LITERATURE REVIEW

2.1 Central nervous system versus peripheral nervous system

There is a distinction between the centrally mediated effects of $\alpha_2$-adrenoceptor agonists and the myriad of pharmacodynamic effects that occur due to activity at peripheral sites. The central effects rely on the ability of these drugs to cross the BBB or blood-spinal cord barrier (BSCB) or both and thereby reach their site of action and mediate their characteristic sedative and antinociceptive effects.

2.1.1 The blood–brain barrier

Sedative and anaesthetic drugs are typically injected or inhaled and, with the exception of epidural and intrathecal administration, these drugs are not administered at their main site of action. The distribution of drugs into the brain tissue is restricted by the BBB. This barrier is constituted by the brain capillary endothelial cells, which surround the cerebral microvessels (Abbott 2006). The barrier exists at three interfaces: the endothelial cells of the blood vessels supplying the brain and spinal cord, as indicated above; the epithelial cells lining the choroid plexus, (i.e. the blood–cerebrospinal fluid barrier); and by the avascular arachnoid epithelium (Abbott et al. 2004). The barrier exists as a structural entity, a transport barrier (Begley & Brightman 2003) and as a metabolic barrier (El-Bacha & Minn 1999). Tight junctions between the endothelial cells prevent the passage of large molecules, ions and polar solutes (Begley & Brightman 2003; Wolburg et al. 2009); instead, these substances need to move via a transcellular route.

2.1.1.1 Transport across the blood–brain barrier

Following the administration of a drug, several factors determine how rapidly the compound reaches its site of action within the CNS. Lipid-soluble molecules readily diffuse through the BBB and thereby enter the brain (Liu et al. 2004), and the rate at which a substance enters the CNS is correlated (to some extent) with its lipid solubility, determined as the logD octanol-buffer partition coefficient at pH 7.4 (Clark 2003). Many drugs used in veterinary anaesthesia are lipid soluble, low molecular weight compounds, such as $\alpha_2$-adrenoceptor agonists, and they can readily cross the BBB and enter the CNS (Salonen 1989).

Factors shown to restrict the entry of substances into the CNS include a high polar surface area and the tendency to form hydrogen bonds (specifically more than six bonds), since this impedes movement from the aqueous phase into the lipid cell membrane (Clark 2003; Gleseson 2008). Other factors may include the presence of rotatable bonds within a molecule and a molecular weight above 450 Da (Abbott 2010). High-affinity protein binding (discussed below) may also affect drug diffusion between plasma and tissues, such as the CNS (Lin 2008). However, these factors are not absolutely predictive of penetration into the CNS. Some drugs with demonstrable CNS activity do not fulfil these criteria (Bodor & Buchwald 2003), whilst other lipid-soluble compounds show a lower CNS penetrance than anticipated based on their logD partition coefficients (Begley 2004). The apparent
inability of some highly lipid soluble molecules to cross BBB is due to the presence of efflux transporters within this barrier, specifically the ABC-transporters (Begley 2004).

There are 48 human genes known to code for ABC proteins and an additional four are reported in the dog (i.e. representing a possible total of 52 genes in the dog) (Dean and Annilo 2005). These proteins are grouped into seven families or subclasses (ABCA to ABCG) (Dean et al. 2001; Hediger et al. 2004). The ABC transporters of greatest significance within the BBB are P-glycoprotein (P-gp, ABCB1, multidrug resistance protein), breast cancer resistance protein (BCRP, ABCG2) and multidrug resistance-associated proteins (MRPs, ABCC1, 2, 4, 5 and 3 and 6) (Begley 2004; Daunhcy et al. 2008; Kamiie et al. 2008). In the BBB, ABC transporters act as efflux pumps, which rely on ATP hydrolysis to transport a wide range of lipid-soluble compounds out of the CNS. As such, their role is to remove xenobiotic molecules and endogenous, potentially neurotoxic substances (Dallas et al. 2006).

Presently the ABC transporter P-glycoprotein is the most clinically relevant to veterinary species. The importance of the ABC transporter P-glycoprotein in the BBB was originally demonstrated in mdr1a(-/-) knockout mice (Schinkel et al. 1994; 1995). Since this discovery, genetic alterations in canine P-gp have also been documented. The administration of P-gp substrates to affected dogs may lead to symptoms of increased sensitivity to opioid drugs such as loperamide and butorphanol (Wandel et al. 2002; Sartor et al. 2004; Mealey 2006) or toxicity when therapeutic doses of avermectin parasiticides are administered to homozygous negative individuals (Mealey et al. 2001). The tranquiliser acepromazine is described as a putative P-gp substrate (Mealey 2006). To date, no studies have investigated the role of the peripheral α2-adrenoceptor antagonist MK-467 as a P-gp substrate or inhibitor in the dog, whilst, species differences exist in the selectivity of this transporter (Takeuchi et al 2006; Mealey et al. 2017).

2.2 Plasma protein binding (PPB)

The impact of PPB on the pharmacodynamic activity of new drugs is an important consideration during drug development (Trainor 2007). Binding by plasma protein may markedly influence the pharmacodynamic effects and efficacy of therapeutic agents. Some drugs that have demonstrable therapeutic activity in vitro have been relatively ineffective in clinical trials (Kageyama et al. 1994; Fischl et al. 1997; Goldberg et al. 1997; Edmondson et al. 2005 and 2006).

The major drug-binding components in plasma include albumin, α1-acid glycoprotein (AGP), lipoproteins and erythrocytes, of which albumin and AGP have been most extensively studied (Bohnert and Gan 2013). Human serum albumin (HSA) is the most abundant of these proteins, accounting for 55% of the total plasma protein content in humans (Lambrinidis et al. 2015). It is a 66 kDa protein consisting of 585 amino acids (Quinlan et al. 2005). Its main physiological role is the transport of fatty acids (Lambrinidis et al. 2015), but it also reversibly binds acidic, neutral and to a lesser extent basic drugs (Vallianatou et al. 2013; Lambrinidis et al. 2015). There are two major overlapping stereoselective
binding sites within the albumin molecule: Sudlow 1 or the warfarin-azapropazone site and Sudlow II or the indole-benzodiazepine site (Sudlow et al. 1975; Fehske et al. 1981; Curry et al. 1999; Petitpas et al. 2001). Alpha1-acid glycoprotein is an acute phase protein, which is highly water soluble and functions as an acid at physiological pH (Fournier et al. 2000). In humans, AGP constitutes 1–3% of total plasma protein (Fournier et al. 2000). A comparative study of bovine, canine and human AGP indicates that binding characteristics differ between these three species. Human AGP consists of three overlapping binding sites: a basic ligand binding site, an acidic ligand binding site and a steroid hormone binding site. Canine and bovine AGP each has basic ligand and steroid hormone binding sites; however, canine and bovine AGP do not contain an acidic ligand binding site. To the author’s knowledge, the amino acid sequence and variants of canine AGP are not defined. Based on the results of fluorescence studies, the hydrophobic nature of the ligand binding pockets within the three AGPs are similar. The overlapping nature of the binding sites suggests that drug–drug interactions may occur in vivo (Matsumoto et al. 2002).

Alterations in the concentration of plasma proteins may result in changes in the percentage of unbound drug, whilst the co-administration of drugs may lead to competition for binding sites on plasma proteins (Ismaili and Dayton 2001). Theoretically, the displacement of a drug from its protein binding site(s) may increase its efficacy (Maillard et al. 2001). Alternatively, this phenomenon may lead to a risk of toxicity. For many therapeutic agents, alterations in protein binding due to drug–drug interactions or disease–drug interactions do not change the exposure of a patient to these compounds. The reasons underlying this arguably surprising finding are comprehensively explained by Rolan (1994) and Benet and Hoener (2002). Briefly, they are dependent upon the route of administration, the extraction ratio of a drug and the site of metabolism or elimination. However, anaesthetic drugs may form an exception to this general rule. Therefore, it is important to know something about the protein-binding characteristics of a drug, its effective plasma concentration, dosing requirements and elimination to understand the impact of alterations in plasma proteins on the drug concentration at the target receptor.

Genetic polymorphisms, which lead to alterations in the plasma albumin concentration, have been identified in certain dog breeds, such as beagles (Ito et al. 2009). In humans, AGP genetic polymorphisms exist with two isoforms, and different binding properties are associated with these variants (Fitos et al. 2006; Nishi et al. 2011); currently, there are no such reports in dogs. Similarities exist between AGP and the efflux transporter P-glycoprotein, both proteins interacting with the same classes of therapeutic drugs (Zsila 2007). The importance of this relationship for future drug development awaits further investigation.

### 2.3 Autonomic nervous system

As alluded to earlier, the ANS controls those physiological processes that are not under voluntary control (Hall 2016). The ANS system is subdivided into sympathetic and parasympathetic branches as proposed by Langley (1921). This division is based on their anatomical location (e.g. sympathetic fibres are present in the thoracic and lumbar
sections of the CNS), the processes they modulate and the neurotransmitters that mediate them. Similarly, to other parts of the nervous system, the ANS also functions by means of reflex arcs with peripheral sensory receptors. For example, vascular baroreceptors and chemoreceptors detect sensory information, which is conducted via afferent fibres to the CNS, particularly the brainstem and hypothalamus, where it is ‘processed’ (Hilton and Spyer 1980; Dampney 1994; Morrison 2001). The response ‘generated’ by the CNS is transmitted to the effector organs or tissues via the efferent nerve fibres of the parasympathetic and sympathetic systems, leading to an efferent response (Saper 2002). However, whilst certain reflexes result in activity within the brain and spinal cord (e.g. sedation, antinociception) other effects take place via peripheral activity (e.g. vasoconstriction) (Kamibayashi and Maze 2000). The differences between centrally and peripherally mediated effects have important clinical implications for drugs acting on or within the ANS.

2.3.1 Sympathetic nervous system

The sympathetic nervous system is said to mediate the ‘flight-or-fight’ response but also functions in association with the parasympathetic system to maintain homeostasis (Cannon 1932). Pre-ganglionic fibres arise from the thoracolumbar region of the spinal cord and ‘travel’ to a ganglion where they synapse. From these synapses, post-ganglionic neurons extend throughout the body to their eventual site of action. Varicosities occur at synaptic clefts along the length of the post-ganglionic sympathetic nerve fibre (Bennett et al. 1998). At the effector cell site, sympathetic nervous system activity is predominantly mediated through adrenergic receptors or adrenoceptors. Adrenoceptors are present within the cell membrane at (junctional) or near (extra-junctional) the sympathetic nerve terminals of the post-ganglionic fibres (Ruffalo 1985).

2.3.2 Endogenous adrenoceptor ligands

The endogenous substances acting at adrenoceptors are termed catecholamines. Structurally, catecholamines consist of a 1,2-dihydroxybenzene or ‘catechol’ group (an organic compound), with a terminal amine group linked by an intermediate ethyl chain. Catecholamines are derived from the amino acid L-tyrosine, which is converted to levodopa by the enzyme aromatic amino acid hydroxylase. Levodopa is converted to dopamine by L-amino acid decarboxylase and to noradrenaline by dopamine β-hydroxylase. Finally, adrenaline is synthesized from noradrenaline via the action of phenylethylamine N-methyltransferase in the adrenal medulla (Axelrod 1962; Nagatsu 1991). Catecholamines are water-soluble substances and approximately 25% are bound to plasma proteins (both albumin and AGP) when they circulate in the bloodstream (Sager et al. 1987; de Vero et al. 1988).

Noradrenaline is the main sympathetic transmitter at the effector cell within the sympathetic nervous system (von Euler 1946; Goldstein et al. 2003). However, both noradrenaline and adrenaline are also secreted from the adrenal medullae (Hopwood 1971). Whilst, acetylcholine is the neurotransmitter at post-ganglionic sympathetic nerve fibres in eccrine glands (Sokolov et al. 1980).
2.3.3 Adrenoceptor structure & differentiation

The identification or differentiation of receptor subtypes within the ANS was proposed by Ahlquist (1948) in a seminal paper entitled: "A study of the adrenotropic receptors." He classified the receptors into two subtypes named alpha and beta. Prior to this work, Cannon and Rosenblueth (1937) proposed the existence of two different neurotransmitters responsible for mediating the respective excitatory and inhibitory effects evident within the sympathetic nervous system. Ahlquist (1948) used synthetic amines similar in structure to adrenaline and evaluated their potency in different tissues. On this basis, he established that a range of functions were mediated via activity at one form of receptor he termed alpha (α), whilst these substances had a differing potency in a separate group of presumptive receptors. Ahlquist (1948) deduced that this difference in efficacy was due to action at a structurally separate receptor, which he subsequently termed beta (β). This initial distinction was therefore based on a form of pharmacological classification. Today, α-adrenoceptors are said to mediate many excitatory functions, such as vasoconstriction, uterine myometrial contraction, urethral contraction and pupillary dilation (Westfall and Westfall 2011). Beta-adrenoceptors, on the other hand, mediate so-called inhibitory functions, for example vasodilation and bronchodilation, but also mediate the stimulation of cardiac contraction and the heart rate (Westfall and Westfall 2011).

Langer (1974) proposed that α-adrenoceptors should be further subdivided into α₁- and α₂-adrenoceptors based on their anatomical location post- or pre-synaptically, respectively. However, this form of classification failed to explain the effects mediated via the two receptor subtypes. An alternative classification system based on function was proposed by Berthelsen and Pettinger (1977), whereby α₁-adrenoceptors mediate excitatory effects and α₂-adrenoceptors mediate inhibitory effects; however, this also proved to be inadequate. Currently, the classification of α-adrenoceptors is based neither on location nor on function, but rather on the pharmacological affinity of each receptor for agonist and antagonist compounds. Specifically, adrenoceptors activated by the compounds B-HT 920, B-HT 933 and UK-14 304 or α-methylnoradrenaline and competitively inhibited by yohimbine, rauwolscine or idazoxan are defined as α₁-adrenoceptors (Ruffalo et al. 1991; Civantos Calzada and de Artauñano 2001). The α₂-adrenoceptor has since been further subdivided into α₂A, α₂B and α₂C (Lorenz et al. 1990; Blaxell et al. 1991; Bylund et al. 1992; Bylund et al. 1994). The α₂D subtype was discovered in the rat submaxillary gland (Michel et al. 1989), but it is now considered to represent the rodent homologue of the human α₂A subtype (Lanier et al. 1991). The α₂A subtype is present in the human, pig and rabbit, whereas the α₂D subtype has been reported in the cow, mouse and rat (O’Rourke et al. 1994; Berlie et al. 1995; Bond et al. 2015; IUPHAR database). This receptor subtype is referred
to as the $\alpha_{2A/D}$ receptor (Bylund et al. 1994; Docherty 1998). Currently, nine adrenoceptor subtypes are recognised: $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{2A/D}$, $\alpha_{2B}$, $\beta_1$, $\beta_2$, and $\beta_3$ (Hieble 2007).

In humans, the three $\alpha_2$-adrenoceptors are encoded by distinct genes located on three different chromosomes: $\alpha_{2A}$ on chromosome 10 (Kobila et al. 1987), $\alpha_{2B}$ on chromosome 2 (Regan et al. 1988) and $\alpha_{2C}$ on chromosome 4 (Lomasney et al. 1990) To the author’s knowledge, similar information for the dog is not currently available. However, there is said to be strong homology of the $\alpha$-adrenoceptor subtype gene sequences across mammalian species (MacDonald et al. 1997).

Adrenoceptors share a similar structure: they are defined as class A G-protein-coupled receptors (GPCRs) with seven-transmembrane protein domains, an extracellular amino terminus and an intracellular carboxyl terminus (Kobila 2011; IUPHAR database). Cytosolic hydrophilic loops link the seven hydrophobic $\alpha$-helices, with the third cytoplasmic loop considered to be the most important in determining receptor function. It contains the domains both for coupling to the G-protein and for phosphorylation by GPCR kinases and later desensitization. The extracellular binding region, G-protein attachment sites and intracellular phosphorylation sites are responsible for the subtype-specific responses. Agonist binding is coupled to the inhibitory heterotrimeric GTP-binding protein $(G_{i/o})$ signalling system (primary transduction mechanism), which leads to the inhibition of adenylyl cyclase activity, an inhibitory effect on the opening of voltage-gated calcium ion channels, the activation of potassium channels and consequent hyperpolarization of the cell membrane, and phospholipase $A_2$ stimulation (Limbird 1988; Bylund and Ray-Prenger 1989; Jones et al. 1991; Surprenant et al. 1992). A secondary transduction mechanism results in adenylyc cyclase stimulation (Eason et al. 1992).

2.3.4 Location and function of $\alpha_2$-adrenoceptors: selected data

The distribution of the $\alpha_2$-adrenoceptor subtypes has been extensively studied in two mammalian species, humans and rats, using assays of adrenoceptor mRNA. The distribution of the $\alpha_2$-adrenoceptors subtypes is outlined in Table 1.

Table 1. The reported distribution of $\alpha_2$-adrenoceptors in humans and rats. Human data derived from Perala et al. 1992; Eason and Liggett 1993; rat data from Handy et al. 1993; Blaxall et al. 1994.

<table>
<thead>
<tr>
<th></th>
<th>Humans</th>
<th>Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_{2A/D}$-adrenoceptor</td>
<td>Brain &gt; spleen &gt; kidney &gt; aorta = lung = skeletal muscle &gt; heart = liver.</td>
<td>Brain &gt; spleen = kidney = aorta &gt; lung = skeletal muscle.</td>
</tr>
<tr>
<td>$\alpha_{2B}$-adrenoceptor</td>
<td>Spleen = kidney = aorta = lung = skeletal muscle = heart = liver.</td>
<td>Kidney &gt;&gt; liver &gt; brain = lung = heart = skeletal muscle.</td>
</tr>
<tr>
<td>$\alpha_{2C}$-adrenoceptor</td>
<td>Brain = kidney &gt; aorta = lung = skeletal muscle = heart = spleen.</td>
<td>Brain &gt;&gt; kidney.</td>
</tr>
</tbody>
</table>

The role of the different $\alpha_2$-adrenoceptor subtypes has been predominantly studied in mice (Figure 1), in which $\alpha_{2A}$-adreceptors mediate sedation and antinociception (Hunter et al. 1997; Lakhani et al. 1997; Stone et al. 1997; Kable et al. 2000), anaesthetic-sparing

![Diagram](image)

**Figure 1.** Selected subtype-specific functions of α₂-adrenoceptor subtypes based on gene-targeted mouse models. Abbreviations: α₂-Ag, α₂-adrenoceptor agonist, α₂A, α₂B, α₂C, α₂-adrenoceptor subtypes. (Knaus et al. 2007).

The α₂-adrenoceptor is sparsely located within the brain, having been identified in the thalamus of the rat (Schein et al. 1994); however, α₂-adrenoceptors in the spinal cord play a role in mediating the analgesic effect of nitrous oxide via descending pathways from supraspinal sites, such as the periaqueductal grey area (Guo et al. 1999; Sawamura et al. 2000). In genetically modified mice, α₂-adrenoceptors are the predominant receptor subtypes mediating arterial vasoconstriction (Link et al. 1996; Makaritsis et al. 1999; Paris 2003). However, α₂A/ß and α₂C-adrenoceptors also cause vasoconstriction of small arteries (Chotani et al. 2004). In venous vascular beds, the major postsynaptic α₂-adrenoceptor is the α₂D-subtype (Paiva et al. 1999).
In the CNS, $\alpha_{2C}$-adrenoceptors influence processes involving memory and behaviour, hypothermia in response to $\alpha_{2}$-adrenoceptor agonists, and they may also regulate dopamine-mediated effects within the brain (Sallinen et al. 1997 and 1998; Bjorklund et al. 1999; Scheinin et al. 2001). The $\alpha_{2C}$-adrenoceptor regulates the feedback of catecholamine release from the chromaffin cells of the adrenal medulla (Brede et al. 2003; Moura et al. 2006; Gilsbach et al. 2007). Within the CNS, both the $\alpha_{2A}$- and $\alpha_{2C}$-subtypes may also serve as heteroreceptors, inhibiting the release of dopamine and serotonin (Scheibner et al. 2001; Bücheler et al. 2002), whilst $\alpha_{2C}$-adrenoceptors, like $\alpha_{2A}$-adrenoceptors, also inhibit noradrenaline release presynaptically within the CNS (Hein et al. 1999; Kable et al. 2000). Peripherally, all $\alpha_{2}$-adrenoceptor subtypes have been shown to contribute to feedback inhibition in vivo (Trendelenberg et al. 2003; Vonend et al. 2007).

In the periphery, the $\alpha_{2A/0}$-adrenoceptor is largely responsible for the inhibition of insulin secretion from $\beta$-cells of pancreatic islets (Niddam et al. 1990; Fagerholm et al. 2004), which leads to hyperglycaemia. Within the kidney, $\alpha_{2A}$ and $\alpha_{2B}$-adrenoceptors are present within the cortical collecting ducts, inhibiting the action of vasopressin and thereby leading to diuresis (Krothapalli & Suki 1984; Wilburn et al. 1998). Alpha $\alpha_{2C}$-adrenoceptors have been identified on renal sympathetic nerves (Taoda et al. 2001). In the myometrium, $\alpha_{2A/0}$ and $\alpha_{2B}$-adrenoceptors exist in equal proportions, as demonstrated in studies investigating the regulation of these adrenoceptors during pregnancy (Adolfsson et al. 1998). $\alpha_{2A}$-autoreceptors mediate the noradrenergic inhibition of gastrointestinal motility by the sympathetic nervous system in vivo. $\alpha_{2A}$-heteroreceptors mediate inhibition of acetylcholine release, neurally mediated contractions and gastrointestinal transit time (Scheibner et al. 2002). Alpha $\alpha_{2}$-adrenoceptors are also located within adipose tissue, where they inhibit lipolysis and subsequently the release of free fatty acids, although this effect is less significant in rodents than humans (LaFontan and Berlan 1993).

Based on this information, the use of drugs that act at specific $\alpha_{2}$-adrenoceptor subtypes would offer some potential clinical benefits. For example, specificity for the $\alpha_{2A}$-adrenoceptor would produce the sedative and analgesic effects but avoid the initial hypertensive effects identified with medetomidine. However, veterinary drugs such as detomidine and dexmedetomidine have little demonstrable specificity for the $\alpha_{2}$ receptor subtypes (MacDonald et al. 1997; Schwartz & Clark 1998). Therefore, this therapeutic approach is currently not a viable proposition, and the alternative possibility of using a peripherally acting $\alpha_{2}$-adrenoceptor antagonist is interesting.

2.5 Characterization of $\alpha_{2}$-adrenoceptor polymorphisms

There are approximately 4000 variations in the genes encoding adrenoceptors. These include single nucleotide polymorphisms and insertion/deletion mutations (Ahles and Engellhardt 2014). Studies in humans have shown that all $\alpha_{2}$-adrenoceptor subtypes are polymorphic. Thus far, the common coding variants for the $\alpha_{2}$-adrenoceptor subtypes have been located within the third intracellular loop of the respective $\alpha_{2}$-adrenoceptor gene. This region is known to play a pivotal role in G protein coupling and kinase-mediated regulation (Chen et al. 1993). Currently, one coding variant has been identified for each of the three $\alpha_{2}$-adrenoceptor subtypes: c.798C>G (p.Asn266Lys) in the $\alpha_{2A}$-adrenoceptor, c.901_909del
(p.Glu301_Glu303del) in the $\alpha_{2b}$-adrenococeptor, and c.964_975del (p.Gly322_Pro325del) in the $\alpha_{2c}$-adrenococeptor. Studies have attempted to identify whether these genetic alterations are associated with changes in human physiology and possibly disease. Two studies examined the association between insulin secretion and the risk of type II diabetes. Impaired insulin secretion was identified in two Scandinavian cohorts (Rosengren et al. 2010) and a subpopulation of Italians (Bo et al. 2012). Three deletion variants have been identified in the $\alpha_{2a}$-adrenococeptor (Small et al. 2001; Salim et al. 2009; Nguyen et al. 2011). All of them map to a region within the third intracellular loop. An association between this variation and an increased risk of cardiac death and coronary problems was identified. However, genome-wide association studies (GWAS) did not confirm this link. Similarly, to $\alpha_{2b}$-adrenococeptor alteration, the human $\alpha_{2c}$-adrenococeptor also contains an in-frame deletion in the third intracellular loop. The impact of this alteration on intracellular signalling remains unclear. Presently, the evidence linking candidate gene studies and GWAS does not indicate an association between $\alpha_{2c}$-adrenococeptor variation and cardiovascular disease. Little information is currently available concerning the impact of genetic variation in these receptors and the efficacy of therapeutic drugs. One study reported increased noradrenaline levels and higher heart rates in response to the $\alpha_{2}$-adrenococeptor antagonist yohimbine in carriers of the $\alpha_{2c}$-adrenococeptor variant (Neumeister et al. 2005).

Current evidence suggests that two $\alpha_{2}$-adrenococeptor variants may have functional relevance: a variant of the $\alpha_{2A}$-adrenococeptor is associated with diabetes and a variant of the $\alpha_{2B}$-adrenococeptor is associated with impaired glucose tolerance/diabetes and cardiovascular disease (Ahles and Engelhardt 2014). To the author’s knowledge there is no information available describing genetic polymorphisms of the $\alpha_{2}$-adrenococeptors in dogs.

2.6 Drugs affecting the $\alpha_{2}$-adrenococeptors

2.6.1 Medetomidine hydrochloride

Medetomidine hydrochloride (IUPAC name: 4-[1-(2,3-dimethylphenyl)ethyl]-1H-imidazole, molecular weight 200) is an $\alpha_{2}$-adrenococeptor agonist (Savola et al. 1986). The molecular structure of this drug consists of a benzene ring with two methyl substituents, which is attached to a dihydro-imidazole ring via a carbon cross-linkage (Figure 2). This bridging atom is a chiral centre and medetomidine is a racemic mixture of two optical enantiomers, dexmedetomidine and levomedetomidine (Vickery et al. 1988; Savola and Virtanen 1991).

![Figure 2. Molecular structure of medetomidine: (RS)-4-[1-(2,3-dimethylphenyl)ethyl]-3H-imidazole. (Vainio et al. 1989).](image-url)
Dexmedetomidine is the pharmacologically active enantiomer (Vickery et al. 1988; MacDonald et al. 1991; Savola & Virtanen 1991; Ansh et al. 1998; Kuusela et al. 2000), whilst levomedetomidine is thought to be pharmacodynamically ineffective (Vickery et al. 1988). However, levomedetomidine may exert an antagonistic effect at higher doses in dogs (Kuusela et al. 2000), and conversely, sedative and analgesic effects are reported at high doses in rats (Savola and Virtanen 1991).

Medetomidine is highly lipophilic, with an apparent octanol-water partition coefficient of 2.8, which is said to result from the aromatic ring and associated substituted methyl groups (Savola et al. 1986). It is a weak base (due to the imidazole moiety) with a pKa value of 7.1, and at physiological pH the non-ionized form predominates (Savola et al. 1986). It is commercially available as the hydrochloride salt due to the enhanced aqueous solubility of this preparation.

Medetomidine exhibits an $\alpha_2:\alpha_1$ ratio of 1620:1 as measured by $^3$H-clonidine and $^3$H-prazosin displacement (Virtanen et al. 1988). On this basis, it is the most selective $\alpha_2$-adrenoceptor agonist drug in clinical use when compared to other members of the group, namely clonidine, xylazine and detomidine (Virtanen et al. 1988). In receptor binding and isolated organ preparations, medetomidine was shown to have little affinity for $\beta_1$, $\beta_2$, $H_1$, $H_2$, 5-HT $1_A$, 5-HT $2_A$, muscarinic, dopaminergic (D$_2$), tryptamine, GABA, mu- and delta-type opioid and benzodiazepine receptors (Virtanen et al. 1988). Dexmedetomidine is defined as a full agonist at the $\alpha_{2B}$-adrenoceptor and a partial agonist at the $\alpha_{2A}$ and $\alpha_{2C}$-adrenoceptors (Peltonen et al.1998).

The molecular structure of medetomidine also defines it as an imidazole derivative. Imidazoline receptors are classified as $I_1$ (central located mediating sympatho-inhibitory effects to lower blood pressure), $I_2$ (an allosteric binding site of monoamine oxidase) and $I_3$ (regulating insulin secretion from pancreatic $\beta$ cells) (Head and Mayorov 2006). There are limited data differentiating the $\alpha_2$-adrenoceptor agonist effects of medetomidine from the imidazoline effects outlined above. One study reports an $\alpha_2/I_1$ receptor specificity of 1:0.0002 for medetomidine in bovine tissue (Ernsberger et al. 1997). The role of $I_1$ receptors in medetomidine mediated blood pressure control would appear to be limited. Classically $\alpha_2$-adrenoceptor agonists inhibit insulin secretion rather than producing an insulinotropic effect and no data could be found concerning medetomidine induced pancreatic effects mediated via $I_3$ receptors. A recent paper describes an inhibitory effect of dexmedetomidine on platelet aggregation mediated via $I_1$ receptors (Kawamoto et al. 2015). There are also reports of dexmedetomidine mediating a protective effect within the CNS via $I_2$ receptors (Zhang et al. 2012). Notwithstanding these studies the main cardiovascular and haemodynamic effects of medetomidine seem to be via activity at $\alpha_2$-adrenoceptors.

The pharmacodynamic properties of medetomidine have been studied for over 30 years and Vainio et al. (1986) described the first use of medetomidine in dogs. Much of the pharmacodynamic and pharmacokinetic information relating to medetomidine was obtained from studies performed in this species.
2.6.1.1 Central pharmacodynamic effects

In rats, dexmedetomidine was found to demonstrate profound sedative properties via activity at centrally located $\alpha_2$-adrenoceptors (Doze et al. 1989a & 1989b; Scheinin and Schwinn 1992). Subsequently, Correa-Sales et al. (1992) showed that the sedative effect could be localized to the locus coeruleus of the brainstem and mediated via $\alpha_{2A}$ receptors at this site (Mizobe et al. 1996; Hunter et al. 1997). The dose-related sedative effects of medetomidine and dexmedetomidine have been demonstrated in dogs (Vainio et al. 1986; Vainio et al. 1989; Vahâ-Vähe 1989; Kuusela et al. 2000), cats (Vainio et al. 1986; Ansah et al. 1998) and humans (Scheinin et al. 1987).

Medetomidine and its active enantiomer, dexmedetomidine, have potent analgesic effects in rats (Vickery et al. 1988; Buerkle and Yaksh 1998), dogs (Vainio et al. 1989; Yliselä and Vainio 1989; Bloor et al. 1992; Sabbe et al. 1994; Tyner et al. 1997), cats (Ansah et al. 1998) and humans (Aantaa et al. 1997). This antinociceptive effect is predominantly mediated through activity within the dorsal horn of the spinal cord (North and Yoshimura 1984) via both presynaptic $\alpha_2$-adrenoceptors located on primary afferent nerve fibres where $\alpha_2$-adrenoceptor agonists reduce glutamatergic transmission (Feng et al. 2002; Pan et al. 2002) and postsynaptic mechanisms (North and Yoshimura 1984, Li and Zhuo 2001). However, $\alpha_2$-adrenoceptor agonists within the brain produce antinociception and modulate antinociception via supraspinal inhibitory descending pathways (Pertovaara et al. 1991; Guo et al. 1996a and b; Nuseir et al. 1999; Sawamura et al. 2000). Epidurally administered dexmedetomidine prevented primary and secondary hyperalgesia when administered to neo/postnatal rat pups and was not diminished by cord transection (Walker & Fitzgerald 2007). The MAC-sparing effects of medetomidine and dexmedetomidine have since been demonstrated in dogs (Vickery et al. 1988; Bloor et al. 1992; Ewing et al. 1993; Pascoe et al. 2006) and rats (Segal et al. 1988). Dexmedetomidine has been shown to have visceral analgesic effects in rats (Ulger et al. 2009).

Aside from the spinal and supraspinal sites of action, other studies have demonstrated that $\alpha_2$-adrenoceptor agonists have peripheral analgesic effects (Ansah and Pertovaara 2007), which are not mediated via $\alpha_2$-adrenergic receptors but by inhibition of the hyperpolarization-activated cation current (Brummett et al. 2011).

2.6.1.2 Other pharmacodynamic effects

In dogs, medetomidine causes an initial increase in systemic vascular resistance (SVR) via stimulation of $\alpha_2$-adrenoceptors on vascular smooth muscle, the response being blocked by the $\alpha_2$-adrenoceptor antagonist idazoxan but not by the $\alpha_1$-adrenoceptor antagonist prazosin (Savola et al. 1986). The vasoconstrictive effect is followed by bradycardia, which is believed to be a baroreceptor-mediated reflex (Bloor et al. 1992) leading to a decrease in the cardiac index (CI) (Flacke et al. 1993). Medetomidine and the active enantiomer dexmedetomidine have been reported to show little effect on myocardial contractility (Housmans 1990; Flacke et al. 1990; Flacke et al. 1992; Pypendop and Verstegen 1998). Medetomidine also inhibits insulin secretion and saliva production, increases growth hormone secretion, promotes diuresis and reduces catecholamine plasma concentrations in a similar manner to other $\alpha_2$-adrenoceptor agonist drugs (Scheinin et al. 1987).
2.6.1.3 Pharmacokinetics of medetomidine

Medetomidine is approximately 85% protein-bound in canine plasma (Salonen 1989). In studies assessing the pharmacokinetics of medetomidine in the dog, the steady state volume of distribution (V_{ss}) and apparent volume of distribution (V_{d}) have been reported as 1.3 L/kg and 2.8 L/kg, respectively (Salonen 1989; Kuusela et al. 2000). Medetomidine elimination half-life (T_{1/2}) was approximately 60 minutes following intravenous administration in vivo (Salonen 1989; Kuusela et al. 2000) and 90 minutes in vitro (Duhamel et al. 2010). Clearance ranged between 1.3 and 1.8 L/kg/hour (Salonen 1989; Kuusela et al. 2000).

Initial studies elucidating the metabolism of dexmedetomidine and medetomidine were performed in the rat (Salonen and Eloranta 1990; Salonen 1991). In the rat and dog, hepatic hydroxylation of the parent compound yields hydroxymedetomidine as the primary metabolite. This step is followed by glucuronidation; alternatively, the hydroxylated metabolite may be converted to the carboxylic acid derivative (Salonen and Eloranta 1990). The primary metabolite of medetomidine is inactive and represents 80–90% of total biotransformation (Salonen 1992). Similar amounts of the glucuronide and carboxylic acid are found in the urine of rats, and biotransformation appears to be similar in dogs (Salonen 1992). In man the cumulative urinary excretion profile of [3H] dexmedetomidine identified two N-glucuronide metabolites, which constituted 34% of all measured metabolites. Aliphatic hydroxylation (mediated mainly by CYP2A6) results in the production of 3-hydroxy dexmedetomidine followed by glucuronidation to 3-hydroxy dexmedetomidine glucuronide. Alternatively, further oxidation may occur with the production of 3-carboxylic acid dexmedetomidine representing 14% of the metabolites (FDA website 1998; EMEA 2011). N-methylation of dexmedetomidine followed by oxidation results in the production of 3-carboxy N-methyl dexmedetomidine. Alternatively, glucuronidation to N-methyl O-glucuronide dexmedetomidine represents 18% of the metabolites. The rate of biotransformation appears to be dependent upon hepatic blood flow (Salonen 1992). This proposal is supported by pharmacokinetic modelling in humans, in which the rate of metabolism of dexmedetomidine is determined by cardiac output and its impact on hepatic blood flow (Dutta et al. 2000). Interestingly, the two enantiomers of medetomidine exhibit different pharmacokinetic characteristics, with values of V_{dss} and clearance being consistently greater for levomedetomidine when compared with dexmedetomidine (Kuusela et al. 2000). In Kuusela’s study the two enantiomers of medetomidine were given individually, meaning that the pharmacodynamic effects of dexmedetomidine did not influence the disposition and elimination of levomedetomidine.

Subsequent in vitro studies using canine, human and rat hepatic microsomes identified species differences in the rates of glucuronidation of medetomidine and its enantiomers (Kaivosari et al. 2002). Glucuronidation of medetomidine by canine liver microsomes is enantioselective, with V_{max}/K_m values being 8-fold higher for levomedetomidine compared with dexmedetomidine (Kaivosari et al. 2002). Whilst canine microsomes were less efficient at conjugating medetomidine when compared with human microsomes, for example, the levomedetomidine V_{max}/K_m ratio was 117 in human microsomes versus 1.9 in canine microsomes. This finding may suggest that hepatic biotransformation would act
as a rate-limiting factor in the dog rather than cardiac output (CO) and hepatic blood flow, as previously suggested in humans (Dutta et al. 2000). To date, there have been no in vivo canine studies investigating this observation. Separately, Baratta et al. (2010) described the interaction of racemic medetomidine and dexmedetomidine with cytochrome P450 enzymes, using canine liver microsomes. Their data suggest that medetomidine acts as an inhibitor of CYP2B11 and may thereby alter the biotransformation of other co-administered anaesthetic and sedative drugs, such as midazolam and ketamine.

2.6.2 Acepromazine

Acepromazine (IUPAC name: (1-{10-[3-(dimethylamino)propyl]-10H-phenothiazin-2-yl} ethanone)) is a phenothiazine tranquiliser that has been used for many years in veterinary anaesthesia prior to the development of the \( \alpha_2 \)-adrenoceptor agonists (Hall et al. 2001). The mechanism underlying the sedative effects of acepromazine is not fully elucidated however phenothiazines are believed to block post-synaptic dopamine receptors within the CNS and may also inhibit the release of dopamine (Carlsson and Lindqvist 1963; Nybäck and Sedvall 1968; Horn and Snyder 1971). Unlike the \( \alpha_2 \)-adrenoceptor agonists, acepromazine has no demonstrable analgesic properties (Wegner et al. 2008). Acepromazine has no specific reversal agents and the treatment of inadvertent overdose can be problematic for this reason, relying on symptomatic therapy.

Acepromazine has several other pharmacodynamic side effects, chief amongst these being effects on the cardiovascular system. It causes a dose-related reduction in arterial blood pressure (Kerr et al. 1972; Parry et al. 1982), which may lead to hypotension when it is used alone (Monteiro et al. 2007) or combined with opioids prior to general anaesthesia (Stepien et al. 1995; Grint et al. 2010). This effect is probably mediated via alpha-adrenergic antagonist activity and a reduction in peripheral vascular resistance (Ludders et al. 1983). In normovolaemic patients, this is generally well tolerated. However, its use is avoided in hypovolaemic animals for this reason. Acepromazine has anti-arrhythmic properties and protects against adrenaline-induced fibrillation (Muir et al. 1975; Dyson and Pettifer 1997). This may be considered advantageous when used for premedication. In healthy horses, its use reduces the risk of anaesthetic mortality (Johnston et al. 1995). In some breeds of dog, caution is advised in dosing, since it may lead to syncope, which is believed to be a vasovagal response (Hall et al. 2001). It reportedly has anti-emetic (Valverde et al. 2004), antispasmodic (Strombeck et al. 1985; Hall et al. 1987; Scrivani et al. 1998) and hypothermic properties (Pugh 1964), as well as anti-histaminergic effects (Moriello and Eicker 1991). Haematological effects include a reduction in packed cell volume and a reduction in platelet aggregation. Reduction in haematocrit is believed to result from dilation of splenic blood vessels following \( \alpha_2 \)-adrenoceptor blockade (Lang et al. 1979; Ballard et al. 1982; Marroum et al. 1994; Leise et al. 2007).

Acepromazine may be used at low doses in animals with compensated cardiac disease resulting from volume overload. The rationale for its use relates to its ability to reduce afterload and thereby improve forward flow from the heart while reducing regurgitant flow (Clutton 2007). However, as indicated below, one disadvantage of acepromazine is its relatively long duration of action (Hashem et al. 1992). Conversely, alpha\(_2\) agonists are currently contra-indicated in these animals due to the increase in SVR and the potentially
detrimental impact on cardiac work. However, medetomidine has also been shown to significantly lower concentrations of adrenaline, noradrenaline and cortisol perioperatively when compared with acepromazine. In this respect, the use of medetomidine may be preferable to acepromazine (Väisänen et al. 2002). The combination of medetomidine and acepromazine has been studied in dogs in which the phenothiazine was used to counteract the peripheral vascular effects of medetomidine (Saponaro et al. 2013).

Data on the pharmacokinetics of acepromazine in dogs are sparse. One report describes the disposition of acepromazine following oral and intravenous administration: $C_{\text{max}}$ plasma concentrations lay between 140–170 ng/mL following an intravenous dose of acepromazine of 1.3–1.7 mg/kg with an approximate $V_{\text{dss}}$ of 20–22 L/kg and a $T_{1/2}$ between 6 and 9 hours. Following oral administration of the same dose, $C_{\text{max}}$ was between 11–15 ng/mL and the $T_{1/2}$ was 16 hours. Oral bioavailability was approximately 20% (Hashem et al. 1992). Acepromazine is thought to be a putative substrate of P-glycoprotein, although no in vitro studies have confirmed this suggestion (Mealey 2006). The median area under the sedation score curve was significantly higher for dogs homozygous for this ABCB1 mutation compared with homozygous normal collies (Deshpande et al. 2016).

2.6.3 Atipamezole (MPV-1248)

Atipamezole [4-(2-ethyl-2, 3-dihydro-1H-inden-2-yl)-1H-imidazole] “is a potent, selective, and specific $\alpha_2$-adrenoceptor antagonist of both centrally and peripherally located $\alpha_2$-adrenoceptors” (Virtanen et al. 1989). In receptor binding experiments, the $\alpha_2/\alpha_1$ selectivity ratio was 8526 compared to 27 for idazoxan, and 40 for yohimbine (Virtanen et al. 1989). Both of the latter drugs have been used as specific $\alpha_2$-adrenoceptor antagonists in pharmacological experiments. Atipamezole reverses the sedative, analgesic and some cardiovascular effects of medetomidine in dogs (Clarke and England 1989; Virtanen et al. 1989; Vainio 1990; Vainio and Vähämäki-Vähä 1990; Vähämäki-Vähä 1990). Presently, it is routinely used in clinical veterinary medicine to antagonise medetomidine-induced sedation and to thereby hasten recovery. It may also be used to ameliorate complications arising from medetomidine administration and inadvertent medetomidine overdose. Low incidences of drowsiness and somnolence were reported approximately half to one hour following atipamezole injection (Vainio and Vähä-Vähä 1990; Vähämäki-Vähä 1990). This phenomenon was also reported by Honkavaara et al. (2008).

In the dog, atipamezole alters the elimination pharmacokinetics of medetomidine. The area-under-the-curve (AUC) and $T_{1/2}$ are decreased, while the clearance is increased (Salonen et al. 1995). Similarly, to medetomidine, atipamezole undergoes hydroxylation within the liver and could compete with the agonist compound (Salonen 1995). However, the increased clearance of medetomidine in the presence of atipamezole does not support this concept. Instead, the differences in elimination are thought to result from the restoration of haemodynamic function following the administration of atipamezole. For example, atipamezole may restore hepatic blood flow and thereby increase biotransformation. Atipamezole may also inhibit the activity of CYP2B11 in vitro and thereby alter the biotransformation of drugs used during anaesthesia in dogs, although this has not been demonstrated in vivo (Baratta et al. 2010).
2.6.4 MK-467

MK-467 (also known as L-659,066) \((2R\text{-}trans\text{-}N\text{-}(2\text{-}1,3,4,7,12b\text{-}hexahydro\text{-}2\text{-}oxo-spiro(2H\text{-}benzofuro,\(2,3\text{-}a\) quinolizine-2,4\text{-}imidazolidin-3\text{-}yl\) ethyl\) methane-sulphonamide\) (Figure 3) is a peripherally acting \(\alpha_2\)-adrenoceptor antagonist. It has an \(\alpha_2\text{:}\alpha_1\) selectivity ratio of 105 (Clineschmidt et al. 1988), although there are no published data on the affinity of MK-467 for the different \(\alpha_2\)-adrenoceptor subtypes. It penetrates the BBB poorly due to its low octanol:phosphate buffer partition coefficient (1.3) and hence poor lipid solubility (Clineschmidt et al. 1988). Brain/plasma ratios of 0.04 and 0.06 have been reported for primates and rats, respectively (Clineschmidt et al. 1988).

![Figure 3. The molecular structure of MK-467 (Provided by Vetcare Oy, Espoo, Finland)](image)

2.6.4.1 Pharmacodynamic effects of MK-467

2.6.4.1.1 Sedation and antinociception

In dogs, MK-467 produced no clinically relevant alterations in composite sedation scores following dexmedetomidine or medetomidine administration (Honkavaara et al. 2008; Restitutti et al. 2011; Rolfe et al. 2012). These findings are similar to those reported in humans (Warren et al. 1991) and sheep (Raekallio et al. 2010). However, the duration of sedation was reduced (Restitutti et al. 2011), the explanations for which are discussed later (see Section 6.).

MK-467 was shown to inhibit dexmedetomidine-mediated analgesia in a model of neuropathic pain, but not in control animals (Poree et al. 1998). It remains unclear whether this represents up-regulation of \(\alpha_2\)-adrenoceptors by peripheral neurons or merely an alteration in the BSCB enabling access of the peripheral antagonist to the dorsal horn of the spinal cord. Using a visceral pain model in rats, MK-467 had no effect on dexmedetomidine-induced antinociception (Unger et al. 2009). There have been no studies investigating the impact of MK-467 on the somatic antinociceptive effects of medetomidine in dogs.

2.6.4.1.2 Cardiopulmonary effects

Intravenous administration of MK-467 in rats has been reported to increase the heart rate (HR) and reduce the mean arterial blood pressure (MAP), with the increase in HR being strongly correlated with plasma noradrenaline concentrations (Szemeredi et al. 1989). These effects result from the inhibition of pre-synaptic \(\alpha_2\)-adrenoceptors on sympathetic nerve terminals and blockade of \(\alpha_2\)-adrenoceptors on vascular smooth muscle (Szemeredi et al. 1989). In dogs, MK-467 increases HR, CO, mean pulmonary arterial pressure, oxygen delivery, and oxygen consumption, and decreases SVR (Enouri et al. 2008). When MK-467 is administered with or prior to medetomidine or dexmedetomidine,
it ameliorates the peripherally mediated cardiovascular effects of the $\alpha_2$-adrenoceptor agonists (Pagel et al. 1998; Enouri et al. 2008; Honkavaara et al. 2008; Honkavaara et al. 2011). Many studies investigating MK-467 have focused on its impact on the cardiopulmonary pharmacodynamic effects of the $\alpha_2$-adrenoceptor agonists medetomidine and dexmedetomidine. There have been no studies investigating the interaction of MK-467 with other commonly used injectable or inhalant anaesthetic drugs in dogs.

### 2.6.4.2 Other effects of MK-467

Initial studies on MK-467 assessed its ability to stimulate insulin secretion from the pancreas and thereby act as a treatment for diabetes mellitus. In humans, MK-467 alone had no effect on the blood glucose concentration; however, it inhibited the effect of clonidine and counteracted the reduction in insulin secretion, the latter effect being dose-related (Warren et al. 1991). In the same study, MK-467 counteracted the fall in the plasma noradrenaline concentration reported after clonidine administration. This is believed to represent a peripheral effect of MK-467, because no changes in sedation, growth hormone levels or salivary secretion occurred in these subjects. Similarly, Schafers et al. (1992) reported no consistent change in fasting blood glucose, insulin or plasma catecholamine concentrations during MK-467 infusion, although heart rate was significantly elevated by MK-467.

MK-467 infusion in healthy human volunteers during exercise increased plasma noradrenaline, insulin and non-esterified free fatty acids, with minimal changes in HR (Sciberras et al. 1994). The increase in plasma insulin had no impact on blood glucose. In dogs, MK-467 prevented the dexmedetomidine-induced increase in the plasma glucose concentration and decrease in insulin (Restitutti et al. 2012). MK-467 inhibits adrenaline-mediated platelet aggregation (Schafers et al. 1992), but less efficaciously than the $\alpha_2$-antagonist yohimbine. MK-467 administered to volume-loaded rats had an antidiuretic effect, leading to fluid retention during the duration of the study (Jackson et al. 1992). Further research has demonstrated that $\alpha_2$-adrenoceptor agonists act postsynaptically within the renal collecting tubules to prevent the binding of vasopressin, thereby leading to diuresis (Krothapalli and Suki 1984). The $\alpha_2$-adrenoceptor antagonist MK-467 prevents this effect and therefore produces antidiuresis. This finding is also supported by the lack of an antidiuretic effect in Brattleboro rats, which are naturally deficient in antidiuretic hormone (Jackson et al. 1992).

### 2.6.4.3 Pharmacokinetics of MK-467

Studies describing the pharmacokinetics of MK-467 are limited. However, Honkavaara et al. (2012) reported some canine pharmacokinetic data: $V_z$ was 0.41 ± 0.13 L/kg, clearance was 7.8 ± 3.4 mL/kg/min, the AUC$_{0-60}$ was 26,600 ± 9100 ng/min/mL and the elimination $T_{1/2}$ was approximately 40 min. However, plasma samples were collected for a maximum of 60 minutes following MK-467 administration; therefore, the pharmacokinetic data may not be a true reflection of the elimination half-life in this species. There are no published accounts of its metabolism and elimination.
Currently, no published information exists on the protein-binding characteristics of MK-467 in the dog, although Sciberras et al. (1994) assumed an MK-467 protein binding of 90% in humans but did not reference this statement. Additionally, nothing is known about the interaction of co-administered drugs on the unbound fraction of MK-467.

### 2.7 Current deficits in the knowledge of MK-467

To date, there have been no reports describing the protein-binding characteristics of MK-467 *in vitro* or *ex vivo* in dogs. In addition, it is anticipated that MK-467 would only be administered in combination with medetomidine or dexmedetomidine. Therefore, it is also relevant to determine the impact of these latter compounds on the unbound and active fraction of MK-467.

The clinical utility of MK-467 lies in its inability to penetrate the BBB, which is believed to result from the compound’s relatively low lipid solubility (Clineschmidt et al. 1988). The access of drugs to the CNS is related not only to their intrinsic lipid solubility, but also to other factors such as the action of efflux transporters present within the BBB, for example P-gp. Several dog breeds express mutations in the gene encoding this transporter. It seems important to assess whether MK-467 is a substrate for this efflux transporter; since this could markedly affect its clinical use in animals known to carry this genetic mutation. I am unaware of any studies reporting this information.

As previously described, MK-467 has little effect on the quality of sedation produced by dexmedetomidine in dogs (Honkavaara et al. 2008; Restitutti et al. 2011; Rolfe et al. 2012). No studies have documented the impact of MK-467 on antinociception. Nor have these pharmacodynamic effects been studied with respect to MK-467 and its impact on the pharmacokinetics of medetomidine.

Previous studies have focused on the impact of MK-467 on the cardiovascular and haemodynamic effects of medetomidine and dexmedetomidine in unanaesthetised dogs (Enouri et al. 2008, Honkavaara et al. 2008; Honkavaara et al. 2011; Rolfe et al. 2012, Salla et al. 2014b). Thus far, no studies have reported the impact of MK-467 on the cardiopulmonary effects of medetomidine prior to or during general anaesthesia in dogs. However, there is one report describing the impact of MK-467 on the cardiopulmonary effects of detomidine in horses during isoflurane anaesthesia (Pakkanen et al. 2015). No studies have described the impact of MK-467 on the disposition of co-administered drugs during general anaesthesia in dogs.

Given the potential benefits of MK-467, it seems important to investigate the current deficits in the literature. Until α₂-adrenoceptor agonist drugs have been developed that target specific α₂-adrenoceptor subtypes, it appears relevant and important to answer these questions and thereby to inform future clinical use.

Based on the current published data and information on MK-467, the following aims were defined for this doctoral study.
3. **AIMS OF THIS STUDY**

1. To determine the protein-binding characteristics of MK-467 in canine plasma and to assess the impact of medetomidine on the unbound fraction. (Study I)

2. To determine whether MK-467, dexmedetomidine and acepromazine are substrates for the efflux transport P-glycoprotein. (Study II)

3. To investigate the effect of MK-467 on the plasma concentrations and disposition of racemic medetomidine and alfaxalone in the dog. (Study III)

4. To evaluate the impact of MK-467 on the sedative and antinociceptive effects of racemic medetomidine in the dog. (Study IV)

5. To characterise the impact of MK-467 and medetomidine on the cardiopulmonary effects of certain injectable and inhalant general anaesthetic drugs in the dog. (Studies III & V)
4. MATERIALS AND METHODS

4.1 Study design

Study I determined the protein-binding characteristics of MK-467 in canine plasma, HSA and human AGP. The influence of medetomidine on the free fraction (\(f_\text{r}\)) of MK-467 was also determined. In study II, we investigated the role of active transport in the membrane transport of acepromazine, dexmedetomidine and MK-467. This study used an in vitro technique with cells transfected with the human MDR1 gene. Studies III, IV and V employed a prospective randomized crossover design. Study IV investigated the impact of MK-467 on the sedative and antinociceptive effects of medetomidine. We investigated the influence of MK-467 on the disposition of racemic medetomidine (studies III and IV) and alfaxalone (study III). Study III examined the effect of MK-467 using a total intravenous anaesthetic technique, whilst study V assessed the effect of MK-467 during inhalant general anaesthesia using isoflurane. In studies III & V, we also determined the influence of MK-467 on the cardiopulmonary effects of medetomidine before and during general anaesthesia.

4.2 In vitro studies

4.2.1 Protein-binding study (Study I)

The protein-binding characteristics of MK-467 in canine plasma, HSA and AGP were assessed using the technique of equilibrium dialysis (Waters et al. 2008) (study I). This technique is based on the passage of the study compound in plasma (or protein solution) across a dialysis membrane. Since only the unbound drug passes across the membrane, the percentage of free drug is calculated from the ratio of the peak area in phosphate buffered saline (PBS), (pH 7.4) phase to the peak area in the plasma phase multiplied by 100.

The influence of medetomidine on the protein binding of MK-467 was also determined in the presence of canine plasma. Stock solutions of MK-467 (Vetcare, Mäntsälä, Finland) were prepared in dimethyl sulfoxide (DMSO) at 10 µM, 100 µM and 1000 µM concentrations. A second set of stock solutions containing medetomidine (Vetcare, Mäntsälä, Finland) (molar ratio 20:1, MK-467:medetomidine) were also prepared. This ratio was derived from previously published plasma concentration data (Kuusela et al. 2000; Honkavaara et al. 2012; Kaartinen et al. 2014).

Study solutions were prepared by adding MK-467 stock solutions to blank canine plasma (mixed male beagle plasma in origin, Innovative research, Novi, MI, USA). In addition, the stock solutions were added to 45 mg/mL HSA in 50 mM phosphate buffer and to 1 mg/mL human AGP in 50 mM phosphate buffer (pH 7.4). (HSA and AGP, Sigma Aldrich, Helsinki, Finland). The final MK-467 concentrations were obtained in each matrix were 0.1 µM, 1 µM and 10 µM. This process was repeated for the MK-467/medetomidine solution. However, these latter solutions were incubated in canine plasma but not HSA or AGP. Each of the solutions were incubated for 4 hours in an equilibrium device (Thermofisher: https://www.thermofisher.com/order/catalog/product/90006) using 150 mM PBS as a receiver side solution for plasma samples and 50 mM phosphate buffer as receiver side solution for
HSA- and AGP-samples. Each study was performed in triplicate at each concentration using each of the three protein solutions.

After incubation, drug samples were collected from each side of the dialysis chamber. Matrices were made similar by adding 50 µL of plasma to the PBS-side samples and 50 µL of PBS to the plasma-side samples. For AGP- and HSA phases, 50 mM phosphate buffer was added to the samples. For AGP-experiments, phosphate buffer phases were diluted with 1 mg/mL AGP; for HSA-experiments phosphate buffer phases were diluted with 45 mg/mL HSA.

All samples were then protein precipitated and the supernatants removed. Drug concentrations were determined using the technique of liquid chromatography and triple-quadrupole mass spectrometry. The unbound fraction was calculated directly from the peak areas measured for each phase, as follows:

$$\text{Unbound fraction \% (f_u) = \frac{100 \times [LC/MS \text{ peak area in PBS phase}]}{LC/MS \text{ peak area in the plasma/HSA/AGP phase}}}$$

4.2.2 P-glycoprotein study (Study II)

In study II, three drugs were investigated as substrates of P-gp: acepromazine, dexmedetomidine and MK-467. Wild type-MDCKII cells and MDCKII cells transfected to express the human ABCC1 gene were used for these studies. The cells were obtained from The Netherlands Cancer Institute (Amsterdam, NL) at passage 20. The three drugs were tested at the following concentrations: acepromazine 100 ng/mL (Plegicil 10 mg/mL, Pharmaxim, Sweden AB, Helsingborg, Sweden), dexmedetomidine 50 ng/mL (Dexdomitor 0.5 mg/mL, Orion Corporation, Espoo, Finland) and MK-467 200 ng/mL and 1000 ng/mL (Merck & Co., Whitehouse Station, NJ, USA). These concentrations were derived from published pharmacokinetic data (Ballard et al. 1982; Hashem et al. 1992; Kuusela et al. 2000; Honkavaara et al. 2012). Cells were seeded onto polycarbonate membrane Transwell® inserts (pore size 3 µm, area 1.12 cm²) in 12-well plates. The cells were grown for a minimum of three to four days prior to each experiment. Experiments were performed in triplicate in both directions, (i.e. n = 3). Transepithelial electrical resistance (TEER) was measured (EVOM-2, WPI Inc., Sarasota, FL, USA) before and after each study period to assess the integrity of the cell layers.

The apparent permeability of the test compounds was measured in the apical-basolateral (AP-BL) and basolateral-apical (BL-AP) directions at 37 C. To assess AP-BL transport, the test solution was added to the apical side of the Transwell filters. Samples were collected from the basolateral chamber at 15, 30, 45, 60 and 90 minutes following drug application. These samples were stored at -20 ºC until they were analysed. At the end of the experiment, a sample was taken from the apical side of the filter for determination of BL-AP transport, the test solution was added to the basolateral chamber and drug samples were taken from the apical side of the filter at the same times points described above. At the end of the experiment, a sample was taken from the basolateral side of the filter. Drug concentrations
Materials and Methods

were measured using liquid chromatography and tandem mass spectrometry as described below (Section 4.4).

The apparent permeability rate of elimination (P\textsubscript{app}) was calculated using the equation (1):

\[
P_{\text{app}} = \frac{dQ/dt}{(A \times C_0 \times 60)}
\]  

(1)

where \(dQ/dt\) is the cumulatively permeated quantity of drug per unit time (ng/s), \(A\) is the filter surface area (1.13 cm\(^2\)) and \(C_0\) is the initial concentration of the test compound on the donor side (ng/mL). The net efflux of the test compound was assessed by calculating the ratio of \(P_{\text{app}}\) in the BL-to-AP direction compared with \(P_{\text{app}}\) in the AP-to-BL direction. An BL-AP:AP-BL ratio greater than 2:1 is believed to indicate net efflux and suggests that carrier-mediated transport may be involved (Brouwer et al. 2013).

Assessment of the cumulative permeability versus time plots showed linearity at the drug concentrations used here. For each drug, ratios were calculated from triplicate wells. However, data from one acepromazine AP-to-BL well were excluded because the filter was leaking. The measured drug concentrations from the test solutions prior to the start of each study were as follows: acepromazine 74.50 ng/mL; dexmedetomidine 50.00 ng/mL and MK-467 296.00 and 1080.00 ng/mL.

4.3 \textit{In vivo studies}

4.3.1 Animals and instrumentation

Eight purpose-bred laboratory beagle dogs, two females and six males, all neutered (weight 13.4 ± 2.1 kg, age approximately 4.5 years) participated in studies III, IV and V. All \textit{in vivo} studies were approved by the National Animal Experiment Board of Finland, license number: ESAVI-2010-07734/Ym-23.

All dogs received routine canine vaccinations and were dewormed during the time these studies were performed. They were housed together and given access to an outdoor area for exercise each day. They were fed a commercial diet. The animals underwent clinical examinations prior to each study and routine haematology and biochemistry profiles were performed prior to the commencement of each study. Feeding was withheld on the morning of each study day until the study was completed.

In study IV, a peripheral venous catheter was placed into a cephalic vein and a central catheter was placed into a jugular vein. No other invasive instrumentation was applied. In studies III and V, additional peripheral venous, arterial and central venous catheters were placed under general anaesthesia. To allow instrumentation animals were anaesthetised. Anaesthesia was induced with propofol (PropoVet 10 mg/mL, Abbott Laboratories Ltd, Berkshire, United Kingdom) administered to effect until intubation was possible. Anaesthesia was maintained with isoflurane (Isoflo, Orion Pharma Ltd, Turku, Finland) in oxygen. After instrumentation, animals recovered from anaesthesia for a minimum period of one hour, thereby allowing the residual effects of the anaesthetic drugs to diminish.
4.3.2  Drug doses

MK-467 was used in all studies. In study III, anaesthesia was induced with alfaxalone (Alfaxan, 10 mg/mL, Jurox (UK) Ltd., Malvern, Worcestershire, UK) at a loading dose of 2.4 mg/kg and maintained using a constant rate infusion (CRI) of 3.6 mg/kg/hour for 60 minutes. This treatment was compared with medetomidine and the combination of medetomidine and MK-467 administered for premedication ten minutes prior to the induction of anaesthesia. Medetomidine (Dorbene 1 mg/mL, Laboratories Syva s.a., León, Spain) was administered intravenously at a loading dose rate of 4.0 µg/kg and then as a CRI at 4.0 µg/kg/hour prior to anaesthesia maintained with alfaxalone (as above). MK-467 was administered at a loading dose of 150 µg/kg intravenously and a CRI of 120 µg/kg/hour in combination with medetomidine.

In study IV an intravenous medetomidine bolus dose of 10 µg/kg was investigated. This treatment was compared with the combination of medetomidine and MK-467 (MMK). MK-467 was administered intravenously at a dose of 250 µg/kg in combination with medetomidine.

In study V, acepromazine was used for premedication at a dose rate of 0.01 mg/kg in combination with butorphanol tartrate (Butordol 10 mg/mL, Intervet International B.V. Boxmeer, Netherlands), at a dose rate of 0.3 mg/kg. The acepromazine and butorphanol treatment served as a control and was compared with medetomidine (10 µg/kg) and medetomidine plus MK-467 (250 µg/kg). Anaesthesia was induced with propofol (PropoVet 10 mg/mL, Abbott Laboratories Ltd, Berkshire, United Kingdom) administered to effect and anaesthesia was maintained with isoflurane (Isoflo, Orion Pharma Ltd, Turku, Finland) in oxygen. All injectable drugs were administered intravenously. The end-tidal isoflurane concentration was maintained at 1.2% (i.e. approximately one times the MAC value of this drug in the dog (Steffey and Howland 1977)).

Following each in vivo study, the dogs received 0.2 mg/kg meloxicam (Metacam 5 mg/mL, Boehringer Ingelheim Vetmedica, Ingelheim/Rhein, Germany) injected subcutaneously to relieve any discomfort caused by tissue damage at the sites of catheter insertion.

4.3.3  Assessment of sedation and antinociception

In study IV, the sedative effect of medetomidine alone and in the presence of MK-467 was determined. Sedation was scored by a blinded researcher at 10, 20, 30, 45, 60 and 90 minutes following drug injection using a composite sedation score adapted from Restitutti et al. (2011) with a maximum score of 12 (see Appendix 1). The sedation score was recorded after application of the nociceptive stimulus. This sequence of testing was used to ensure a minimal effect on the electroencephalogram (EEG) prior to application of the nociceptive stimulus.

Electroencephalographic activity was recorded as a method of monitoring changes in cortical oscillatory activity (study IV). Recordings were taken immediately after drug administration (baseline measurements, i.e. prior to drug injection, were not taken due to movement artefacts) and then pre- and post-stimulation using a somatic nociceptive
stimulus. Electroencephalographic recordings were made using a portable wireless amplifier (Embla titanium, Broomfield, CO, USA) connected to a laptop computer. Five needle electrodes were placed in the skin overlying the cerebrum prior to drug administration at five positions. The reference electrode was inserted over the external occipital protuberance. Acceptable electrode placement was confirmed by an electrode impedance of less than 5 kΩ. In association with each EEG recording, a digital video recording was simultaneously made using a webcam (Labtec Inc., Logitech International S.A, Lausanne, Switzerland). Prior to analysis, the digital video recordings were used to identify movement artefacts in the EEG traces. If movement was evident, this section of the EEG trace was removed. EEG data were collected in two-second epochs over one minute prior to and after nociceptive stimulation. Fast Fourier transformation of the EEG data generated values in five frequency bands defined as: delta (δ) 0–3.99 Hz, theta (θ) 4.00–7.99 Hz, alpha (α) 8.00–11.99 Hz, sigma (σ) 12.00–13.99 and beta (β) 14.00–24.99 Hz. High- and low-pass filters were applied to raw EEG traces at 40 Hz and 0.3 Hz, respectively. Only the EEG data for the initial 30 minute after drug administration were used for analysis; at later times, the movement effects were too great to obtain reliable frequency information.

In study V, the level of sedation and depth of anaesthesia were measured by using the bispectral index (BIS) and electromyography (EMG). The BIS electrode was placed over frontal-temporal position as described by Campagnol et al. (2007). Additionally, the level of sedation (at baseline and at 5, 10 and 15 minutes after premedication) was evaluated by an experienced blinded observer using a modified sedation score where 0 represented no sedation and 16 very deep sedation (Kuusela et al. 2001). In addition, the induction dose of propofol necessary to allow endotracheal intubation was recorded. The quality of induction and ease of intubation were subjectively evaluated using a scale from 1 (smooth) to 4 (rough/failure to achieve intubation) (Maddern et al. 2010). Recovery quality was assessed using a scale of 1 (smooth) – 5 (extreme excitement), modified from Lozano et al. (2009). In addition, extubation, rising, sternal and walking times were recorded. The assessor was blinded to the treatments.

Antinociception was specifically assessed in study IV and responsiveness to a nociceptive stimulus was also assessed in study III. However, assessment of antinociception was not a primary aim of study III. In study IV, the analgesic effect of medetomidine was assessed by the same blinded researcher at 0, 10, 20, 30, 45, 60 and 90 minutes after drug injection. A standardized nociceptive pressure stimulus was applied to the nail bed of the fifth digit of the left hind limb. This stimulus consisted of a haemostat closed to the first ratchet with the intention of applying a constant pressure stimulus during the test period. The time for limb withdrawal (LWT) was measured in seconds from the time the clamp was applied to a maximum of 60 seconds. If there was no response, the withdrawal time was recorded as a total time of sixty seconds. Time delays for withdrawal of the limb and for the animal to lift its head (HLT) were recorded.

In study III, a nociceptive pressure tester (Selitto Randell Paw Pressure tester, IITC Life Science Inc., Woodland Hills, CA, USA) was used to assess the responsiveness of the animals during anaesthesia. An increasing pressure (in mmHg) was applied to the ventral
surface of the tail using the tester. It was not feasible to assess limb withdrawal due to the number of peripheral catheters used during each study day. Baseline measurements were taken before drug administration and assessments were repeated at 15, 30, 45, 60 and 70 minutes following premedication. Using baseline data, the responses were graded into one of three categories: 1 = no response, 2 = change in respiratory rate, heart rate or blood pressure (10-20% from baseline) and 3 = >20% change in these parameters or significant limb or body movement. The observers were not masked to treatments.

4.3.4 Cardiopulmonary monitoring

In studies III and V, the influence of MK-467 on the pharmacodynamic effects of medetomidine were assessed.

Heart rate was recorded from lead II of the electrocardiogram (ECG), which was also used to assess cardiac rhythm. When this was not in place, HR was measured by auscultation of the thorax. Systolic, diastolic, and mean arterial pressures, central venous pressure and the inspired fraction of oxygen were continuously recorded using a multichannel monitor (S/5 Anesthesia Monitor, GE Healthcare, Helsinki, Finland). The pressure transducers (Gabarith PMSET, Becton Dickinson, Sandy, Utah, USA) were zeroed to atmospheric pressure prior to each experiment. The dogs were positioned in lateral recumbency, and the manubrium of the sternum was used as zero reference. The gas analyser was calibrated before the experiments using the calibration gas supplied by the manufacturer (Quick Cal Calibration gas, GE Healthcare, Hatfield, UK). Cardiac output was measured with the lithium indicator dilution method (LidCO Plus Hemodynamic Monitor, LidCO Ltd., Cambridge, UK) as described by Mason et al. (2001). A standard dose 0.075 mmol/L of LiCl was used in each dog. Standard values of 10 g/L haemoglobin and 140 mmol/L of sodium were used for initial measurements and were later corrected with measured values obtained from simultaneously drawn arterial blood gas samples. Arterial and central venous blood gas samples were drawn anaerobically into pre-heparinised syringes (Pico50™, Radiometer, Copenhagen, Denmark) via the arterial and central venous catheters, stored on iced water for no longer than 15 minutes and analysed (ABL 855, Radiometer, Copenhagen, Denmark). Oxygen (\(\text{PaO}_2\)) and carbon dioxide (\(\text{PaCO}_2\)) partial pressures as well as arterial pH, lactate, and haemoglobin concentrations were recorded. Haemoglobin oxygen saturation was calculated as described by Reeves et al. (1982). Subsequently, the CI, systemic vascular resistance index (SVRI), oxygen delivery, oxygen consumption and oxygen extraction ratio were calculated by using standard equations (Haskins et al. 2005). The rectal temperature was measured with a digital thermometer. Before the induction of anaesthesia, the respiratory rate (\(f_r\)) was measured by counting the chest movements over one minute and subsequently from the respiratory gas monitor. In addition, in study V the end-tidal isoflurane concentration was measured using the multi-parameter monitor described above.

4.4 Drug analysis and pharmacokinetic analysis

The drugs analysed and the study in which these analyses were performed are presented in Table 2.
Materials and Methods

Table 2. Summary of the individual drugs analysed in studies I, II, III and IV.

<table>
<thead>
<tr>
<th>Study number</th>
<th>Drugs analysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>MK-467</td>
</tr>
<tr>
<td>II</td>
<td>Acepromazine, dexmedetomidine &amp; MK-467</td>
</tr>
<tr>
<td>III</td>
<td>Alfaxalone, dexmedetomidine, levomedetomidine &amp; MK-467</td>
</tr>
<tr>
<td>IV</td>
<td>Dexmedetomidine, levomedetomidine &amp; MK-467</td>
</tr>
</tbody>
</table>

In studies III, IV and V all drugs were administered via a peripheral venous catheter. In studies III and IV, blood samples were collected from a preplaced jugular catheter. Blood samples were centrifuged and the plasma harvested and stored at -20 °C until analysis.

The respective drug concentrations were determined using liquid chromatography and tandem mass spectrometry (LC-MS/MS). MK-467 concentrations (studies I, II, III and IV) were analysed using the technique previously described by Honkavaara et al. (2012). Dexmedetomidine (study II) analysis used the technique described by Snapir et al. (2006).

Concentrations of acepromazine (reference standard: acepromazine, Sigma-Aldrich) (study II) were determined with LC-MS/MS after diluting 20 μL of incubation buffer sample with 180 μL of a mixture of 5% acetonitrile in water containing 0.1% formic acid. Chlorpromazine hydrochloride (Sigma-Aldrich) was used as an internal standard in the analysis. Reversed-phase separation with a SunFire™ C18 column (2.1 x 150 mm, 3.5 μm, Waters Corporation) and a gradient solvent system (0.1% formic acid in water and acetonitrile) was used. Quantitative detection was performed in multi-reaction monitoring mode with a triple quadrupole mass spectrometer (4000 QTrap, MDS Sciex). For acepromazine and chlorpromazine, the respective precursor ions (m/z) scanned were 327.0 and 319.2. The fragment ions (m/z) monitored and used for quantitation were 58.0 and 86.4 respectively. The chromatograms were analyzed and processed using Applied Biosystems / MDS Sciex software ( Analyst® version 1.4.2). The linear ranges of the assays were from 0.02 to 20 ng/mL for acepromazine. The inter-assay accuracies of the accepted quality control samples (at three different concentration levels / analyte) ranged from 87% to 112%.

The concentration of alfaxalone (study III) in canine plasma samples was analyzed with the study drug product as the reference substance (Jurox (UK) Ltd). Sample preparation involved protein precipitation with acetonitrile, and prednisolone was used as the internal standard. After reversed-phase separation was (Gemini C18 column, 2.0 x 150 mm; 5 μm, Phenomenex). Quantitative detection was performed in multiple-reaction mode by use of a triple quadrupole mass spectrometer (API 4000, MDS Sciex). The scanned ion pairs (m/z) were 333.3 and 297.4 for alfaxalone and 361.2 and 343.4 for prednisolone. The linear concentration range for the determination of alfaxalone was from 100 ng/mL to 4,000 ng/mL. The chromatograms were analyzed and processed using Applied Biosystems / MDS Sciex software ( Analyst® v. 1.4.1) (Bennett et al. 2016c).
Materials and Methods

The concentrations of dexmedetomidine and levomedetomidine in canine plasma (studies III and IV) (reference standard: racemic medetomidine, Ferion 0y) were analysed with high performance LC-MS/MS using diphenylimidazole (Sigma-Aldrich) as internal standard. After chiral separation with a Chiralpak AGP column (4 x 100 mm, 5 μm, Chiral Technologies), quantitative detection was performed in multiple reaction monitoring mode with a triple quadrupole mass spectrometer (API 4000, MDS Sciex). For dexmedetomidine and levomedetomidine the precursor ions (m/z) were 201.1 and 221.1 for diphenylimidazole. The fragment ions (m/z) monitored and used for quantification were 95.1 for dexmedetomidine and levomedetomidine and 194.0 for diphenylimidazole. Reverse phase separation was performed and the chromatograms were analysed and processed using Applied Biosystems / MDS Sciex software (Analyst® version 1.4.2) (Bennett et al. 2016a).

Pharmacokinetic variables were calculated using Phoenix 64, WinNonLin software, version 6.3, (Pharsight, Princeton, Nj, USA). In study III, the area under the curve to the last sampling time (AUC$_\text{last}$) and T$_{1/2}$ of alfaxalone were calculated, alone and in the presence of medetomidine and MK-467. In addition, the AUC$_\text{last}$ was calculated for dexmedetomidine, levomedetomidine and MK-467. The following settings were applied: non-compartmental analysis (NCA), linear trapezoidal linear/log interpolation, best fit slope and uniform weighting. Pharmacokinetic data were examined, and only results where the elimination phase correlation coefficient of the regression line was greater than 0.90 are presented here and were used in further statistical analysis. The steady-state clearance of alfaxalone was calculated from the mean alfaxalone plasma concentrations between 30 and 60 minutes for each dog and each treatment. These times were selected because the alfaxalone plasma concentrations were considered to be stable during this time period. These ‘steady-state’ concentrations were then used to calculate clearances using the following formula: Clss = dose/steady state drug concentration. The steady state volume of distribution (Vss) was calculated using the formula: elimination half-life = ln2 (Vss/Clss).

In study IV, plasma dexmedetomidine, levomedetomidine and MK-467 concentration–time data were recorded using similar settings to those described in study III. The Vp, elimination T$_{1/2}$, clearance and area under the curve to infinity (AUC$_\text{inf}$) were then calculated for dexmedetomidine, levomedetomidine and MK-467.

4.6 Statistical analysis

Data are expressed as the mean ± standard deviation (SD) or median (minimum – maximum range) for parametric or non-parametric/ordinal data, as appropriate.

In studies I and II, due to the small number of replicates, statistical analyses were not feasible. In studies III and V, repeated measures analysis of covariance was used to assess the effect of treatment, time and dog effects in the models used. The change from the baseline measurement was calculated for all of the response variables, and the change was used as a response in the modelling. The differences between treatments were evaluated with repeated measures analysis of covariance models. The model included
a baseline covariate, the main effects of treatment, time point and period (the order of treatments) and two-way interactions of treatment*time point and period*time point as fixed effects. The main effect of subject and two-way interactions of subject*time point and subject*period were included as random effects.

The estimates of treatment effects were calculated both over time and for each time point from the fitted models. For the overall and time-specific treatment differences, 95% confidence intervals and p-values were calculated. Statistical analyses were performed by 4Pharma Ltd. using SAS® System for Windows, version 9.3 (SAS Institute Inc., Cary, NC, USA).

In studies III and IV, pharmacokinetic parameters were analysed using paired t-tests, the Mann-Whitney rank sum test or Friedman’s two-way analysis of variance, whilst nociceptive responses in study III were analysed using Friedman’s two-way analysis of variance.

In study IV, the effects of treatment, time and subject on measured outcomes (sedation, LWT, HLT and drug concentrations) were analysed using a linear mixed model of variance. ‘Treatment’ and ‘time’ were considered to be fixed effects and ‘subject’ was taken as a random effect. Two-way interactions of treatment*time and order of treatment*time were also assessed as fixed effects within the model. Pairwise comparisons were made using the package “multcomp” with adjustments made for multiple comparisons (Torsten et al. 2008). Model fit was assessed using quantile-quantile (Q-Q) plots.

Statistical analyses were performed using several statistical software packages including PASW version 18, (SPSS, Chicago, IL, USA), SPSS Statistics for Windows, versions 20 and 22 (IBM SPSS Inc., Chicago, IL, USA) and R software (R Core Team, 2014). The significance level was set at p < 0.05.
5. RESULTS

5.1 Protein binding characteristics of MK-467 (study I)

Data on the protein-binding characteristics of MK-467 are displayed in Table 3.

<table>
<thead>
<tr>
<th>MK-467 Drug concentration µM</th>
<th>f_u in canine plasma (%)</th>
<th>f_u in canine plasma + medetomidine (%)</th>
<th>f_u in HSA (%)</th>
<th>f_u in AGP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>27.6 (3.5)</td>
<td>27.5 (0.4)</td>
<td>50.1 (2.5)</td>
<td>56.3 (3.7)</td>
</tr>
<tr>
<td>1.0</td>
<td>26.6 (0.9)</td>
<td>26.6 (0.9)</td>
<td>49.4 (1.2)</td>
<td>54.6 (5.6)</td>
</tr>
<tr>
<td>10.0</td>
<td>42.4 (1.2)</td>
<td>41.0 (2.4)</td>
<td>56.7 (0.5)</td>
<td>65.3 (0.4)</td>
</tr>
</tbody>
</table>

Saturation of MK-467 protein binding was inferred by the increase in f_u as the concentration of MK-467 increased from 1.0 to 10 µM, in canine plasma, HSA and AGP. Using this dose ratio, medetomidine had no apparent effect on the protein binding of MK-467 in this in vitro study.

5.2 Transport of dexmedetomidine, acepromazine and MK-467 by P-glycoprotein (study II)

Transport ratios for acepromazine and dexmedetomidine are shown in Table 4.

<table>
<thead>
<tr>
<th></th>
<th>Acepromazine</th>
<th>Dexmedetomidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-MDCKII</td>
<td>1.17:1.0</td>
<td>0.98:1.0</td>
</tr>
<tr>
<td>BL-AP:AP-BL ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDR1-MDCKII</td>
<td>1.51:1.0</td>
<td>1.15:1.0</td>
</tr>
<tr>
<td>BL-AP:AP-BL ratio</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MK-467 showed no detectable passive movement in either cell line (i.e. movement in an AP-BL direction) at the two drug concentrations used. Therefore, no efflux ratios could be calculated. However, BL-AP MK-467 transport was measured in both cell lines.
5.3 Effect of MK-467 on plasma concentrations of co-administered drugs and pharmacokinetic parameters (studies III and IV)

Table 5 presents the mean plasma dexmedetomidine and levomedetomidine concentrations measured during CRI, with and without MK-467 (study III). For dexmedetomidine alone, AUC\text{last} was 184 (28) min.ng/mL and 87 (16) min.ng/mL when combined with MK-467. For levomedetomidine, AUC\text{last} was 103 (15) min.ng/mL, which was significantly greater than the value of 50 (11) min.ng/mL in the presence of MK-467. AUC\text{last} values for dexmedetomidine with and without MK-467 were significantly greater than those for levomedetomidine with and without MK-467 (by 80 and 76%, respectively).

Table 5. Mean plasma dexmedetomidine (Dex) and levomedetomidine (Levo) concentrations ± SD (ng/mL) following the administration of racemic medetomidine alone and with MK-467 (Dex+MK-467, Levo + MK-467). The medetomidine loading dose was 4 µg/kg and the constant rate infusion 4.0 µg/kg/hour. MK-467 was administered at a loading dose of 150 µg/kg and a constant rate infusion of 120 µg/kg/hour (from Bennett et al. 2016c).

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>100</th>
<th>110</th>
<th>130</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dex</td>
<td>2.58</td>
<td>2.27</td>
<td>2.49</td>
<td>2.61</td>
<td>1.74</td>
<td>0.67</td>
<td>0.49</td>
<td>0.42</td>
<td>0.37</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>(0.75)</td>
<td>(0.31)</td>
<td>(0.47)</td>
<td>(0.46)</td>
<td>(0.96)</td>
<td>(0.18)</td>
<td>(0.13)</td>
<td>(0.12)</td>
<td>(0.10)</td>
<td>(0.09)</td>
</tr>
<tr>
<td>Dex + MK-467</td>
<td>1.10</td>
<td>1.02</td>
<td>1.12</td>
<td>1.04</td>
<td>1.017</td>
<td>0.46</td>
<td>0.37</td>
<td>0.30</td>
<td>0.27</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>(0.16)</td>
<td>(0.20)</td>
<td>(0.19)</td>
<td>(0.37)</td>
<td>(0.31)</td>
<td>(0.13)</td>
<td>(0.07)</td>
<td>(0.10)</td>
<td>(0.08)</td>
<td>(0.05)</td>
</tr>
<tr>
<td>Levo</td>
<td>1.38</td>
<td>1.26</td>
<td>1.46</td>
<td>1.55</td>
<td>1.02</td>
<td>0.34</td>
<td>0.24</td>
<td>0.17</td>
<td>0.18</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>(0.48)</td>
<td>(0.15)</td>
<td>(0.23)</td>
<td>(0.26)</td>
<td>(0.64)</td>
<td>(0.09)</td>
<td>(0.07)</td>
<td>(0.08)</td>
<td>(0.05)</td>
<td>(0.04)</td>
</tr>
<tr>
<td>Levo + MK-467</td>
<td>0.59</td>
<td>0.57</td>
<td>0.64</td>
<td>0.61</td>
<td>0.61</td>
<td>0.26</td>
<td>0.20</td>
<td>0.16</td>
<td>0.16</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>(0.10)</td>
<td>(0.14)</td>
<td>(0.13)</td>
<td>(0.22)</td>
<td>(0.21)</td>
<td>(0.08)</td>
<td>(0.03)</td>
<td>(0.04)</td>
<td>(0.04)</td>
<td>(0.03)</td>
</tr>
</tbody>
</table>

Figure 4 presents the plasma concentrations of dexmedetomidine and levomedetomidine measured in study IV. Administration of MK-467 approximately halved the plasma concentrations of dexmedetomidine and levomedetomidine when compared with the concentrations following medetomidine administration alone. Co-administration of MK-467 was also associated with statistically significant effects on the disposition of both enantiomers of medetomidine: $V_z$ and clearance were increased, and $T_{1/2}$ and AUC were reduced when racemic medetomidine was co-administered with MK-467, as shown in Table 6. Dexmedetomidine plasma concentrations were consistently greater than the levomedetomidine concentrations at each time point.
Figure 4. Mean plasma drug concentrations ± SD (ng/mL) of dexmedetomidine (Dex), levomedetomidine (Levo) when racemic medetomidine was administered alone and in combination with MK-467. The doses of medetomidine were 10 μg/kg and MK-467 250 μg/kg respectively (Study IV) (Bennett et al. 2016a).

Table 6. Mean values ± SD of apparent volume of distribution ($V_z$), clearance (Cl), area under the curve to infinity ($\text{AUC}_{\text{inf}}$) and elimination half-life ($T_{1/2}$) for racemic medetomidine alone and in the presence of MK-467. * Statistically significant difference from enantiomer alone; † statistically significant difference from dexmedetomidine, p < 0.05 (Study IV) (Bennett et al. 2016a).

<table>
<thead>
<tr>
<th>Drug</th>
<th>$V_z$ (L/kg)</th>
<th>Cl (mL/min/kg)</th>
<th>$\text{AUC}_{\text{inf}}$ (min.ng/mL)</th>
<th>$T_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dex</td>
<td>1.5 ± 0.2</td>
<td>18.6 ± 4.4</td>
<td>291 ± 107</td>
<td>61.3 ± 18.6</td>
</tr>
<tr>
<td>Dex + MK-467</td>
<td>2.2 ± 0.3†</td>
<td>44.5 ± 14.4*</td>
<td>122 ± 36*</td>
<td>36.9 ± 10.6*</td>
</tr>
<tr>
<td>Levo</td>
<td>2.8 ± 0.3†</td>
<td>28.2 ± 6.1†</td>
<td>187 ± 54†</td>
<td>71.3 ± 14.6†</td>
</tr>
<tr>
<td>Levo + MK-467</td>
<td>3.8 ± 0.5†</td>
<td>60.4 ± 18.6*</td>
<td>90 ± 26*</td>
<td>46.7 ± 13.4*</td>
</tr>
</tbody>
</table>

Figure 5 presents the mean alfaxalone plasma concentrations when alfaxalone was administered alone or in combination with medetomidine or medetomidine and MK-467. The addition of MK-467 nullified the increase in alfaxalone plasma concentrations associated with medetomidine administration. Plasma concentrations decreased prior to the end of the infusion at 70 minutes. Medetomidine administration led to a significant
increase in the AUC\textsubscript{last} of alfaxalone (220,000 (162,000 – 270,000) versus 450,000 (350,000 – 601,000) min.ng/mL) and a significant decrease in clearance (19.7 (14.3–27.0) versus 8.8 (8.0–10.2) mL/kg/min) when compared with alfaxalone administration alone (Table 7).

![Graph](image)

**Figure 5.** Plasma alfaxalone concentrations (geometric mean and 95% confidence intervals) in 8 Beagles during anaesthesia with alfaxalone alone (loading dose, 2.4 mg/kg; CRI, 3.6 mg/kg/h); medetomidine (loading dose, 4.0 μg/kg; CRI, 4.0 μg/kg/h), followed by alfaxalone (as described); or medetomidine (as described) and MK-467 (loading dose, 150 μg/kg; CRI, 120 μg/kg/h), followed by alfaxalone (as described) (from Bennett et al. 2016c).

**Table 7.** Geometric mean and range (minimum and maximum) values of AUC\textsubscript{last}, Cl\textsubscript{ss}, Vd\textsubscript{ss}, and T\textsubscript{1/2} of alfaxalone for 3 treatments (i.e. alfaxalone alone, medetomidine-alfaxalone combination, and medetomidine-alfaxalone combination plus MK-467) in 8 healthy adult Beagles. *Significant difference between alfaxalone and medetomidine-alfaxalone treatments (p < 0.05). †Significant difference between medetomidine-alfaxalone and medetomidine-alfaxalone and MK-467 treatments (p < 0.05). Where n = 7 for alfaxalone alone, n = 4 for medetomidine-alfaxalone, and n = 7 for medetomidine-alfaxalone and MK-467, (Bennett et al. 2016c).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AUC\textsubscript{last} (ng\cdot min/mL)</th>
<th>Cl\textsubscript{ss} (mL/kg/min)</th>
<th>Vd\textsubscript{ss} (L/kg)</th>
<th>T\textsubscript{1/2} (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfaxalone</td>
<td>220,000 (162,000–270,000)*</td>
<td>19.7 (14.3–27.0)*</td>
<td>1.1 (0.6–1.9)</td>
<td>39.3 (20.1–57.1)</td>
</tr>
<tr>
<td>Medetomidine-alfaxalone</td>
<td>450,000 (350,000–601,000)†</td>
<td>8.8 (8.0–10.2)†</td>
<td>0.70 (0.4–1.1)</td>
<td>54.4 (30.2–82.9)</td>
</tr>
<tr>
<td>Medetomidine-alfaxalone and MK-467</td>
<td>240,000 (191,000–270,000)</td>
<td>18.4 (16.3–23.1)</td>
<td>0.93 (0.8–1.2)</td>
<td>35.6 (26.8–39.4)</td>
</tr>
</tbody>
</table>
MK-467 plasma concentrations as measured in studies III and IV are presented in Table 8 and Figure 6 respectively. In study III, the geometric mean value of \( \text{AUC}_{\text{last}} \) for MK-467 was 36,000 (20,400 – 50,000) min.ng/mL. In study IV, the \( V_z \) of MK-467 was 1.0 (± 0.9) L/kg, clearance was 10.6 (± 6.9) mL/min/kg, \( \text{AUC}_{\text{inf}} \) was 31,600 (± 15,100) min.ng/mL and \( T_{1/2} \) was 65.6 (± 20.1) minutes.

**Table 8.** Mean MK-467 plasma concentration ± SD (ng/mL) following a loading dose of 150 \( \mu \)g/kg and a CRI of 120 \( \mu \)g/kg/hour (Bennett et al. 2016c).

<table>
<thead>
<tr>
<th>Study III</th>
<th>Time (minutes)</th>
<th>MK-467 Plasma concentration (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>MK-467</td>
<td>436.9</td>
<td>414.6</td>
</tr>
<tr>
<td>Concentration</td>
<td>(119.3)</td>
<td>(114.7)</td>
</tr>
</tbody>
</table>

**Figure 6.** Mean MK-467 plasma concentration ± SD (ng/mL) following a bolus dose of 250 \( \mu \)g/kg IV (Bennett et al. 2016a).

### 5.4 Sedation and antinociception (studies III, IV and V)

In study III, the response to a nociceptive stimulus was assessed and no differences were detected between treatments at earlier time points. Between 45 and 70 minutes, animals receiving medetomidine-alfaxalone showed significantly less responsiveness to the nociceptive stimulus compared with alfaxalone alone. At 45 minutes, the response score following the medetomidine-alfaxalone MK-467 combination was significantly lower than alfaxalone alone.
Results

Table 9. Median nociceptive response scores and minimum and maximum range for 3 treatments (i.e., alfaxalone alone, medetomidine-alfaxalone combination, and medetomidine-alfaxonole plus MK-467) in 8 healthy adult Beagles at baseline and 15, 30, 45, 60, and 70 minutes after premedication.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>70 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alfaxalone</td>
<td>3 (2-3)</td>
<td>1</td>
<td>2</td>
<td>2*†</td>
<td>2*</td>
<td>2*</td>
</tr>
<tr>
<td>Medetomidine-alfaxalone</td>
<td>3 (2-3)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Medetomidine-alfaxalone plus MK-467</td>
<td>3 (2-3)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Significant difference between alfaxalone alone and medetomidine-alfaxalone combination. †Significant difference between alfaxalone alone and medetomidine-alfaxalone plus MK-467, p < 0.05 (From Bennett et al. 2016c).

In study IV sedation scores after medetomidine were significantly greater than those after MMK-467 at 45, 60 and 90 minutes following drug administration. Thus, the duration of sedation was shorter after MMK than after medetomidine.

Table 10. Change from baseline (as per cent of maximal possible effect, MPE = Maximal possible effect) for limb withdrawal time (LWT), head lift time (HLT) and sedation score. Medians and (minimum-maximum range). Data were obtained from eight beagles. # Statistically significant difference from baseline (p<0.05). *Statistically significant difference from MED (p < 0.05), (From Bennett et al. 2016a).

<table>
<thead>
<tr>
<th>T10</th>
<th>T20</th>
<th>T30</th>
<th>T45</th>
<th>T60</th>
<th>T90</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MED</td>
<td>100*</td>
<td>80.7*</td>
<td>33.0*</td>
<td>0.42</td>
<td>0.42</td>
</tr>
<tr>
<td>(0.8 – 100)</td>
<td>(0.8 – 100)</td>
<td>(0 – 100)</td>
<td>(-1.75 – 25.9)</td>
<td>(-3.51 – 8.62)</td>
<td>(-3.51 – 8.62)</td>
</tr>
<tr>
<td>MMK</td>
<td>0*</td>
<td>0*</td>
<td>0*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(0 – 88.1)</td>
<td>(0 – 11.9)</td>
<td>(0 – 15.3)</td>
<td>(0 – 1.7)</td>
<td>(-1.4 – 15.3)</td>
<td>(-1.4 – 0.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MED</td>
<td>100*</td>
<td>100*</td>
<td>58.6*</td>
<td>5.3</td>
<td>9.2</td>
</tr>
<tr>
<td>(12.6 – 100)</td>
<td>(3.4 – 100)</td>
<td>(2.5 – 100)</td>
<td>(-23.4 – 32.8)</td>
<td>(-25.5 – 18.6)</td>
<td>(-25.5 – 14.3)</td>
</tr>
<tr>
<td>MMK</td>
<td>11.8</td>
<td>8.5*</td>
<td>9.3</td>
<td>5.9</td>
<td>0</td>
</tr>
<tr>
<td>(5.01 – 100)</td>
<td>(1.7 – 43.9)</td>
<td>(1.7 – 52.6)</td>
<td>(0.8 – 36.8)</td>
<td>(0 – 13.6)</td>
<td>(0 – 3.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MED</td>
<td>58.3*</td>
<td>62.5*</td>
<td>58.3*</td>
<td>50.0*</td>
<td>33.3*</td>
</tr>
<tr>
<td>(50.0 – 91.7)</td>
<td>(50.0 – 75.0)</td>
<td>(50.0 – 66.7)</td>
<td>(41.7 – 50.0)</td>
<td>(12.5 – 50.0)</td>
<td>(6.3 – 33.3)</td>
</tr>
<tr>
<td>MMK</td>
<td>54.2*</td>
<td>50.0*</td>
<td>45.8*</td>
<td>16.7*</td>
<td>8.3*</td>
</tr>
<tr>
<td>(36.4 – 75.0)</td>
<td>(18.2 – 75.0)</td>
<td>(33.3 – 66.7)</td>
<td>(8.3 – 50.0)</td>
<td>(-9.1 – 33.3)</td>
<td>(-9.1 – 16.7)</td>
</tr>
</tbody>
</table>

Limb withdrawal times (study IV) were significantly longer after medetomidine at T10, T20 and T30 than after MMK. After medetomidine there were significant differences between the baseline and T10, T20 and T30. After MMK, there were no significant differences
between the baseline and later time points. Head lift times were significantly longer after medetomidine at T20 compared with MMK. There were no other significant differences in HLT between the treatments. After medetomidine there were significant differences between the baseline and T10, 20 and 30. After MMK, there were no significant differences between the scores at baseline and at later time points.

Composite sedation scores in study V are shown in Table 11. These values did not differ significantly from each other.

**Table 11.** Composite sedation scores at baseline (T0) and 5, 10 and 15 minutes following premedication (study V). Premedication consisted of medetomidine, medetomidine and MK-467 and acepromazine and butorphanol. Data are shown as median (minimum – maximum range) (Salla et al. 2014a).

<table>
<thead>
<tr>
<th>Composite sedation score</th>
<th>T0</th>
<th>T5</th>
<th>T10</th>
<th>T15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medetomidine</td>
<td>0 (0 – 2)</td>
<td>9 (5 – 10)</td>
<td>9 (7 – 13)</td>
<td>9 (7 – 14)</td>
</tr>
<tr>
<td>Medetomidine/MK-467</td>
<td>0 (0 – 3)</td>
<td>8 (7 – 11)</td>
<td>8 (7 – 13)</td>
<td>10 (6 – 11)</td>
</tr>
<tr>
<td>Acepromazine/Butorphanol</td>
<td>0 (0 – 3)</td>
<td>8 (3 – 10)</td>
<td>8 (4 – 11)</td>
<td>10 (5 – 13)</td>
</tr>
</tbody>
</table>

Bispectral index values were significantly lower with medetomidine when compared with acepromazine/butorphanol and medetomidine/MK-467. There were no apparent differences in the depth or plane of subsequent anaesthesia. Muscular activity as measured by EMG was significantly lower following medetomidine when compared with medetomidine/MK-467 and acepromazine/butorphanol (data not shown).

### 5.5 Cardiopulmonary effects (studies III and V)

Cardiopulmonary data obtained during studies III and V are shown in Tables 12 and 13 respectively. The presence of MK-467 attenuated the vasoconstrictive and hypertensive effects of medetomidine and diminished the bradycardia and reduction in the CI associated with the administration of medetomidine.

In study III, the MAP was greatest with medetomidine-alfaxalone treatment and lowest with the medetomidine-alfaxalone MK-467 combination, although with the latter combination MAP was always greater than 70mmHg. In study V, MAP was again significantly higher with medetomidine and lowest with acepromazine/butorphanol premedication during isoflurane anaesthesia.
**Table 12.** Mean ± SD of heart rate, MAP, CI, SVRI, \( f_r \), \( \text{PaO}_2 \), and \( \text{PaCO}_2 \) in dogs (n=8) under alfaxalone anesthesia (ALF). Premedication consisted of medetomidine (MA) or medetomidine and MK-467 (MA and MK) or none (ALF), (Bennett et al. 2016c).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Baseline</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>70 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>ALF</td>
<td>79 ± 16</td>
<td>129 ± 26*†‡</td>
<td>104 ± 27*†‡</td>
<td>95 ± 12*†‡</td>
<td>101 ± 30*†‡</td>
<td>84 ± 14*†‡</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>79 ± 20</td>
<td>77 ± 17§</td>
<td>61 ± 10*</td>
<td>55 ± 15*§</td>
<td>52 ± 10*§</td>
<td>48 ± 10*§</td>
</tr>
<tr>
<td></td>
<td>MA-MK</td>
<td>82 ± 16</td>
<td>105 ± 17*</td>
<td>74 ± 12</td>
<td>71 ± 10</td>
<td>68 ± 12*</td>
<td>70 ± 9</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>ALF</td>
<td>105 ± 14</td>
<td>104 ± 16†‡</td>
<td>103 ± 12†‡</td>
<td>110 ± 15†‡</td>
<td>112 ± 13†‡</td>
<td>106 ± 12†‡</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>111 ± 14</td>
<td>130 ± 12§</td>
<td>109 ± 13§</td>
<td>106 ± 12§</td>
<td>106 ± 12§</td>
<td>105 ± 11§</td>
</tr>
<tr>
<td></td>
<td>MA-MK</td>
<td>104 ± 14</td>
<td>92 ± 12*</td>
<td>78 ± 9*</td>
<td>78 ± 8*</td>
<td>79 ± 8*</td>
<td>79 ± 8*</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>ALF</td>
<td>3.71 ± 1.09</td>
<td>4.91 ± 1.07†</td>
<td>4.05 ± 0.66*†</td>
<td>3.99 ± 0.78*</td>
<td>3.89 ± 0.99*†</td>
<td>4.06 ± 1.72*†</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>3.99 ± 1.04</td>
<td>2.31 ± 0.73§</td>
<td>1.84 ± 0.18*§</td>
<td>1.80 ± 0.34*§</td>
<td>1.85 ± 0.33*§</td>
<td>2.13 ± 0.34*§</td>
</tr>
<tr>
<td></td>
<td>MA-MK</td>
<td>3.78 ± 0.71</td>
<td>4.45 ± 1.03*</td>
<td>3.40 ± 0.72*</td>
<td>3.41 ± 0.74*</td>
<td>3.19 ± 0.66*</td>
<td>3.19 ± 0.76*</td>
</tr>
<tr>
<td>SVRI (dynes/cm²/m²)</td>
<td>ALF</td>
<td>2340 ± 669</td>
<td>1820 ± 530†</td>
<td>2070 ± 291†</td>
<td>2270 ± 594†</td>
<td>2450 ± 749†</td>
<td>2390 ± 988†</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>2320 ± 701</td>
<td>4530 ± 1240*§</td>
<td>4530 ± 870*§</td>
<td>4620 ± 1115*§</td>
<td>4500 ± 1278*§</td>
<td>3860 ± 979*§</td>
</tr>
<tr>
<td></td>
<td>MA-MK</td>
<td>2220 ± 413</td>
<td>1740 ± 670</td>
<td>1900 ± 575</td>
<td>1880 ± 558</td>
<td>2040 ± 625</td>
<td>2080 ± 628</td>
</tr>
<tr>
<td>( f_r ) (breaths/min)</td>
<td>ALF</td>
<td>18 ± 6</td>
<td>8 ± 5*†</td>
<td>15 ± 6†</td>
<td>16 ± 7†</td>
<td>18 ± 11†‡</td>
<td>18 ± 11†‡</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>19 ± 7</td>
<td>3 ± 3*</td>
<td>7 ± 2*</td>
<td>7 ± 3*</td>
<td>7 ± 3*</td>
<td>7 ± 4*</td>
</tr>
<tr>
<td></td>
<td>MA-MK</td>
<td>18 ± 5</td>
<td>4 ± 2*</td>
<td>10 ± 5*</td>
<td>10 ± 5*</td>
<td>11 ± 4*</td>
<td>11 ± 5*</td>
</tr>
<tr>
<td>( \text{PaO}_2 ) (mmHg)</td>
<td>ALF</td>
<td>100 ± 5</td>
<td>455 ± 117*†‡</td>
<td>558 ± 36*</td>
<td>542 ± 55*</td>
<td>535 ± 76*</td>
<td>542 ± 79*</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>102 ± 3</td>
<td>220 ± 106*§</td>
<td>537 ± 56*</td>
<td>540 ± 54*</td>
<td>554 ± 43*</td>
<td>545 ± 56*</td>
</tr>
<tr>
<td></td>
<td>MA-MK</td>
<td>101 ± 8</td>
<td>362 ± 124*</td>
<td>547 ± 24*</td>
<td>550 ± 28*</td>
<td>537 ± 57*</td>
<td>568 ± 23*</td>
</tr>
<tr>
<td>( \text{PaCO}_2 ) (mmHg)</td>
<td>ALF</td>
<td>34 ± 2</td>
<td>43 ± 5*†‡</td>
<td>37 ± 2†‡</td>
<td>36 ± 4†</td>
<td>36 ± 3†</td>
<td>35 ± 3†</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>34 ± 1</td>
<td>56 ± 5*</td>
<td>47 ± 6§</td>
<td>46 ± 7§</td>
<td>45 ± 3§</td>
<td>48 ± 5§</td>
</tr>
<tr>
<td></td>
<td>MA-MK</td>
<td>33 ± 3</td>
<td>52 ± 5*</td>
<td>40 ± 3*</td>
<td>39 ± 3*</td>
<td>39 ± 3*</td>
<td>39 ± 3*</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>ALF</td>
<td>7.40 ± 0.02</td>
<td>7.33 ± 0.04*†‡</td>
<td>7.37 ± 0.01*†</td>
<td>7.37 ± 0.04*†</td>
<td>7.36 ± 0.03*†</td>
<td>7.37 ± 0.03*†‡</td>
</tr>
<tr>
<td></td>
<td>MA</td>
<td>7.39 ± 0.01</td>
<td>7.23 ± 0.03*</td>
<td>7.27 ± 0.04*§</td>
<td>7.28 ± 0.05*§</td>
<td>7.28 ± 0.03*§</td>
<td>7.27 ± 0.04*§</td>
</tr>
<tr>
<td></td>
<td>MA-MK</td>
<td>7.40 ± 0.02</td>
<td>7.25 ± 0.03*</td>
<td>7.35 ± 0.02*</td>
<td>7.33 ± 0.02*</td>
<td>7.33 ± 0.02*</td>
<td>7.33 ± 0.02*</td>
</tr>
</tbody>
</table>

* statistically significant difference from baseline. † statistically significant difference between ALF and MA; ‡ statistically significant difference between MA and MA and MK (p < 0.05), § statistically significant difference between ALF and MA and MK (p < 0.05).
Table 13. Mean ± SD of heart rate, MAP, CI, SVRI, \( f_r \), \( PaO_2 \) and \( PaCO_2 \) in dogs (n=8) ten minutes after premedication (T10) and under isoflurane anaesthesia (T40-80). Dogs were premedicated with medetomidine (MED), medetomidine and MK-467 (MMK) and acepromazine and butorphanol (AB) (Salla et al. 2014a).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Baseline</th>
<th>10</th>
<th>40</th>
<th>60</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>MED</td>
<td>97 ± 12</td>
<td>37 ± 7</td>
<td>82 ± 12</td>
<td>94 ± 8</td>
<td>97 ± 9</td>
</tr>
<tr>
<td></td>
<td>MMK</td>
<td>100 ± 19</td>
<td>81 ± 14*†</td>
<td>116 ± 16*†</td>
<td>111 ± 17*†</td>
<td>110 ± 11*†</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>93 ± 19</td>
<td>94 ± 26</td>
<td>91 ± 15</td>
<td>91 ± 14</td>
<td>95 ± 13</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>MED</td>
<td>117 ± 16</td>
<td>133 ± 13‡</td>
<td>94 ± 18‡</td>
<td>82 ± 18‡</td>
<td>77 ± 17‡</td>
</tr>
<tr>
<td></td>
<td>MMK</td>
<td>112 ± 22</td>
<td>91 ± 9*†</td>
<td>66 ± 14*†</td>
<td>65 ± 15*†</td>
<td>64 ± 12*†</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>115 ± 22</td>
<td>81 ± 11</td>
<td>53 ± 13</td>
<td>52 ± 9</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>CI (L/min/m²)</td>
<td>MED</td>
<td>4.31 ± 0.87</td>
<td>1.26 ± 0.37‡</td>
<td>2.54 ± 0.59</td>
<td>2.89 ± 0.68</td>
<td>3.20 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>MMK</td>
<td>5.56 ± 1.70</td>
<td>4.26 ± 2.31*†</td>
<td>4.19 ± 0.85*†</td>
<td>3.88 ± 0.53</td>
<td>3.61 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>4.12 ± 0.41</td>
<td>4.36 ± 1.17</td>
<td>3.03 ± 0.68</td>
<td>3.17 ± 0.74</td>
<td>3.35 ± 0.47</td>
</tr>
<tr>
<td>SVRI (dyne sec/cm²/m²)</td>
<td>MED</td>
<td>2173 ± 485</td>
<td>8428 ± 3083‡</td>
<td>2912 ± 988‡</td>
<td>2279 ± 888‡</td>
<td>1965 ± 775</td>
</tr>
<tr>
<td></td>
<td>MMK</td>
<td>1645 ± 495</td>
<td>1975 ± 853*</td>
<td>1255 ± 386*</td>
<td>1292 ± 302</td>
<td>1472 ± 684</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>2154 ± 410</td>
<td>1600 ± 569</td>
<td>1310 ± 306</td>
<td>1238 ± 225</td>
<td>1122 ± 207</td>
</tr>
<tr>
<td>( f_r ) (breaths/min)</td>
<td>MED</td>
<td>18 ± 5</td>
<td>12 ± 5</td>
<td>7 ± 3</td>
<td>9 ± 3</td>
<td>11 ± 3</td>
</tr>
<tr>
<td></td>
<td>MMK</td>
<td>18 ± 6</td>
<td>10 ± 3</td>
<td>7 ± 3</td>
<td>11 ± 6</td>
<td>13 ± 9†</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>18 ± 5</td>
<td>12 ± 6</td>
<td>8 ± 5</td>
<td>8 ± 3</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>( PaO_2 ) (mmHg)</td>
<td>MED</td>
<td>93 ± 9</td>
<td>87 ± 12</td>
<td>555 ± 17</td>
<td>557±23</td>
<td>558±24</td>
</tr>
<tr>
<td></td>
<td>MMK</td>
<td>94 ± 6</td>
<td>85 ± 7</td>
<td>553 ± 28</td>
<td>568 ± 20</td>
<td>571 ± 20†</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>95 ± 3</td>
<td>89 ± 5</td>
<td>549 ± 21</td>
<td>556 ± 14</td>
<td>548 ± 15</td>
</tr>
<tr>
<td>( PaCO_2 ) (mmHg)</td>
<td>MED</td>
<td>35 ± 3</td>
<td>32 ± 4‡</td>
<td>45 ± 3‡</td>
<td>44 ± 3‡</td>
<td>43 ± 4‡</td>
</tr>
<tr>
<td></td>
<td>MMK</td>
<td>36 ± 1</td>
<td>39 ± 3*</td>
<td>44 ± 5†</td>
<td>43 ± 5*†</td>
<td>41 ± 5*†</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>36 ± 2</td>
<td>38 ± 2</td>
<td>48 ± 3</td>
<td>48 ± 2</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Arterial pH</td>
<td>MED</td>
<td>7.40 ± 0.02</td>
<td>7.40 ± 0.03‡</td>
<td>7.31 ± 0.02‡</td>
<td>7.32 ± 0.02‡</td>
<td>7.33 ± 0.02‡</td>
</tr>
<tr>
<td></td>
<td>MMK</td>
<td>7.39 ± 0.02</td>
<td>7.37 ± 0.03*</td>
<td>7.34 ± 0.03*†</td>
<td>7.35 ± 0.03*†</td>
<td>7.36 ± 0.04*†</td>
</tr>
<tr>
<td></td>
<td>AB</td>
<td>7.38 ± 0.03</td>
<td>7.36 ± 0.02</td>
<td>7.29 ± 0.03</td>
<td>7.29 ± 0.02</td>
<td>7.29 ± 0.03</td>
</tr>
</tbody>
</table>

*statistically significant difference between MMK and MED (p<0.05), † between MMK and AB (p<0.05) and ‡ between MED and AB (p<0.05).
Results

In study III, all treatments were associated with an initial reduction in \( f_r \). Hypercapnia and respiratory acidosis occurred with medetomidine-alfaxalone, whilst MK-467 diminished the respiratory depressant effects of the sedative/anaesthetic combination. Initial mean \( \text{PaO}_2 \) values were significantly lower with medetomidine-alfaxalone. In study V there were no differences in \( f_r \) or \( \text{PaO}_2 \) between the three treatments. However, during anaesthesia, mild hypercapnia occurred with all treatments. This was most marked following acepromazine/butorphanol premedication, whilst MK-467 appeared to counteract the respiratory depressant effect of medetomidine and isoflurane.

5.6 Induction and recovery quality (study V)

In study V, the induction dose of propofol was 1.5 ± 0.3 mg/kg, 2.1 ± 0.5 and 2.3 ± 0.9 mg/kg after medetomidine, medetomidine and MK-467 and acepromazine/butorphanol, respectively, and these values were significantly different from each other. Induction of anaesthesia was generally smooth and uneventful after all treatments. Although, one individual following medetomidine and two animals with acepromazine/butorphanol showed mild paddling, twitching or excitement after induction of anaesthesia. Recovery quality was also smooth with all treatments but one animal with medetomidine and acepromazine/butorphanol and three animals with medetomidine/MK-467 showed mild trembling or paddling. The times to extubation, righting, sternal recumbency and walking were all significantly shorter with medetomidine/MK-467 combination than medetomidine or acepromazine/butorphanol, see Table 14.

Table 14. Mean (± SD) time in minutes for extubation, righting, sternal recumbency and walking after isoflurane anaesthesia in dogs premedicated with medetomidine (MED), medetomidine/MK-467 (MMK) and acepromazine/butorphanol (AB).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Extubation</th>
<th>Righting</th>
<th>Sternal</th>
<th>Walking</th>
</tr>
</thead>
<tbody>
<tr>
<td>MED</td>
<td>10 ± 3</td>
<td>20 ± 15</td>
<td>22 ± 15</td>
<td>33 ± 16</td>
</tr>
<tr>
<td>MMK</td>
<td>7 ± 2*†</td>
<td>9 ± 2*†</td>
<td>10 ± 4*†</td>
<td>15 ± 5*†</td>
</tr>
<tr>
<td>AB</td>
<td>18 ± 10‡</td>
<td>22 ± 9</td>
<td>25 ± 9</td>
<td>39 ± 11</td>
</tr>
</tbody>
</table>

* statistically significant difference between MMK and MED (p < 0.05), † between MMK and AB (p < 0.05) and ‡ between MED and AB (p < 0.05), (Salla et al. 2014a.).
6. DISCUSSION

6.1 Protein-binding characteristics of MK-467

The three concentrations of MK-467 used in this study, 0.1, 1.0 and 10 µM, are equivalent to drug concentrations of 45.5, 455, and 4550 ng/mL (study I). These concentrations are comparable to those measured in vivo: more specifically, 1100 to 4280 ng/mL reported by Honkavaara el al. (2012) after intravenous injection and 100 to 800 ng/mL (approximately) during CRI (Kaartinen et al. 2014). The data of this study (I) showed an increase in the free or unbound drug fraction (f_u) between 1.0 and 10 µM MK-467, suggesting that saturation of the protein-binding sites occurred. No measurements were made at intermediate drug concentrations, and it thus remains unclear at which value unbound drug fraction starts to increase. Theoretically, protein-binding saturation may also occur in vivo with the possibility of an increased pharmacodynamic effect. In section 2.2, it was stated that this phenomenon rarely arises clinically however, anaesthetic drugs may constitute an exception to this general rule. This assertion is based on the algorithm described by Rolan (1984). Specifically, where a drug is greater than 90% protein bound, has a narrow therapeutic index, a high extraction ratio and an intravenous route of administration then there remains a possibility of clinically significant drug-drug interactions. One such example is reported by Hiraoka et al. (2004); with respect to the intravenous anaesthetic agent propofol. Whether similar criteria are relevant to the administration of MK-467 remains unclear. Currently, there are no published data describing the mechanism of biotransformation or elimination of MK-467 in any species (i.e. the hepatic extraction ratio is unknown). However, the volume of distribution of MK-467 has been reported in the dog, and dexmedetomidine had no impact on the disposition of MK-467 or its clearance when the two drugs were administered together (Honkavaara et al. 2012). Similar data describing the impact of medetomidine (rather than dexmedetomidine) on the disposition of MK-467 were unavailable at the time of study I. Given that differences in the metabolism of dex- and levomedetomidine exist (Kaivosaari et al. 2002), it cannot be assumed that the racemate behaves in the same manner as the active enantiomer. Therefore, medetomidine may alter the clearance of MK-467. An ex vivo study with co-administration of medetomidine and MK-467 would help to elucidate the relationship between the latter drug’s pharmacodynamic effects whilst its distribution, metabolism and elimination were occurring.

Between 0.1 and 1 µM, MK-467 was approximately 72–74% protein bound, suggesting that alterations in plasma protein concentration would have relatively little impact on the pharmacodynamic effects of MK-467 (study I). The free or unbound drug is recognized as the pharmacologically active proportion of the drug within the blood (Zeitlinger et al. 2004). At a steady state, the impact of changes in protein binding are limited. As indicated above, the intravenous administration of drugs as boluses, as is the case for injectable anaesthetic agents, may be an exception to this rule. In this scenario, the f_u value may change when other drugs are co-administered, leading to alterations in the pharmacodynamic effects of the original drug or possibly signs of toxicity. Here, we investigated the effect of medetomidine on the binding characteristics of MK-467. We selected a medetomidine:MK-467 dose ratio of 1:20, which is equivalent to 0.005–0.5 µM medetomidine or plasma concentrations between approximately 1 and 100 ng/mL. Kuusela
et al. (2000), reported plasma drug concentrations up to 70 ng/mL (approximately) when a medetomidine dose of 40 µg/kg was administered intravenously to dogs. The plasma concentration reported by Kuusela et al. (2000) was less than the highest concentration used in this in vitro study. In our study, medetomidine had no detectable effect on MK-467 protein binding. It appears improbable that medetomidine would have a more dramatic effect in vivo, because intravenous bolus doses above 40 µg/kg are rarely used clinically. Conversely, medetomidine itself is reportedly 85% protein bound (Salonen, 1992), although later reports indicate that this value may be higher (EMEA 2002) and it would be interesting to determine whether MK-467 influences the protein-binding characteristics of medetomidine.

This study was performed using canine plasma, HSA and human AGP. MK-467 is approximately 51% protein bound by HSA and 45% bound by AGP. Since it was impossible to obtain canine AGP, we report the binding characteristics of whole canine plasma here. MK-467 is believed to act as both an acid and a base (personal communication from the manufacturer), with several differing pKa values, suggesting that it might be capable of binding to both proteins; this is supported by our data using human proteins. It might be anticipated that both canine albumin and AGP are likewise capable of binding this compound, although this cannot be stated definitively. Given that protein binding was relatively low, it again seems unlikely that changes in the concentration of either protein would lead to significant alterations in the pharmacodynamic effects of this drug in vivo.

The limitations of this study are discussed in section 6.6. In summary, saturation of protein binding may occur at MK-467 concentrations previously reported in vivo (Honkavaara et al. 2012; Kaartinen et al. 2014), and medetomidine had no detectable effect on the f_u value of MK-467 at clinically relevant concentrations. MK-467 is moderately protein-bound and there are no data describing the clearance mechanism of MK-467. Theoretically changes in the unbound or free drug fraction of MK-467 may be minimal in nature, However, further ex vivo studies may be required, to determine how alterations in plasma protein concentrations may alter the pharmacodynamic effects of MK-467.

6.2 MK-467, dexmedetomidine and acepromazine as P-glycoprotein substrates.

In study II, we investigated the role of active transport in the transcellular movement of MK-467, dexmedetomidine and acepromazine using cells transfected with human P-glycoprotein. The potential clinical benefits of MK-467 rely on its limited ability to penetrate the BBB (Clineschmidt et al. 1988). Should MK-467 be identified as a substrate for P-gp, this might preclude its use in dogs known to be homozygous negative for this genetic mutation. Passive movement (i.e. AP-BL movement) of MK-467 was undetectable at the drug concentrations used in study II; however, transport of MK-467 occurred in the BL-AP direction in both cell lines This finding suggests that MK-467 may be a substrate for a carrier-mediated mechanism; however, our data do not allow us to establish the identity of this transporter. The MDCKII cells express additional endogenous transporters. Conrad et al. (2001) reported the expression of MRP 1 and 2 in canine kidney, while Goh et al.
(2002) reported the presence of these proteins in MDCKII cells. Therefore, MK-467 may serve as a substrate for one of these transporters. However, there have been no reports of genetic mutations in MRP1 and 2 transporters leading to enhanced efficacy or toxicity of therapeutic agents in dogs. Our results are also confounded by the fact that WT-MDCKII cells express P-gp (Goh et al. 2002), and since we observed transport in both cells lines, we cannot rule out the possibility that P-gp played some role here. Further studies are required to answer this question more conclusively. Presently, it remains unclear whether MK-467 can be used safely in dogs carrying a mutation in the MDR1 gene. Further studies are required to answer this question. The author suggests that MK-467 be used with caution in dogs known to have this condition until more information is available.

We chose to study the transport of acepromazine (study II) because homozygous mdr 1(-/-) dogs may exhibit increased sensitivity to this drug (Mealey 2006). An BL-AP:AP-BL transport ratio of 2:1 or greater is believed to represent active transport (Brouwer et al. 2013). Formerly, this ratio was 1.5:1.0 (Karlsson et al. 1993), and our results are therefore equivocal. A recently published paper reported the inhibition of endogenous P-gp in cells transfected with the human MDR1 gene (Li et al. 2013), and this may have played some role in our study. It remains unclear whether acepromazine may serve as a P-gp substrate. Deshpande et al. (2016) compared the effects of acepromazine in known homozygous negative collies with heterozygotes and homozygous unaffected individuals. Comparison of AUC values for sedation scores, indicated that homozygous affected dogs had significantly higher values than other groups; although individual sedation scores at each time point did not significantly differ from each other. More research is required before the mechanism underlying this effect is fully explained. Nonetheless, given the relatively long elimination half-life of acepromazine (Hashem et al. 1992) and lack of specific antagonists, it seems advisable to use it judiciously in animals that are homozygous for this genetic abnormality.

We detected no evidence that \( \alpha_2 \)-adrenoceptor agonists, such dexmedetomidine, act as P-gp substrates (study II). This is supported by the work of Salonen (1989) indicating that medetomidine is a highly lipid soluble drug, freely permeable across the BBB.

### 6.3 Drug disposition

With the exception, of study V, all other studies included analyses of drug concentrations and in the case of in vivo data, pharmacokinetic analyses. Arguably the most striking finding of the in vivo studies (studies III and IV) was the influence of MK-467 on the plasma concentration of co-administered drugs (i.e. racemic medetomidine and alfaxalone). In study III, higher AUC\(_{\text{last}}\) values were observed for alfaxalone during medetomidine/alfaxalone infusion compared with alfaxalone CRI alone. Medetomidine administration reduced alfaxalone clearance (study III), which was probably mediated by either a reduction in hepatic perfusion or a reduction in the rate of metabolism, or possibly both. To the author’s knowledge the hepatic extraction ratio of alfaxalone has not be reported. Alfaxalone reportedly undergoes biotransformation in the liver (Sear and McGiven 1981). For drugs with a high hepatic extraction ratio, a reduction in hepatic blood flow would likely influence drug clearance. Although, changes in hepatic perfusion were not measured
in study III; previously, Lawrence et al. (1996) reported non-significant alterations in hepatic perfusion following dexmedetomidine administration, although the anaesthetic techniques used differed between that study and study III. Restitutti et al. (2013) used the technique of contract-enhanced ultrasonography to measure hepatic organ perfusion and found no significant difference between control and dexmedetomidine treatments. However, this may also relate to some discrepancy between the peak drug effect and the timing of the measurements. Notwithstanding these considerations, the reduction in alfaxalone clearance (study III) is probably secondary to medetomidine-induced reductions in the CI, as proposed by Dutta et al. (2000), rather than a direct effect on hepatic vasculature. Administration of atipamezole (a peripherally and centrally acting \( \alpha_2 \)-adrenoceptor antagonist) increased medetomidine clearance, presumably by inhibiting vasoconstriction, preventing the subsequent reduction in CI and thereby maintaining hepatic perfusion (Salonen et al. 1995). Similarly, to atipamezole, MK-467 counteracted the effect of medetomidine on alfaxalone clearance (study III), probably via the same mechanism as that proposed for atipamezole. In vitro, medetomidine has been shown to inhibit CYP2B11-mediated metabolism of co-administered drugs (Baratta et al. 2010), and this interaction may also occur in vivo. Although, medetomidine may alter the metabolism of alfaxalone; it seems improbable that MK-467 would counteract this effect. This suggests that alfaxalone clearance is probably more dependent on changes in CI than alterations in hepatic enzyme activity per se. However, further work is required to answer this question more completely.

In studies III and IV, AUC_{last} and AUC_{inf} values of dex- and levomedetomidine were significantly reduced by the administration of MK-467. In study IV MK-467 approximately doubled the \( V_z \) and clearance of the two enantiomers. Again, this is probably secondary to the impact of MK-467 on the cardiovascular effects of medetomidine (Pagel et al. 1998; Enouri et al. 2008; Honkavaara et al. 2011; Rolfe et al. 2012). In an earlier paper, Bührer et al. (1994) demonstrated that dexmedetomidine reduced the dose requirement of thiopental due to a reduction in the distribution volume and distribution clearance of the barbiturate, and not via a centrally mediated sedative or antinociceptive effect per se. Many papers have previously reported dose-sparing effects of co-administered drugs in the presence of medetomidine (Bufalari et al. 1997; Kojima et al. 2002; Sano et al. 2003; Ko et al. 2006). It remains unclear whether these reductions are due to a pharmacodynamic action of the \( \alpha_2 \)-adrenoceptor agonist, an alteration in drug disposition due to the \( \alpha_2 \)-adrenoceptor agonist or a combination of both processes. Further studies are necessary to clarify the answer to this question.

In both study III and IV, AUC_{last} and AUC_{inf} values were significantly higher for dexmedetomidine compared with levomedetomidine, which is arguably an unexpected finding, not previously reported in vivo. It may be due to differences in hepatic drug biotransformation since the in vitro work of Kaivosaari et al. (2002) indicated that glucuronidation of medetomidine is enantioselective. Values of \( V_{max}/K_{max} \) being eight times higher for levo- compared with dexmedetomidine. A comparative clinical study of racemic medetomidine and dexmedetomidine reported a shorter duration of action following administration of dexmedetomidine in cats (Bruniges et al. 2015). Likewise, an equine clinical study recorded a greater dosing requirement when dexmedetomidine was
compared with medetomidine (Sacks et al. 2015). This alteration in the dose requirement may result from lower dexmedetomidine plasma drug concentrations rather than a lack of drug efficacy. The preferential hepatic metabolism of levomedetomidine is thought to contribute to the higher dexmedetomidine plasma concentrations when the racemic compound is used. The authors are not aware of any in vivo studies that definitively confirm this hypothesis.

MK-467 disposition was previously described by Honkavaara et al. (2012). They report mean values for AUC_{0-60} of 26,600, volume of distribution of 0.41 L/kg, clearance of 7.8 ml/kg/min and T_{1/2a} of 39 minutes. However, Honkavaara et al. (2012) focused on the initial phase of drug distribution with samples taken at 1, 3, 5, and 10 minutes following intravenous injection, whilst the final sample was drawn at 60 minutes. Our data are not directly comparable to those of Honkavaara et al. (2012), since the first plasma sample was not taken until 10 minutes following drug injection and our sampling time was longer than reported in the earlier study. Our data probably reflect more accurately the true T_{1/2} (65.6 ± 20.1 minutes), whilst the data reported by Honkavaara et al. (2012) might be considered as estimates of the T_{1/2}.

In study V, no plasma samples were taken for drug analysis. This was in part due to the limitations imposed by the withdrawal of arterial and venous samples for blood gas analysis and blood removed during the measurement of CO using the lithium dilution technique. Notwithstanding these factors, it would have been interesting to determine drug disposition for a number of reasons: there is little published information on the pharmacokinetics of acepromazine and butorphanol in dogs; the impact of MK-467 administration on dexmedetomidine and levomedetomidine plasma concentrations during inhalant anaesthesia has not been reported; also the influence of the different premedicant drugs on the plasma concentrations of propofol has not been reported previously. As described in study III, medetomidine administration had a significant effect on the alfaxalone plasma concentration. Similarly, in study V, the dose of propofol was significantly lower with medetomidine when compared with acepromazine/butorphanol and medetomidine/MK-467. This influence of medetomidine on the induction dose of propofol has previously been reported by a number, of authors (Bufalari et al. 1997; Kojima et al. 2002; Sano et al. 2003; Ko et al. 2006). However, in none of these studies were propofol plasma concentrations reported, and therefore the true explanation for the effect of medetomidine remains elusive.

6.4 Sedation, antinociception during general anaesthesia.

According to the composite sedation scores and the EEG frequency data, MK-467 did not alter the quality of sedation produced by (dex)-medetomidine (study IV), as reported previously (Honkavaara et al. 2008; Restitutti et al. 2011; Rolfe et al. 2012); however, its use was found to reduce the duration of effect of this drug. As composite sedation scores are somewhat subjective and the scoring system has not been validated, the use of EEG frequency data provided a more objective assessment of cortical oscillatory activity. The EEG frequency data were analysed within five frequency bands, but no attempt was
made to determine the change in spectral edge frequency (SEF) over time or between treatments (study IV). Previously, Itamoto et al. (2002) found no change in SEF using a medetomidine dose of 20 μg/kg given intramuscularly and only identified an increase in the lower frequency band using a medetomidine dose of 80 μg/kg intramuscularly. It seems improbable that further EEG analysis would have been sufficiently sensitive to detect changes related to MK-467 within the brain tissue (study IV), although this cannot be stated conclusively. Since no differences were identified between treatments in study IV, it may be inferred that MK-467 did not penetrate the BBB of these animals. However, there have been no studies demonstrating or reporting the brain:plasma ratio of MK-467 in dogs to date. An alternative technique for assessing the penetration of the BBB is the measurement of drug concentrations in cerebrospinal fluid (CSF). However, Mealey et al. (2008) questioned this approach in a canine P-gp knockout model, because the expression of the efflux transporter P-gp within the blood–CSF barrier did not mirror that in the BBB model; whereas Lin (2008) emphasized that the CSF drug concentration may be an accurate predictor of the unbound drug fraction within the brain. No published studies have reported MK-467 concentrations in CSF in dogs.

Bispectral index has also been used as a tool to assess sedation and the level of consciousness in dogs given MK-467, and thereby to infer the penetration of the BBB (study V) (Restitutti et al. 2011). The lower BIS values associated with medetomidine administration in study V may reflect higher dexmedetomidine plasma drug concentrations in the absence of MK-467. As previously mentioned, we were unable to collect plasma samples for drug analysis during study V, so the impact of MK-467 on medetomidine plasma concentrations and pharmacokinetics was not determined. Notwithstanding the potential influence of MK-467 on BIS, (based on the findings in study V), it is assumed that MK-467 has a limited ability to penetrate the BBB in dogs. In a previous study by Van Soens et al. (2009), medetomidine induced a greater reduction in BIS measurements when compared with the combination of acepromazine and methadone. This may reflect the different mechanism or site of action of these drugs. Acepromazine is considered to act at dopaminergic receptors, unlike the action of α₂-adrenoceptor agonists (Savola et al. 1986). Muscular activity and EMG values may also interfere with BIS. In study V, these differed between treatments: medetomidine less than medetomidine/MK-467 less than acepromazine/butorphanol. Therefore, it remains unclear whether higher BIS values were due to a lower level of sedation or greater degree of muscular activity. Despite the differences in BIS between treatments, clinically there were no significant differences in the sedation scores in study V. Previously, Restitutti et al. (2011) also reported lower BIS values when dexmedetomidine was compared with the combination of dexmedetomidine and MK-467. Again, the differences in BIS values did not represent clinical differences in sedation scores. Although, the higher BIS values reported by Restitutti et al. (2011) with MK-467 may also indicate differences in plasma dexmedetomidine drug concentrations, as reported by Honkavaara et al. (2012).

The reduction in the duration of sedation (study IV) is believed to result from the effect of MK-467 on dexmedetomidine disposition, thereby increasing the clearance of the active enantiomer and ultimately shortening its duration of action. This finding has been described in the preceding section. It again highlights the relationship between the
cardiovascular pharmacodynamic effects of the $\alpha_2$-adrenoceptor agonist and their effect on drug disposition.

The antinociceptive effect of medetomidine (as demonstrated by the increase in LWT) was evident following the intravenous administration of a 10 µg/kg dose, whilst MK-467 eliminated the antinociceptive effect of the $\alpha_2$-adrenoceptor agonist, which was an unexpected finding (study IV). Alpha $\alpha_2$-adrenoceptor agonists act at supraspinal (Guo et al. 1996a), spinal (Sabbe et al. 1994) and peripheral locations (Brummett et al. 2011) to mediate analgesia; however, the main site of action is the dorsal horn of the spinal cord (North and Yoshimura 1984; Li and Zhuo 2001; Feng et al. 2002; Pan et al. 2002). Rather like the brain, which is surrounded by the relatively impenetrable BBB, the spinal cord is similarly surrounded by the BSCB. The structure of the BBB was outlined in section 2.1.1, and the nature of this barrier is believed to impede the passage of MK-467 to supraspinal binding sites. It was assumed that MK-467 would also have a limited ability to traverse the tissue layer surrounding the spinal cord. The integrity of the BSCB may be altered during trauma or infection (Bartanusz et al. 2011), and in a neuropathic pain model (sciatic nerve ligation), MK-467 inhibited the antinociceptive effect of dexmedetomidine in neuropathic animals but not in control animals (Poree et al. 1998). Poree et al. (1998) suggested that spinal nerve ligation may cause local disruption within the BSCB at that location, thereby allowing MK-467 to gain access to the dorsal horn. In this case, it would imply that MK-467 cannot penetrate the BSCB in healthy animals. Alternatively, there may be an increase in the numbers of $\alpha_{2A}$ and $\alpha_{2C}$-adrenoceptors within the dorsal root ganglion at the site of ligation, allowing MK-467 access to receptors mediating the response to nociceptive stimuli (Birder and Perl 1999). Currently, it remains somewhat unclear whether MK-467 is able to cross the BSCB ordinarily and thereby inhibit the antinociceptive effect of (dex) medetomidine at that site.

On the other hand, following co-administration of MK-467, the lack of antinociception (between 10 and 30 minutes following administration) may be attributed to the reduction in dexmedetomidine plasma concentrations. Previously, authors have documented plasma drug concentrations associated with sedation and antinociception (Kuusela et al. 2000; van Oostrom et al. 2011). The minimum reported antinociceptive dexmedetomidine plasma concentration is 2 ng/mL (van Oostrom et al. 2011). In study IV dexmedetomidine plasma concentrations were initially greater than this value following medetomidine administration, however, they were below this ‘critical’ value in the presence of MK-467. Arguably, had a higher dose of medetomidine been used, this apparent lack of efficacy may not have been observed. Further studies targeting dexmedetomidine plasma concentrations above the ‘critical antinociceptive value’ with and without MK-467 may help to explain these results.

Administration of medetomidine in combination with alfaxalone CRI (study III) was associated with a significant reduction in the responsive to the nociceptive stimulus when compared with the other treatments. Again, MK-467 administration was associated with lower dexmedetomidine and alfaxalone plasma concentrations, presumably due to altered clearance and possibly also distribution. It remains unclear whether this difference in response is due to the intrinsic analgesic effect of dexmedetomidine or the hypnotic
action of alfaxalone as MK-467 decreased the plasma concentration of both drugs. Alternatively, MK-467 co-administration may ameliorate the dose-sparing effect of (dex-) medetomidine on the anaesthetic induction drug - alfaxalone. The clinical significance of this finding remains unclear. Further studies are required to define the ideal plasma drug concentrations and drug doses necessary for antinociception and anaesthesia using this total intravenous drug combination.

In study V, the recovery time was shorter in the presence of MK-467. Again, this was probably related to the impact of the peripheral \( \alpha_2 \)-adrenoceptor antagonist on medetomidine pharmacokinetics. MK-467 may also alter the pharmacokinetics of the injectable anaesthetic propofol. However, since it was not feasible to take plasma samples for drug analysis, this cannot presently be confirmed.

6.5 Cardiopulmonary effects

The cardiovascular effects of medetomidine premedication, namely hypertension, bradycardia, a reduction in CI and increase in CVP (studies III and V), have been reported by other authors (Bloor et al. 1992; Pypendop and Verstegen 1998). The co-administration of MK-467 ameliorated these effects (studies III, V), as previously described (Pagel et al. 1998; Enouri et al. 2008; Honkavaara et al. 2011; Rolfe et al. 2012), but unlike the earlier studies, we also reported the response during general anaesthesia (studies III and V) and thereby add to the published literature on the use of MK-467 in dogs. In study III, MAP was diminished by the addition of MK-467; however, values were always clinically acceptable (i.e. MAP greater than 70 mmHg). During study V, the use of MK-467 diminished MAP, this effect was less marked than the reduction caused by premedication with acepromazine/butorphanol. Therefore, the combination of medetomidine and MK-467 may offer an alternative to the use of acepromazine in certain clinical conditions. Further studies are warranted to investigate its use in animals with pre-existing cardiovascular abnormalities or those individuals with a higher physical status assignment (ASA III or greater). It would also be important to determine the efficacy of vasoactive substances such as dopamine, dobutamine and noradrenaline in the presence of MK-467, because these drugs are commonly used during clinical anaesthesia. In studies III and V, it could be argued that the administration of MK-467 led to more cardiovascular or haemodynamic stability when compared with the other treatments. For this reason, MK-467 may offer some clinically important beneficial effects when used in combination with \( \alpha_2 \)-adrenoceptor agonists, not only for sedation but also as premedication prior to general anaesthesia.

6.6 Methodological considerations and limitations of the studies

In study I, we reported the impact of medetomidine on the protein-binding characteristics of MK-467. Arguably, the effect of MK-467 on the \( f_u \) of medetomidine should also be determined, because MK-467 is known to alter the clearance of dexmedetomidine, which in turn may alter the \( f_u \) of the latter drug in vivo. We used only one drug ratio of 1:20 medetomidine:MK-467, and a greater range of ratios should preferably have been
investigated, since the two drugs have differing pharmacokinetic characteristics (e.g. \( V_z \) and \( T_{1/2} \)), and in vivo the reported plasma drug concentrations and their respective ratios alter following administration (Honkavaara 2012). Finally, reported protein-binding percentages of medetomidine (85%) (Salonen 1992) and dexmedetomidine (92%) differ slightly from one another (EMEA 2002). Although, these data represent studies undertaken over a 10-year time frame during which methodologies may have changed. It is also unclear whether medetomidine is principally bound by albumin, AGP or by both substances. This information may be clinically useful given the known differences in these two plasma proteins.

Study II has several limitations. Specifically, for each study drug, three repetitions (i.e. \( n = 3 \)) were performed, while conventionally three separate experiments on different days would normally be required. The drug concentrations we selected were based on known plasma drug concentrations using clinically relevant doses. Arguably, it would have been more meaningful to study a wider range of drug concentrations to mimic changes that might occur in vivo, and to assess when or whether transporter activity was saturated. Nonetheless we obtained different results with the three drugs studied, we believe this provides some useful information.

Although both acepromazine and MK-467 may act as substrates for an active transport carrier, we were unable to establish the identity of this transporter, because specific P-gp inhibitors such as rhodamine 123 were not used. Neither did we use a known positive control, such as verapamil, to confirm P-gp activity in the MDR1 transfected cells. Furthermore, we did not confirm transporter expression by Western blot analysis (study II).

Other investigators have questioned the use of cells transfected with the human P-gp gene, since it may not reflect the activity of its canine counterpart (Takeuchi et al. 2006). Species differences exist in P-gp activity, with a reported canine-to-human P-gp efflux correlation coefficient ratio of 0.665 (Takeuchi et al. 2006). It is hard to assess whether these results can be applied to the dog. An alternative in vitro model used canine peripheral blood lymphocytes to study the transport of avermectins (Griffin et al. 2005). This technique has the advantage of using canine cells (i.e. from the species of interest). The use of cells from known mdr1 -/- homozygous dogs would provide a tool for further investigating this question by determining the differences between affected and non-affected animals. Recently a model using MDCK cells transfected with canine P-gp has been developed (Mealey et al. 2017). These in vitro techniques offer some practical advantages over the in vivo studies. However, the use of in vitro studies cannot truly predict what will happen in the whole animal.

In study IV, a simple haemostat was used as the nociceptive stimulus, which did not allow the applied pressure to be quantified or maintained at a constant level during application. Nonetheless, preliminary tests were performed in an attempt to establish the pressure needed to produce a response in a conscious animal within one to two seconds following application. The same device was used in all animals by the same operator, who was blinded to the treatment. Therefore, we believe the risk of bias to be small.

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In study V, the selected doses of acepromazine, butorphanol and medetomidine were derived from other publications describing their MAC-sparing properties (Heard et al. 1986; Ewing et al. 1993). We chose doses with equivalent MAC-sparing effects. An end-tidal isoflurane concentration of one times MAC (1.28%) was selected on this basis (Steffey and Howland 1977). We did not attempt to assess the depth of anaesthesia objectively. However, there was no need to administer additional injectable drugs during anaesthesia; nor was there any need to increase the vaporizer setting with any treatment. Subjectively, no differences in the depth of anaesthesia were evident between treatments during the study, and we therefore propose that the doses of sedative drugs were appropriate.

In studies III and V, CO was measured using the technique of lithium dilution. It should be noted that only one CO measurement was made at each time point due to the associated increase in the plasma lithium concentration following each measurement (Mason et al. 2001). This may lead to a decrease in the accuracy of measurements due to an increasing serum lithium concentration following repeated injections over a short time span (Mason et al. 2002a). A serum lithium value greater than 0.2 mmol/L is given as the cut-off value by the manufacturer. However, there are no published data confirming this to be the optimal maximum value for serial CO measurements using this technique. Lithium chloride is reported to have a half-life of 11.2 hours in beagle dogs (Rosenthal and Koritz 1989). Using the technique of thermal dilution, it is standard practice to perform three measurements at a given time point and then to take the mean of these values, assuming they lie within 10% of each other. The use of lithium dilution may therefore be somewhat less representative of cardiovascular function at a given time point. However, measurements made with the former technique correlate well with the thermodilution method (Mason et al. 2001) during hypotensive and hypertensive conditions (Morgaz et al. 2014). While thermodilution requires the placement of a pulmonary catheter with its attendant risks (Connors et al. 1985; Hadian and Pinsky 2006), lithium dilution employs a less invasive technique (Mason et al. 2002b) with arguably fewer associated risks.

It should also be noted that the accuracy or reliability of the lithium sensor may be affected by drugs such as acepromazine and medetomidine (Ambrisko et al. 2013), with potential effects on calculated values derived from these measurements. The results reported by Ambrisko et al. (2013) were obtained in vitro using concentrations of 500 ng/mL medetomidine and 100 ng/mL acepromazine. These drug concentrations led to a calculated bias of 32.7% and 6.5% for medetomidine and acepromazine, respectively. The significance of this finding is difficult to assess, because the medetomidine concentration is far greater than the plasma drug concentrations measured in vivo in our studies (III and IV). However, plasma acepromazine concentrations of 100 ng/mL have been reported in vivo (Hashem et al. 1992), but only in association with doses higher than that used in study V. Currently, there is no published information on the interaction between alfaxalone or MK-467 and the lithium electrode, and it is therefore unclear whether this may have affected the accuracy of these readings. In general, the potential interaction between the lithium chloride electrode and the drugs used in studies III and V were probably limited compared to the magnitude of the effect of MK-467 on CO.
6.7 Clinical implications and future studies

Arguably the most notable findings of these studies were (i) the alteration in the disposition of medetomidine and its enantiomers following the administration of MK-467 and (ii) the relationship between the pharmacodynamic effects of the enantiomers of medetomidine and their impact on some of the pharmacokinetic parameters of co-administered injectable drugs.

We have obtained some information on the protein-binding characteristics of MK-467 in vitro. However, further research is warranted ex vivo to determine how metabolism and clearance may also affect this factor. It would also be useful to determine whether or how MK-467 affects the protein-binding characteristics of medetomidine.

Based on our data, it remains unclear whether MK-467 acts as a substrate for P-glycoprotein. It is impossible currently to state that MK-467 can be used safely in animals, which are mdr1 -/- for this transporter. However, our data suggest that MK-467 may be actively transported by other transporters, and further studies may be warranted to fully elucidate this process. Even though MK-467 does not seem to be a substrate for this efflux transporter, other factors may also lead to alterations in the permeability of the blood–brain barrier (e.g. age, specifically in neonates and elderly animals, uncontrolled hypothyroidism, meningitis, trauma and oedema), which may affect the clinical usefulness of this drug.

If MK-467 is used in clinical veterinary anaesthesia, there may need to be a re-evaluation of the appropriate doses of medetomidine and dexmedetomidine, since the associated plasma concentrations differ significantly when these drugs are administered in combination with the peripheral α₂-adrenoceptor agonist. The same may also be true for injectable anaesthetic drugs such as alfaxalone, particularly if it is used for total intravenous anaesthesia.

MK-467 has been shown to inhibit many of the unwanted and potentially deleterious effects of medetomidine. This may widen the scope for the use of medetomidine in clinical veterinary anaesthesia. Further studies are required to determine the impact of medetomidine and MK-467 in animals with some forms of cardiovascular disease, endocrine disease (specifically type I and type II diabetes mellitus) and in animals with impaired hepatic function or renal insufficiency.

In the future, the identification of drugs with activity at the α₂-adrenoceptor subtypes would again allow the desired drug effects without the unwanted effects. Until this is possible, the use of MK-467 may provide a clinical alternative in haemodynamically or systemically unwell animals.
7. **CONCLUSIONS**

1. Medetomidine does not alter the protein-binding characteristics of MK-467 at the concentration used in this study.

2. MK-467 may be a substrate for a cellular efflux transporter other than P-gp. However, the evidence for acepromazine as a P-gp substrate is equivocal. Dexametomidine showed no active transport and is therefore unlikely to be a substrate for an active transport mechanism.

3. Medetomidine altered the disposition of the intravenous anaesthetic drug alfaxalone and reduced its clearance. MK-467 normalised the plasma drug concentration of medetomidine and hereby moderated the pharmacodynamic effects of the two drugs. MK-467 reduced dexametomidine and levomedetomidine plasma concentrations by approximately half and increased their rate of elimination.

4. MK-467 did not reduce the composite sedation scores produced by medetomidine nor did it alter EEG effects of medetomidine. However, it reduced the duration of sedation. MK-467 significantly reduced the antinociceptive effect of medetomidine, which was probably due to its effect on dexametomidine plasma concentrations.

5. MK-467 ameliorated the cardiopulmonary effects of medetomidine during inhalant and total intravenous anaesthesia.
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REFERENCES


References


Brummett CM, Hong EK, Janda AM, et al. (2011) Perineural dexmedetomidine added to ropivacaine for sciatic nerve block in rats prolongs the duration of analgesia by blocking the hyperpolarization-activated cation current. Anesthesiology 115, 836-43.


References


References


References


References


References


Mason DJ, O’Grady M, Woods JP, et al. (2002b) Comparison of a central and a peripheral (cephalic vein) injection site for the measurement of cardiac output using the lithium-dilution cardiac output technique in anesthetized dogs. *Canadian Journal of Veterinary Research* 66, 207-10.


References


Pan YZ, Li DP, Pan HL. (2002) Inhibition of glutamatergic synaptic input to spinal lamina II (o) neurons by presynaptic alpha (2)-adrenergic receptors. Journal of Neurophysiology 87, 1938-47.


Pugh, DM. (1964) “Acepromazine in veterinary use.” *Veterinary Record* 76, 439-43.


References


References


References


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APPENDIX 1

Composite sedation scoring (0-12):

1. General Appearance (0-4)
   0 Normal,
   1 Head drooping,
   2 Mild sedation,
   3 Moderate sedation,
   4 Profound sedation

2. Palpebral reflex (0 – 3)
   0 Normal,
   1 Slightly reduced,
   2 weak
   3 Absent

3. Eye position (0-1)
   0 Central,
   1 Rotated

4. Jaw and tongue relaxation (0-4)
   0 Normal,
   1 Clenches jaws together;
   2 Opens jaws but with resistance,
   3 Slight resistance to tongue pulling,
   4 No resistance