“Pharmacology of Salicin Derivatives in Sheep”

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Dedicated to my husband

Chandrakant Mathurkar

Abstract

Sheep suffer from pain during various husbandry practices as well as during injury or diseases such as footrot. This pain could be potentially minimised with the use of analgesics such as non-steroidal anti-inflammatory drugs (NSAID). Unfortunately, there are very few registered NSAIDs for sheep. Thus, registered analgesics for cattle, for instance ketoprofen and meloxicam are used in sheep. Again, the high cost of analgesics and associated potential side effects such as reduced fertility, gastric irritation, gastric ulcers etc. evident in other species usually limits their use in sheep. Fear of residues in meat may stop some farmers from using analgesics. Considering these problems, this study was designed as a groundwork to explore a possible and potential use of natural, inexpensive analgesic for sheep.

Salicylic acid, a derivative of salicin, is a NSAID used effectively in humans as an analgesic since ancient times in the form of willow bark and leaves. During this research study, the pharmacokinetics of salicylic acid in sheep was analysed after administration of the sodium salt of salicylic acid (sodium salicylate/NaS) intravenously and orally at different dose rates. The analgesic efficacy of salicylic acid in sheep was also studied after administration of sodium salicylate at different dose rates by measuring mechanical and thermal nociceptive thresholds. The minimum therapeutic plasma concentration of salicylic acid for analgesia in sheep ranged from 25 to 30 μg/mL, which was achieved for about 30 minutes by a 200 mg/kg intravenous dose of NaS. During this study it was discovered that thermal nociceptive threshold testing is unable to detect any analgesia from salicylic acid and ketoprofen in sheep. However, mechanical nociceptive threshold testing efficiently detected the analgesic effects of salicylic acid and the positive control, ketoprofen.

The seasonal variation of willow salicin content (principal precursor of salicylic acid in willow) was studied over a year. The salicin in willows in New Zealand is higher during the summer months as compared to the winter months of the year, and appears greater in areas subject to drought. The analgesic efficacy of willow leaves can be assessed by feeding the willow leaves to lame sheep as they readily eat willow leaves. However, to assess the analgesia produced by willow in sheep, further research is warranted.

Keywords: Salicin, sheep, salicylic acid, analgesia, HPLC, nociceptive testing, willow.

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List of Abbreviations

µg	Microgram/ micrograms
ADME	Absorption, Distribution, Metabolism, Excretion
AIC	Akaike information criterion
ANOVA	Analysis of variance
A/P	Associate professor
AUC	Area under curve/ Area under concentration-time curve
AUMC	Area under the moment curve
BC	Before Christ
BIC	Bayesian information criterion
C	Concentration of the drug in the plasma
C_0	Concentration of drug at time zero
Ca^{++}	Calcium ions
CGRP	Calcitonin gene related peptide
CINODs	COX inhibition nitric oxide donors
Cl	Clearance
C_{max}	Maximum concentration in the plasma
CMPS-SF	Glasgow composite measure pain scale short form
CNS	Central nervous system
COX	Cycloxygenase
COX-1	Cycloxygenase 1
COX-2	Cycloxygenase 2
COX-3	Cycloxygenase 3
COXIBs	COX-2 selective inhibitors
CYP450	Cytochrome P450
D	Dose
Da	Dalton
DF	Descending facilitation
DI	Descending inhibition
DLF	Dorsolateral funiculus
DNA	Deoxyribonucleic acid
DP1	PGD receptor

DRG	Dorsal root ganglia
EEG	Electroencephalography
EP	E prostanoid receptor
F	Bioavailability
FDA	The Food and Drug Administration
FP	PGF receptor
Fig	Figure
G/g	gram/grams
GABA	Gamma amino butyric acid
GCMPS	Glasgow composite measure pain scale
GI	Gastro-intestinal
G-proteins	Guanosine nucleotide-binding proteins
HPLC	High performance liquid chromatography
Hr/hr	Hour
Hrs/hrs	Hours
HVA	High voltage activated
IASP	International Association for the Study of Pain
IP	PGI receptor
I/V	Intravenous/intravenously
IVABS	Institute of Veterinary, Animal and Biomedical Sciences
K_{12}/ K_{21}/ K_{10}	Inter-compartmental constants
K_a	Absorption constant
KA	Kainate
K_{el}/K_{10}	Elimination rate constant
Kg/kg	Kilogram/kilograms
L	Litre/Litres
LATU	Large Animal Teaching Unit
LLE	Liquid-liquid extraction
LOD	(Lower) limit of detection
LOX	Lipoxygenase
LTMR	Low-threshold mechanoreceptor
LTP	Low term potentiation

LVA	Low voltage activated
M^{++}	Magnesium ions
mg	Milligram/milligrams
Min	Minute/minutes
mL	Millilitre/millilitre
MNT	Mechanical nociceptive threshold testing
MRP2	Multi-drug-resistance-associated-protein type 2
MRT	Mean residence time
MS	Mass spectrometry
N	Newton/Newtons
Na^{+}	Sodium ions
NaS	Sodium salicylate
NCA	Non-compartmental analysis
NFκB	Nuclear transcription factor
NMDA	N-methyl-D-Aspartate
NO	Nitric oxide
NRS	Numerical rating scale
NS	Nociceptive specific
NSAID	Non-steroidal anti-inflammatory drug
NTS	Nucleus tractus solitaris
PAG	Peri-aquaductal grey matter
PD	Pharmacodynamics
PG	Prostaglandin
PGD	Prostaglandin D_2
PGF	Prostaglandin F_2
PGG	Prostaglandin G_2
PGI	Prostaglandin I_2
PK	Pharmacokinetics
PKC	Protein kinase
PN	Parabrachial nucleus
PPAR-γ	Peroxisome proliferator-activated receptor-gamma
PTFE	Polytetrafluoroethylene

R^2/r^2	Correlation coefficient
R-COH	Enolic acids
R-COOH	Carboxylic acids
RPM/rpm	Revolutions per minute
RSD	Relative standard deviation
RVM	Rostral ventromedial medulla
SA	Salicylic acid
SD	Standard deviation
SDS	Simple descriptive scale
SEP	Somatosensory evoked potentials
SMT	Spinomesecephalic
SP	Substance P
SPE	Solid phase extraction
SRT	Spinoreticular
STT	Spinothalamic
$T_{1/2}$	Half-life
T_{max}	Time at which plasma drug concentration is maximum
TNF	Tumour necrosis factor
TNT	Thermal nociceptive threshold testing
TP	Thromboxane receptor
TT	Theotepa
TTX-r	Tetrodotoxin-resistant
TXA_2	Thromboxanes
VAS	Visual analogue scale
V_d	Volume of distribution
VOCC	Voltage operated calcium channels
WDR	Wide dynamic range
A	Alpha
B	Beta
Δ	Delta

CONTENTS

1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Sheep welfare in New Zealand is important from both ethical and economical perspectives. As stated in the Animal Welfare Act 2006, one of the five freedoms of animal welfare is "freedom from pain, injury or disease". Sheep pain (resulting from routine husbandry practices or pathological conditions such as footrot) is under treated due to variety of reasons, including lack of ability to reliably recognise it, lack of knowledge on use of analgesics, their high cost and fear of residues in the meat. Also, a limited number of products are available in the market for management of pain in sheep (Lizarraga and Chambers, 2012, Martinsen *et al.*, 2011, Chambers *et al.*, 2002).

Therefore, discovery of new analgesics for sheep is essential. In this research work, the analgesic efficacy of salicin derivative sodium salicylate (sodium salt of salicylic acid) in sheep was investigated by exploring its pharmacokinetics and pharmacodynamics. The pharmacokinetics were analysed to assess the disposition of the drug in sheep. The pharmacodynamics of sodium salicylate was evaluated using pain threshold testing. Finally, of salicin concentration in willow leaves (natural source of salicylic acid) and its seasonal variation was explored with the further aim and possibility to establish the use of willow leaves as an analgesic in sheep.

1.2 LITERATURE REVIEW

1.2.1 Pain

Pain is the universal experience for all human beings (and probably all mammals) though the intensity and kind of pain experienced by each individual may differ (Beecher, 1957). The pain is not only physical/sensory but emotional experience as well (Anil *et al.*, 2005).

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or

described in terms of such damage" (ISAP, 1979). This definition is adequate for normal adult human beings as it is reliant upon self-reporting but the requirement of language or verbal communication confines its use in non-speaking humans and animals (Anand and Craig, 1996).

Pain is complex phenomenon comprising of sensory, emotional components (Hellebrekers, 2000). Understanding the mechanisms of pain is consequently important for the management of painful disorders in humans and especially in animals due to their incapability to communicate their experiences verbally. Hence, identification of pain mechanisms has been recommended as the key to analgesia (Farquhar-Smith, 2008). The types, mechanism and pathways of pain are discussed further below.

Pain can be considered clinically as acute or chronic depending upon its duration; it can also be contemplated as musculoskeletal/somatic, visceral and neuropathic on the basis of its location (Hsu and Riedesel, 2013, Lorenz, 2011). Livingston and Chambers (2000) in accordance with Woolf and Chong (1993) considered simply two types of pain for more convenient and easier understanding as physiological and pathological pain.

Physiological pain is the pain which is perceived as a noxious stimulus and is often defensive pain to protect from the tissue damage and is usually acute pain. Perception of this type of pain is proportional to the intensity of noxious stimulus (Livingston and Chambers, 2000). Pathological pain refers to the perception that is greater than noxious stimulus with involvement of inflammatory changes due to peripheral stimulus causing tissue damage and this pain can be acute or chronic. The pain without inflammatory process or lesions is also a pathological pain which involves the damage or improper functioning of nervous system (Loeser and Treede, 2008, Livingston and Chambers, 2000). For instance, acute or inflammatory pain is produced by the tissue damage (leading to process of inflammation); visceral pain doesn't require inflammation and only distensions of organs (such as distension of colon) can cause pain. Similarly, phantom pain is represented in a limb or any other body part which does not exist/ amputed/removed. Tooth pain can also be a result of damaged nerve. Ischemic pain is different in its own way (Julius and Basbaum, 2001, Gebhart, 1996). The explanation for such pain covers many theories such as neuromatrix theory, body schema theory and others (Giummarra *et al.*, 2007, Katz and Melzack, 1990). Therefore, to

present all aspects of pain is beyond the scope of this thesis. Hence, inflammatory pain will be given prominence in this thesis.

The inflammatory pain pathway is usually initiated by noxious stimulus at the peripheral level, then progresses to spinal and central mechanisms where pain signals reach the higher centres of the brain and then pain perception as well as inhibitory/descending modulatory pathway is produced (Figure 1.1)

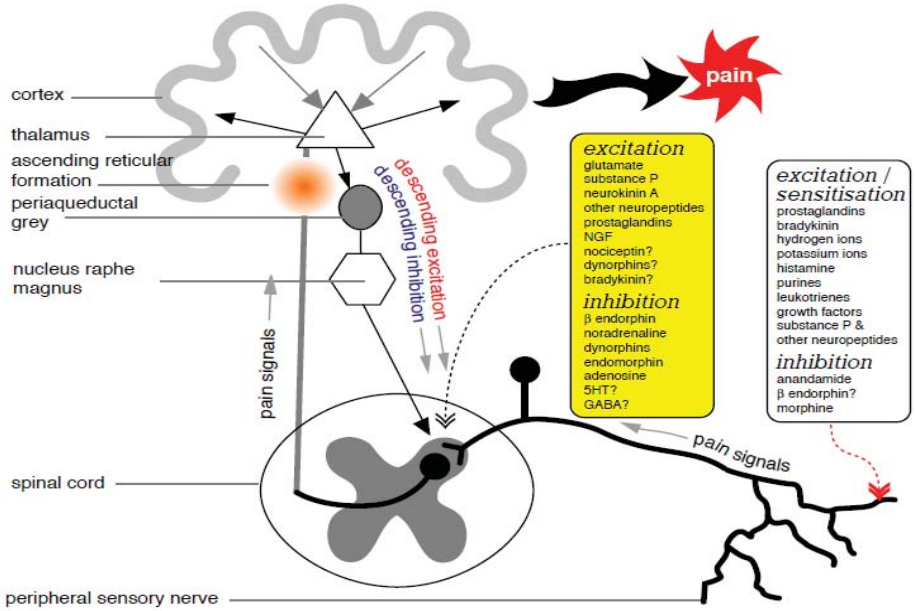

Fig 1.1: *Mechanism of pain (Courtesy: Associate Professor Paul Chambers)*

1.2.1.1 Peripheral /afferent pathway

The pain signal arises when some intense noxious stimuli (mechanical, thermal or chemical) is detected by the nociceptive receptors extensively found in the skin, mucosa, membranes, deep fascias, connective tissues of visceral organs, ligaments and articular capsules, periosteum, muscles, tendons, and arterial vessels (Almeida *et al.*, 2004). Activation of these nociceptive receptors by an external noxious stimulus and its conversion to electrical activity is termed transduction (Bridgestock and Rae, 2013, Kidd and Urban, 2001). The process of detection of noxious stimuli is called nociception (Latremoliere and Woolf, 2009, Sherrington, 1906).

The nociceptive receptors correspond to peripheral free nerve endings/fibres otherwise referred as nociceptors (Basbaum *et al.*, 2009, Basbaum and Jessell, 2000). Nociceptors conduct the noxious stimuli/impulse to the dorsal horn of the spinal cord (Kidd and Urban, 2001). These nociceptors/fibres originate from the nerve cell bodies and their diameter is categorised as large, medium or small depending upon the size and type of nerve cells they belong (Table 1.1). Aδ-fibres are thinly myelinated, medium diameter and fast conducting; activation results in acute sharp pain with a withdrawal reflex. This type of pain is also called first pain and is usually a protective or defensive mechanism for animal or human to prevent tissue damage (Diesch, 2010, Livingston and Chambers, 2000). C-fibres are non-myelinated, small diameter and slow conducting fibres resulting in dull, burning or longer lasting pain (Julius and Basbaum, 2001, Livingston and Chambers, 2000). Another type of fibres are Aβ-fibres with large diameters and rapidly conducting fibres which respond usually to normal innocuous (e.g. light touch or proprioception) (Basbaum *et al.*, 2009). In addition to noxious stimuli, peripheral sensitisation occurs primarily by post-translational reorganisation of key receptors and ion channels (Costigan and Woolf, 2000). To exemplify, phosphorylation of TTX-r (tetrodotoxin-resistant) sodium channel by protein kinase A (PKC-A) and protein kinase C (PKC- C) increases sodium currents to provoke a depolarising stimulus to produce additional excitation, and the activation threshold of the neurons is lowered (Tate *et al.*, 1998). Also, alterations in voltage-gated sodium channels play significant role in the pathogenicity of chronic inflammatory as well as neuropathic pain (Amir *et al.*, 2006).

Table 1.1: Different types of afferent sensory peripheral fibres involved in the conduction of impulses (Innocuous/noxious) to the spinal cord[a]

Fibres	Diameter	Velocity of conduction*	Myelinated	Function/information carried
Aα	13-20μm	120-60	Yes	Proprioception
Aβ	6-12 μm	75-35	Yes	Proprioception, superficial touch, deep touch, vibration
Aδ	1-5 μm	30-6	Yes	Pain, temperature (cool)
C	0.2-1.5 μm	2-0.5	No	Pain, temperature (warm), itch

*[a]Edited from: (Pappagallo, 2005, Burke-Doe). * Unit for measuring velocity of conduction is metre/second.*

Tissue injury causing damage to cells also causes secretion of numerous compounds resulting in inflammation in the vicinity of peripheral fibres. This inflammatory secretion

has a variety of components which act as inflammatory mediators (Besson, 1999, Dray, 1997a) such as prostaglandins, bradykinin, hydrogen ions, potassium ions, histamine, purines, leukotrienes, growth factors, substance P and neuropeptides responsible for excitation or sensitisation of the peripheral nerve fibres and collectively referred as "inflammatory soup" (Dickenson, 2008, Julius and Basbaum, 2001, Livingston and Chambers, 2000). Inflammatory mediators contribute to nociception either by exciting or sensitising afferent nerve fibres (Table 1.2) which affects the conduction of nociceptive impulses. A small number of afferent fibres called "silent" or "sleeping" nociceptors can be traced in the skin, joints and visceral organs and these are usually unresponsive to intense stimuli; however, when influenced by inflammatory mediators, these nociceptors become sensitised and responsive to sensory stimuli (Dray, 1997b). The constant application of the noxious stimulus for a longer duration results in increased nociceptive responses of that tissue and such a state is called hyperalgesia (Short, 1998); while nociceptive responses produced by the surrounding tissues is referred to as secondary hyperalgesia (Simone, 1992). Inflammation of peripheral tissues causes spontaneous pain and hyperalgesia (Ikeda *et al.*, 2006). Sometimes normal non-noxious stimulus too produces nociceptive responses and such state is called as allodynia (Short, 1998). The nociceptive impulses carried by afferent nerve fibres are conducted to the spinal cord where they are further processed by numerous chemicals such as different neurotransmitters, ion channels, amino acids etc. and dispatched further to the higher brain centres.

1.2.1.2 Spinal cord

Dorsal horn grey matter neurons of the spinal cord acquire sensory information from primary afferents of the sensory receptive neurons that innervate the skin and deeper tissues of the body and that respond to definite types of noxious and non-noxious stimuli (Todd, 2010, Caspary and Anderson, 2003, Costigan and Woolf, 2000). Noxious stimuli are transduced to electrical activity by these highly receptive neurons (Farquhar-Smith, 2008, Costigan and Woolf, 2000). Impulses from the nociceptive afferent fibres (sensory fibres) Aδ mechanoreceptive and C polymodal at first instance synapse in the spinal cord in the grey matter of the dorsal horn. However, these noxious signals are also carried to the ventral horn to form spinally mediated reflex arc (withdrawal response through motor neuron) as the

ventral half of the spinal cord regulates motor output (Caspary and Anderson, 2003, Livingston and Chambers, 2000).The nociceptive afferents terminate in a specifically distributed pattern of the dorsal horn that is determined by their sensory modality and the part of the body which they innervate and is also an important site of drug action (Todd, 2010, Pappagallo, 2005, Livingston and Chambers, 2000). The dorsal horn of the spinal cord is divided into anatomically and electrophysiologically distinct laminae (I to X) (Figure 1.2) (Basbaum *et al.*, 2009, Basbaum and Jessell, 2000, Rexed, 1952). Aδ nociceptors project to lamina I and to the deeper dorsal horn (lamina V). Similarly, low threshold, rapidly conducting Aβ afferents, those which respond to light touch, project into deeper laminae (III to VI) (Colvin and Power, 2005). On the contrary, C nociceptors project more superficially to laminae I and II (Dickenson, 2008, Pappagallo, 2005).

Table 1.2: Inflammatory mediators at periphery/site of injury[b]

Inflammatory mediator	**Origin/source**	**Effect on nociceptors**
Protons (H^+)	Hypoxia of muscle	Activation
Nitric oxide	Sensory neurons	Activation
Adenosine	ATP	Sensitisation
Kinins: Bradykinin Kallidin	 Blood cells Kininogen	 Activation Activation
Prostanoids: Prostaglandins, Leukotrienes, Hydroxy-acids	Mast cells release Arachidonic acid by cyclooxygenase and lipoxygenase enzymes	Sensitisation
5-Hydroxytryptamine	Platelets and mast cells	Activation
Histamine	Mast cells	Activation
Potassium ions	Damaged cells	Activation
Growth factor	Macrophages	Sensitisation
Substance P	Sensory nerve endings	Sensitisation
Neuro peptides	Sensory nerve endings	Sensitisation

[b]*Edited from: (Kongara, 2008, Costigan and Woolf, 2000, Carr and Goudas, 1999, Dray, 1997b)*

All these nociceptors use glutamate as their primary neurotransmitter which is present all over the central nervous system, though the actions of glutamate are modulated by the different neuropeptides in the dorsal root ganglia (DRG) through which the nociceptors enter

the spinal cord dorsal horn. Other neuromodulators, such as calcitonin gene- related peptide (CGRP), galanin, vasoactive intestinal polypeptide and somatostatin, also have crucial roles at the first synapse in the dorsal spinal cord. They modulate the impulses in the spinal cord to either send them to brain centres or to the motor neuron in the ventral horn (Wilcox *et al.*, 2005).

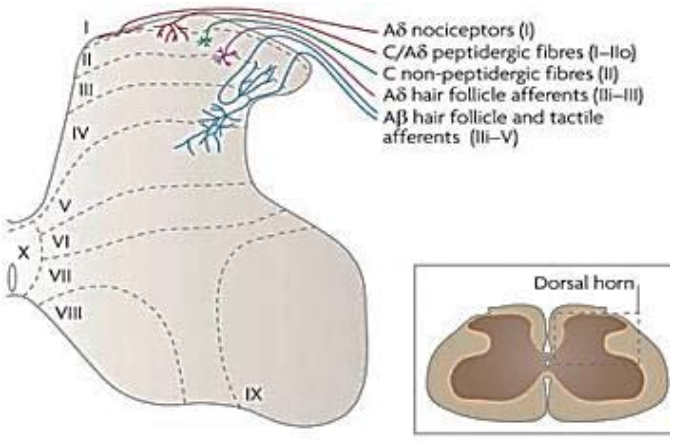

Fig 1.2: *Laminar distribution of spinal dorsal horn, edited from:(Todd, 2010). All mammals are thought to be similar.*

The activity of these pain-projection neurons is also influenced by local inhibitory interneurons in the spinal cord and by supra-spinal and brainstem-to-spinal cord mechanisms.

The dorsal horn laminae consist of various neurons (interneurons, and projection neurons) which play important role in the relay of sensory input within and beyond (supraspinal /higher brain centres) the spinal cord (McMahon *et al.*, 2013). These neurons are also considered on the basis of the specific type of sensory information they receive; e.g. The neurons receiving sensory input from Aδ and C fibres to which they respond by firing action potential are called as nociceptive specific (NS) whereas neurons responding to the input from Aβ fibres are called proprioceptive/ low-threshold mechanoreceptive (LTMR) neurons. A third type of neurons, called wide dynamic range neurons (WDR), receive sensory input from all three types of fibres and they are also responsible for exhibiting 'wind-up' where repetitive stimulation of the WDRs cumulatively evokes their response (D'Mello and

Dickenson, 2008, Herrero and Max Headley, 1995). It has been observed that somatosensory neurons in the spinal dorsal horn of fully conscious sheep had wide dynamic range properties and over 60 % neurons exhibited these properties. These neurons were found in both superficial as well as deeper laminae of the spinal dorsal horn (Herrero and Max Headley, 1995).

The interneurons based in lamina I to III have their axons within spinal cord itself. Almost all the neurons in the lamina II are interneurons; equally, most of the parts of lamina I and II (substantia gelatinosa) are also comprised of interneurons (MuirIII, 2002). Interneurons can be divided into two classes as inhibitory (GABA or glycine as main transmitter) and excitatory (glutamatergic) interneurons (Todd, 2010, Pappagallo, 2005).

The incoming information is processed by complex circuits involving excitatory and inhibitory interneurons, and is transmitted to projection neurons for further dispatch to a number of brain areas, such as the brainstem and thalamic nuclei, including the ventral posterior nucleus, the intralaminar nucleus and the parafascicular nucleus (Todd, 2010, Milligan and Watkins, 2009).

Projection neurons are predominantly found in lamina I and scattered in Lamina III to VI with a very few in lamina II (Hylden *et al.*, 1989, Lima and Coimbra, 1988). Most of the neurons that contribute to spinothalamic, spinoreticular and spinomesencephalic tracts are found primarily in lamina I, the outer layer of lamina II and in laminae IV, V and VI of the dorsal horn (Fein, 2012, Farquhar-Smith, 2008). However, lamina I to III are actively involved in the processing of nociceptive information as majority of afferents terminates in this particular zone especially in lamina I (Todd, 2010, Yu and Chan, 2003).

Therefore, the spinal cord is the first site where sensory and nociceptive signals are modulated and depending upon the signal, the summated output is further dispatched beyond the spinal cord (Melzack and Wall, 1965) as in the gate control theory proposed by Melzack and Wall in 1965 (Livingston and Chambers, 2000). Amplification of pain-related information in the spinal dorsal horn lamina I contributes to inflammatory pain (Ikeda *et al.*, 2006). Inflammation causes release of neuromodulators, including substance P and

neurotransmitter such as glutamate in spinal dorsal horn and they play very important role in modulation of pain impulses (Milligan and Watkins, 2009, Ikeda *et al.*, 2006).

Calcium (Ca^{++}) ions also play an important role in numerous biological processes including the overall process of pain. Even a momentary upturn in cytoplasmic Ca^{++} concentration can cause neurotransmitter release and the modulation of cell membrane excitability. The passage of Ca^{++} ions through membrane channels, transport by ion pumps, or release of Ca^{++} ions from internal stores are the bases for cytoplasmic concentration changes (Prado, 2001). The Ca^{++} ion influx is controlled by three ways: first, voltage operated calcium channels (VOCC), secondly, receptor activated calcium channels and lastly, the ligand gated non-specific calcium channels (Barritt, 1999). VOCC are further divided into two types as high voltage activated (HVA) and low voltage activated (LVA); HVA has several subtypes as L, N, P, Q, R and T and each having specific function (Bourinet *et al.*, 2014).

Intracellular Ca^{++} ion influx is increased in response to acute activation of primary afferent terminals leading to glutamate release. Further persistent stimulation of these afferents increases intracellular Ca^{++} provoking substance P (SP) release and more secretion of glutamate. Also, these afferents use glutamate and substance P as their transmitters to transmit the nociceptive information (Bear *et al.*, 2007, Kangrga and Randic, 1990, De Biasi and Rustioni, 1988). N-type Ca^{++} channels are mainly responsible for neurotransmitters release such as calcitonin gene related peptide (CGRP), glutamate and SP at both peripheral as well as dorsal horn synaptic level (Bourinet *et al.*, 2014).

The glutamate binds to several receptors subtypes with different affinity. These receptors are NMDA (N-Methyl-D- Aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid), kainate (KA) receptors for fast excitatory transmission (Pin and Duvoisin, 1995) and metabotropic glutamate receptors that are coupled to G-proteins (guanosine nucleotide-binding proteins) (Costigan and Woolf, 2000) for slower synaptic transmission (Ferraguti and Shigemoto, 2006). NMDA receptors have overall seven sub-units; glycine binding Glu1 and two glutamate binding Glu2 (Glu2A, Glu2B, Glu2C, Glu2D) and Glu3 (Glu3A, Glu3B) (Bourinet *et al.*, 2014, Paoletti *et al.*, 2013, Lizarraga and Chambers, 2006, Dingledine *et al.*, 1999). Amongst these receptors, NMDA has been shown to play a significant role in central sensitivity and hyperalgesia (Besson, 1999). Excitatory synaptic

transmission in the nociceptive pathways is amplified by NMDA receptors (Vanegas and Schaible, 2007). NMDA receptors have important functions in the dorsal horn neurons including wind-up of the dorsal horn neurons and modulation of the flexion reflex (Daw *et al.*, 1993). However, under normal circumstances at resting membrane potential, NMDA receptors are blocked by magnesium ions (Mg^{++}) and these Mg^{++} ions are dislodged primarily by depolarisation of sufficient amplitude and co-release of the glutamate by the influence of Ca^{++} ion channels (N-type), resulting in activation of NMDA receptors (Blanke and VanDongen, 2009, Dickenson, 2008, Besson, 1999). All fibres including C-fibres mediate pain transmission largely through activation of AMPA receptors by the action of glutamate as a substance of release at the primary afferent synapse; while, NMDA receptors are activated by persistent and sufficiently intense stimuli (Dickenson, 2011). NMDA receptors have a non-specific cation channel, both $Ca^{++}$and sodium ions (Na^{+}) are allowed to enter neurons after NMDA receptor activation (Dickenson, 2011, Dickenson, 2008). Though, Na^{+} and K^{+} ion channels are opened by AMPA and KA receptors; a large influx of Ca^{++} ions is the major mechanism by which NMDA receptor produces its effects (D'Mello and Dickenson, 2008, Budai, 2000). As described above, the amplified response of the spinal dorsal horn neurons (WDRs) to the C-fibre stimulation because of the stimulus being persistent is known as “wind-up” and this is considered to be the cause of central hypersensitivity.

Molecular cloning of metabotropic glutamate receptors have led to discovery of eight subunits (mGlu1 to mGlu8). These receptors are also involved in many brain functions involving synaptic plasticity for example long-term potentiation (LTP) and long-term depression (LTD) which is related to memory and learning (Pin and Duvoisin, 1995).

1.2.1.3 Ascending pathways of pain to higher centres

After processing and modulation of noxious stimuli at the spinal cord dorsal horn, they are transmitted to the higher centres of the brain for further processing i.e. pain perception and modulation. The principle pathways, spinothalamic (STT) and spinoreticular (SRT) and spinomesencephalic (SMT) transmit noxious stimuli to the brain (Schaible, 2006).

Axons of second order neurons in lamina IV to VI (collectively called as nucleus proprius) after crossing the midline forms the anterolateral pathway which combines with axons from second order dorsal horn neurons in the lamina I and forms the spinothalamic tract (Purves *et al.*, 2001). This is the principle ascending pathway from the dorsal horn of the spinal cord (Hodge Jr and Apkarian, 1990). It projects to the lateral complex of thalamus, nuclei of posterior medial and intralaminar complex of thalamus and to the medial central nucleus of the thalamus (Teixeira, 1990, Kerr, 1975).

Axons for SRT arise from lamina V, VII, VIII mainly along with lamina I and X (Almeida *et al.*, 2004). This tract ascends towards brainstem to medial rhombencephalic reticular formation, the lateral and dorsal reticular nucleus, the nucleus reticularis gigantocellularis and others (Schaible, 2006). Some of these project to the intralaminar nuclei of thalamus, ventral thalamus and hypothalamus; however, the functional prominence of this pathway is because of its involvement in establishing connections to brainstem (Almeida *et al.*, 2004).

The spinomesencephalic tract also uses most of the STT neurons in the dorsal horn of spinal cord along with laminae VII (in the ventral horn) and X (in the middle) (Schaible, 2006, Yezierski and Mendez, 1991).This tract mainly projects to the lateral and venterolateal periaqueductal grey matter regions (PAG) along with dorsal PAG and very sparse projections to the medial region of thalamus (Almeida *et al.*, 2004).

Apart from these pathways, some additional spinal projection paths exist such as the spinohypothalamic tract which projects directly to the medial and lateral hypothalamus from deeper laminae of dorsal horn of spinal cord. This pathway is functional in emotional, somatosensory and painful stimuli (Burstein *et al.*, 1990). The spino-parabrachio-amygdalar, tract originates from the neurons in the superficial laminae (I and II) of the dorsal horn and to some extent from the deeper laminae (X) near central canal and projects directly to the parabrachial area or in the amygdala (Jasmin *et al.*, 1994). This pathway is involved in the emotional components of the pain (Moffat and Rae, 2011). The spino-cervical tract is also evident in some species (such as cats, rats and monkeys where lateral cervical nucleus has been identified) which ascends from dorsolateral funiculus. Neurons of this tract typically process mechano-sensory input but some of the neurons receive nociceptive input as well (Schaible, 2006, Willis Jr and Coggeshall, 2004).

1.2.1.4 Pain perception and processing at higher centres of brain/pain matrix

Pain signals are received to the higher centres of the brain via ascending pathways mentioned above. The cortex is considered the prime pain perception centre though it is further subdivided in to several regions (Brooks and Tracey, 2005). However, some authors consider the thalamus more important (Albe-Fessar *et al.*, 1985). The entire process of pain perception and processing is complex and involves many factors and therefore is termed as pain matrix currently (Tracey and Mantyh, 2007). The pain matrix is divided into medial and lateral systems depending upon the pathways involved in the processing, inhibition and facilitation of pain signals in different area of brain (Brooks and Tracey, 2005). Various imaging studies confirm the involvement of somatosensory (primary and secondary), insular, anterior cingulate and prefrontal cortices along with thalamus during acute pain (Apkarian *et al.*, 2005). Also in chronic pain, specifically pre-frontal, frontal and anterior insular cortex are activated (Tracey and Mantyh, 2007).

However, Tracey and Mantyh, (2007) suggest consideration of some novel modes of investigation such as structural imaging, spinal cord imaging, imaging microglial activation and genetics to confirm the precise involvement of brain centres in particular type of pain perception.

The neurotransmitters engaged in the activation of supraspinal centres are aspartate and glutamate (Kelly *et al.*, 2001).

1.2.1.5 Descending pathways/control of pain

Axons descending from the brainstem can modulate pain at the level of dorsal horn of the spinal cord (McMahon *et al.*, 2013, Todd, 2010).

The descending pathways of pain were initially thought to inhibit pain and therefore the concept of endogenous analgesia was proposed, but eventually various research studies established the presence of descending inhibition (DI) and descending facilitation (DF) contributing to descending control of pain (Gebhart, 2004).

The principle centres for the descending control of the pain are the PAG and rostral ventromedial medulla (RVM) (Heinricher *et al.*, 2009). The pathways for DI or DF

originating from higher brain centres (thalamus and cortex) pass through the RVM. They also receive afferent input from PAG, nucleus tractus solitaris (NTS) and parabrachial nucleus (PN) to form spinobulbospinal loops (Moffat and Rae, 2011) during chronic pain states.

Modulation of pain at RVM and also at spinal cord level is governed by various transmitters, receptors along with group of neurons (in RVM, on and off cells) to either facilitate or inhibit pain (Palazzo *et al.*, 2008, Vanderah *et al.*, 2001, Fürst, 1999). Various descending pathways of pain modulation have been studied to date and the involvement of each neurotransmitter, receptor and the neuronal circuitry is known (Todd, 2010, Bee and Dickenson, 2009, Millan, 2002).

Further descending pathways from the supra-spinal centres originate from the higher brain centres (thalamus, hypothalamus, anterior cingulate, cortex etc.) and the central relay and modulatory centre for them is the RVM (Heinricher *et al.*, 2009). The descending projections from the RVM pass to the dorsolateral funiculus (DLF) and the dorsal horn where they synapse with primary afferent neuron terminals, intrinsic interneurons, ascending tract neurons and terminals of the further descending tract neurons (Bee and Dickenson, 2009).

Primary transmitters involved in the various descending modulations are histamine, acetylcholine, GABA, neuropeptides, neurotensins, galanin, SP and glutamate, 5-HT, noradrenaline depending upon serotonergic and counteracting noradrenergic pathways (Benarroch, 2008, Millan, 2002). Pain modulation occurs through endogenous opioids (endorphins) and opioid receptors at different brain regions (especially in RVM) also contribute to the overall nociception processing (Basbaum and Fields, 1984).

Central action of NSAIDs in pain modulation/inhibition is evident in descending pain pathway in RVM by altering responses of on and off cells (Vanegas *et al.*, 2010).

1.2.2 Pain in animals

Although, the mechanisms of pain in animals and humans are similar, pain in animals is difficult to understand and detect accurately. There have been many arguments about animal pain over past years.

Animals lack speech and therefore for years, the dispute on "can animals feel pain" was on-going (Musk, 2013, Paul-Murphy *et al.*, 2004). However, now it is almost universally accepted that animals do feel pain but the expression of pain varies in different species (Rutherford, 2002). The IASP (1979) suggested that the inability to communicate does not mean that the individual is not experiencing pain.

Pain detection and amelioration are therefore important components in animal care and welfare (Anil *et al.*, 2005, Barnett, 1997). It is reported that animal and human pain are similar in terms of physiological, pathological and emotional components (Panksepp, 2005, Yaksh *et al.*, 1999, Bennett and Xie, 1988, Berkowitz, 1983). Many of the pain management strategies for humans are based on animal models (Morton and Griffiths, 1985). This has been possible due to the similar neuronal pathways and neurotransmitter receptors in animals and humans (Livingston, 2010).

It has been suggested that new-born and young animals may experience pain more intensely than do older animals (Moss *et al.*, 2007, Johnson *et al.*, 2005, Mellor and Gregory, 2003). On the contrary it has been argued that the infants under the age of one year do not feel pain (Derbyshire, 2003).

According to the Animal Welfare Act (1999), a painful procedure is defined as any procedure that reasonably would be expected to cause more than slight and momentary pain or distress in a human being (AWIC, 2000). It has been argued that animals should receive the benefit of the doubt (Anil *et al.*, 2005).

Many husbandry procedures in animals such as castration, tail docking, disbudding or destruction of the horn bud, dehorning, branding, debeaking and even management practices such as shackling, transport, milking, housing etc. may result in acute pain, (Sneddon and Gentle, 2000) compromising animal welfare (Grant, 2004).

In addition to the routine surgical and other practices, farmed animals tend to acquire injuries from fighting and various activities. The systemic conditions such as pneumonia, enteritis, arthritis, mastitis, foot rot etc. are also painful leading to acute or chronic pain (Molony and Kent, 1997). Acute pain is usually a short term pain associated with the development of protective mechanisms to prevent further processing of pain (Greisen *et al.*, 1999). However,

on-going acute pain which ultimately results in chronic pain is not beneficial. Chronic pain in farmed animals leads to poor appetite, poor growth and production (Molony *et al.*, 1995, Dantzer and Mormède, 1983). Therefore, the welfare as well as production of the animals is compromised and in such conditions, analgesic treatment and proper animal care is warranted (Stafford and Mellor, 2005, Anil *et al.*, 2005). The qualitative and/or quantitative assessment of pain in animals is essential for the management of painful conditions and welfare improvement (Fitzpatrick *et al.*, 2006).

1.2.2.1 Pain in sheep

Sheep are susceptible to various diseases and either infectious or non-infectious diseases can compromise welfare of sheep by generating pain (Fitzpatrick *et al.*, 2006).

1.2.2.1.1 Lameness

Sheep lameness is caused due to pain and this pain is associated with many conditions such as foot and mouth disease to footrot (Winter, 2008). However, 80 per cent of lameness in sheep is caused by footrot and interdigital dermatitis in UK (Kaler and Green, 2009). Also in many other countries of the world, lameness due to footrot is reported and lameness in sheep is global problem (FAW, 2011). Therefore, assessment of lameness and its treatment/management is a requirement for improving sheep welfare.

1.2.2.1.2 Footrot

Footrot is an acute, sub-acute or chronic disease of sheep which mainly affects sheep feet (Murnane, 1933). The causative agent of this disease is *Dichelobacter nodosus*, (*D. nodosus*) which is active after initial infection of the feet with Fusobacterium necroforum (F. necroforum) bacteria which causes interdigital infection followed by footrot lesions (Winter, 2008).

Pre-disposing factors for footrot are wet conditions (mud during winter, faeces), humidity (temperatures above 10°C) which deteriorate interdigital skin and provide favourable conditions to *F. necroforum* to proceed with further damage which allows entry of *D. nodosus* (Morck *et al.*, 1994). The damp conditions are also responsible for the transmission of the disease between sheep (Green and George, 2008). Potentially the bacteria produce

protease enzyme which acts on the hoof tissue and causes under-running hoof leading to virulent footrot (Winter, 2008). Another mild or benign footrot type is also developed by the similar causative agent but there is no damage to hoof as the strain of *F. nodosus* is not virulent and only inflammation of interdigital skin is observed (Egerton *et al.*, 1969).

Samples from footrot lesions confirm the presence of both *D. nodosus* and *F. necroforum* with several strains where *D. nodosus* with type IV fimbriae are responsible for virulence due to production of serine proteases (Kennan *et al.*, 2011). General clinical signs observed in sheep during footrot are severe lameness, poor feed intake, isolation from the herd, loss of body condition and ultimately lowered growth and production (wool, meat) etc. causing impact on the welfare and economics (Bennett and Hickford, 2011).

Typical footrot treatment consists of parenteral antibiotics, foot baths of either formalin (2-3%) or zinc sulphate (10-20%) solution, hoof trimming in very severely affected feet, isolation of the affected animals and provision of clean and dry environment to prevent the transmission of the disease within the herd (Winter, 2008). Also, vaccination for prevention of footrot is available. However, the presence of multiple strains of causative bacteria restricts the utility of these vaccines (Schwartzkoff *et al.*, 1993).

Pain due to footrot can be managed with other treatment strategies such as use of analgesic (NSAIDs) drugs as they have demonstrated efficacy to minimise pain due to lameness (Welsh and Nolan, 1995).

1.2.3 Assessment of pain in animals

The assessment of animal pain is a vital aspect of veterinary medicine and animal welfare. However, pain is an individual experience and its measurement is exceedingly challenging (O Callaghan *et al.*, 2003), as there are intraspecies and interspecies variations in responses to painful stimuli. Even the responses of the same animal may not be the same in all cases (Anil *et al.*, 2002). The individual variation may be associated with stage of development (age), sex, genetic variation, environment, emotional status and prior pain experience etc. (Nielsen *et al.*, 2008, Johnson *et al.*, 2005, Mellor and Stafford, 2000).

Therefore, to assess pain in animals in the absence of verbal communication, the researcher has to rely on other approaches to confirm or quantify the nature and intensity of the painful or nociceptive experience (Livingston, 2010). Bufalari (2007), has suggested the inclusion of neurological, cardiovascular, respiratory, skeletal, endocrine, digestive and urinary systems for the evaluation of pain (Bufalari *et al.*, 2007). Apparently, direct and indirect indicators such as behavioural, physiological and/or clinical responses can be considered for assessing pain in animals (Landa, 2012).

1.2.3.1 Behavioral responses

Behavioural responses of animals due to pain involve changes in postures or gait, vocalisation, temperament and others such as alteration in urination and defecation frequency (Morton and Griffiths, 1985), changes in responses to nociceptive thresholds (Ley *et al.*, 1989) changes in locomotion such as licking, lying down, shaking head, flicking ears, lameness etc. (Duncan, 2006, Molony and Kent, 1997), changes in facial expressions (Guesgen, 2015, Love *et al.*, 2011). Also, change in feeding pattern (loss of appetite) can be observed during pain (González *et al.*, 2008).

Many techniques have been suggested for pain assessment on the basis of behavioural changes in animals during pain. Researchers have developed different pain scales for different animals to score the pain. Criteria selected for the measurement of pain varies and are conditional as on the study and animals (Bufalari *et al.*, 2007). Pain scales are considered subjective methods (Rutherford, 2002). The most commonly used pain scales are the simple descriptive scale (SDS), numerical rating scale (NRS) and visual analogue scale (VAS) in animals (Holton *et al.*, 1998a). There are also various composite pain scales published, the Glasgow composite measure pain scale (GCMPS) followed by Glasgow composite measure pain short form (CMPS-SF) has been developed to measure acute pain in dogs and its use in cats has also been validated (Brondani *et al.*, 2011, Reid *et al.*, 2007, Holton *et al.*, 2001).

1.2.3.1.1 Simple descriptive scale (SDS)

The simple descriptive scale is the most basic of all the pain scales (Carpenter *et al.*, 2004). This scale is usually based on limited scores for respective intensities of pain starting from ‘no pain’ (0 score) and progressing towards ‘mild pain’ (1), ‘moderate pain’ (2) and ‘severe

pain' (3) (Holton *et al.*, 1998b) in dogs. Similarly in cats, depending upon the particular behavioral parameter (such as lameness, feeding pattern, pain on palpation, weight bearing capacity, general demeanour etc.), each parameter is scored from minimum 1 to maximum 4 (Clarke and Bennett, 2006, Lascelles *et al.*, 2001).

Advantages of this scale as stated by (Clarke and Trim, 2013):

1. Basic scale and easy to use
2. Time saver/quick assessment.

Disadvantages of this scale as stated by (Clarke and Trim, 2013, Kongara, 2008):

1. Limited pain scores/intensities
2. Inter-observer variability (subjective).

1.2.3.1.2 Numerical rating scale (NRS)

Numerical rating scale is occasionally mentioned under simple descriptive scale by some researchers (Quinn *et al.*, 2007). However, when simple numerical scale is considered, it usually has a score from 0 to 10 where 10 is the worst pain (Clarke and Trim, 2013). Another NRS which is more advanced considers multiple behavioral parameters where each parameter is scored and the summation of all the scores is considered as the final pain score which can exceed the score of 10 up to the extent of parameters selected (Bussières *et al.*, 2008). NRS is often used to score lameness in animals (Quinn *et al.*, 2007, Pritchett *et al.*, 2003, Ley *et al.*, 1989).

The advantages of NRS are (Clarke and Trim, 2013, Fitzpatrick *et al.*, 2006):

1. It has advantage of scoring multiple parameters with marginal scores
2. More accurate than SDS
3. Easy to integrate the scores with accuracy.

Disadvantages of NRS are (Clarke and Trim, 2013, Mathews, 2000):

1. Time consuming
2. Lack of specificity e.g. if vocalisation is one of the parameters to score pain, then even if animal vocalises due to other reason than pain, may lead to over-scoring and vice versa.

1.2.3.1.3 Visual analogue scale (VAS)

Visual analogue scale uses a line usually 100mm long where one end is marked with 0 meaning 'no pain' and another extreme end as 'worst pain' having score of 100 (Lascelles *et al.*, 1997). In this scale observer marks the pain of the patient or animal on the line according to the severity perceived. This scale is widely used to measure post-operative pain in animals after surgical procedures (Dodman and Clark, 1992, Mbugua *et al.*, 1988).

Advantage of VAS are (Kongara, 2008, Chapman *et al.*, 1985):

1. Sensitive, popular and reproducible
2. Provides scope for multiple parameters as it does not include descriptive parameters.

Disadvantages of VAS include (Mathews, 2000):

1. Trained and experienced observer is required
2. Inter-observer variability.

Apart from these scales, many other modifications of these scales have been proposed by researchers and those scales as well are gaining popularity in assessment of animal pain. Examples of such scales are CPS (composite multifactorial pain scale) for horses UMPS (University of Melbourne pain scores) for dogs, GCMPS for dogs, CMPS-SF dogs, etc. (Bussières *et al.*, 2008, Reid *et al.*, 2007, Firth and Haldane, 1999, Holton *et al.*, 1998b).

Other behavioural tests have also been discovered which are demonstrated as reliable indicators of pain assessment in animals such as place preference where animals such as livestock avoid the places where painful husbandry practices were performed on them (Millman, 2013) and self-selection of analgesics where animals prefer pain relieving drug/solution over normal/placebo drug; for instance, Colpaert *et al.* (1980) in their experiment with rats suffering from adjuvant-induced arthritis observed the preference of rats for consumption of analgesic solution over normal sugar solution which was preferred by control (healthy animals). Such self-selection of analgesic was also observed by Danbury *et al.* (2000) in lame broiler chickens when they preferred analgesic carprofen containing feed over normal feed.

1.2.3.2 Changes in responses to nociceptive thresholds

Animals can feel pain and can react to it, if a stimulus capable of activating nociceptive receptors located subcutaneously in animals is perceived.

The significance of term "nociceptive" is the stimulus which the animal perceives as noxious and can produce a pain with or without tissue damage (Criado, 2010). The nociception can be restricted to produce acute pain resulting in action by an animal or human to prevent further pain or it can damage the tissue to produce chronic pain (inflammatory soup) (Loeser and Melzack, 1999). To measure the changes in response to nociceptive thresholds, four types of nociceptive stimuli are used. They are mechanical, thermal, chemical (not used often due to technical difficulties) and electrical (Le Bars *et al.*, 2001). Among the four types of nociceptive stimuli, mechanical and thermal nociceptive stimuli are commonly used in the farm animals such as sheep, cattle, horses etc. (Poller *et al.*, 2013, Love *et al.*, 2011, Whay *et al.*, 1998, Welsh and Nolan, 1995, Chambers *et al.*, 1994, Chambers *et al.*, 1990). Electrical stimuli are less often used as they activate all neurons, rather than those specific for pain. Therefore, in this review, mechanical and thermal nociceptive threshold testing will be discussed in detail.

1.2.3.2.1 Mechanical nociceptive threshold testing (MNT)

Mechanical nociceptive testing (MNT) usually involves external application of pressure to produce a noxious stimulus in an animal. This stimulus is usually quantifiable and the animal responds by lifting its leg (if the stimulus is applied to leg) or flicking of skin, vocalising, flicking of ear or tail, changing gait or posture and sometimes standing still, without any movement etc. depending upon the species of animal or location of the stimulus (Vivancos *et al.*, 2004, Le Bars *et al.*, 2001, Chambers *et al.*, 1990, Ley *et al.*, 1989). The most commonly used device for MNT, especially in farm animals is a pneumatically driven blunt pin in a specific region on the animal's body to create a noxious stimulus which is terminated as soon as the animal responds (Chambers *et al.*, 1990, Ley *et al.*, 1989).

During MNT, after application of stimulus, selective nociceptors are activated in response, which includes two types of Aδ and polymodal C fibre nociceptors (Julius and Basbaum,

2001). They transmit the noxious stimuli to dorsal and ventral spinal cord as well as to the higher brain centres, so that pain perception and the response to pain is generated.

In animals, MNT is mostly used to test the efficacy of analgesic drugs as it is reliable, reproducible and does not damage the tissue (Dixon *et al.*, 2010). Dixon et al. has used the MNT in cats to test the analgesic efficacy of different NSAIDs as well as opioids (Dixon *et al.*, 2010, Dixon *et al.*, 2007, Steagall *et al.*, 2007, Dixon *et al.*, 2002). In horses, the efficacy of different classes of drugs such as NSAIDs, opioids, α2-adrenergic agonists and other analgesics have been tested with MNT (Love *et al.*, 2012, Love *et al.*, 2011, Moens *et al.*, 2003, Chambers *et al.*, 1990). In cattle, apart from analgesic efficacy, the severity of lameness and its impact on the nociceptive thresholds has also been evaluated by using MNT (Tadich *et al.*, 2013, Whay *et al.*, 1998). Also, pain assessment and analgesic efficacy of various classes of drugs in other mammals such as pigs (Nalon *et al.*, 2013, Janczak *et al.*, 2012, Sandercock *et al.*, 2009), donkeys (Grint *et al.*, 2014) , rabbits (Tanelian and Beuerman, 1984) and birds (Hothersall *et al.*, 2011) using MNT has been reported.

Advantages of MNT are (Le Bars et al., 2001):

1. The responses produced by this method are directly proportional to the intensity of stimulus
2. This method produces precise results to evaluate analgesic efficacy of drugs and is reproducible.

Disadvantages of MNT include (Le Bars *et al.*, 2001, Hargreaves *et al.*, 1988):

1. Associated with technical difficulties in freely moving animals
2. Both high and low mechanoreceptors in cutaneous and non-cutaneous tissue are activated, so the contribution of each in behavioural response is not distinguishable.

1.2.3.2.2 Thermal nociceptive threshold testing (TNT)

Thermal nociceptive threshold testing applies thermal stimulation (usually heat) using different sources such as a thermode, infrared radiation (usually a laser) and hot water (Dixon *et al.*, 2002, Veissier *et al.*, 2000, Hardy *et al.*, 1965).

Thermal nociception activates the cutaneous thermoceptors including Aδ and polymodal C fibre nociceptors.

A thermode based device is usually mounted on either on animal's leg or ear and then the temperature gradually increased until a response is evoked through either lifting of leg, flicking of ear, tail, etc. (Dixon *et al.*, 2002).

A laser based device has a beam of known intensity which is projected on the skin surface of the animal at a constant distance and the response from the animal is similar to that of the thermode device (Veissier *et al.*, 2000).

Thermal nociceptive threshold testing is useful tool in pain assessment and it is widely used in different animals to evaluate analgesic efficacy, particularly for opioids, in animals such as cattle (Herskin *et al.*, 2003, Veissier *et al.*, 2000, Machado Filho *et al.*, 1998), pigs (Di Giminiani *et al.*, 2013, Herskin *et al.*, 2009), rabbit (Tanelian and Beuerman, 1984) birds (Hothersall *et al.*, 2011) and also in fishes (Nordgreen *et al.*, 2009).

There are particular advantages and disadvantages of thermode, laser and hot water as every device generates heat by a different mechanism and therefore the time and distribution of heat in the animal's skin is dissimilar (Le Bars *et al.*, 2001).

Advantage of TNT (Le Bars *et al.*, 2001)

1. Specific/selective receptors (physiological) can be activated and therefore properties of targeted receptors can be studied.

Disadvantage of TNT includes (Le Bars *et al.*, 2001):

1. Skin surface and thermode contact limits the conduction of heat while this can be overpowered by the use of laser technique. However, the cost is usually prohibitive.

1.2.3.3 Physiological responses

Measurement of physiological parameters includes plasma cortisol (gluco-corticoid hormone) levels during stress or painful procedures in animals. They are often used as markers for assessment of pain (Stafford *et al.*, 2002, Mathews, 2000, Mellor *et al.*, 2000). Some researchers have also measured β-endorphin, lactate, tumour necrosis factor alpha, interleukin-1beta, C-reactive protein, serum amyloid A and haptoglobin levels in blood (Moya *et al.*, 2008, Mears and Brown, 1997, Shutt *et al.*, 1988). However, plasma cortisol

remains the preferred and reliable physiological parameter (Landa, 2012). Physiological responses which can alter due to pain include pulse, temperature, respiration, blood pressure, etc. It may be useful during normal and painful conditions to relate and compare the parameters before and during pain (Bussières *et al.*, 2008, Mellor *et al.*, 2000). All of these physiological parameters are responses to stress, rather than pain, and stress can also be induced by non-painful stimuli such as handling.

Neurophysiological responses can also be monitored during painful conditions with electroencephalography (EEG) which provides an overall indication of cortical activity. This can give an indication of pain as the cerebral cortex is involved in pain perception and processing (Murrell and Johnson, 2006, Jongman *et al.*, 2000). Some other neurophysiological techniques such as bispectral index (BIS, a number derived from the EEG) and somatosensory evoked potentials (SEPs) have their own advantages and disadvantages (Murrell and Johnson, 2006). However, many studies have been conducted in different animals using spectral analysis of the EEG to evaluate the efficacy of many analgesic and anaesthetic drugs, e.g. in horses (Murrell *et al.*, 2005), cattle (Gibson *et al.*, 2009), sheep (Jongman *et al.*, 2000), dogs (Kongara *et al.*, 2012) and pigs (Haga and Ranheim, 2005). The advantages of EEG recording are that it is a non-invasive technique and data recording is not limited to certain data points but is continuous (Murrell and Johnson, 2006, Supèr and Roelfsema, 2005, Teplan, 2002) although the data is analysed in packets. However, it is technically difficult, particularly in awake animals, and prone to artefacts which can be exaggerated by the mathematical processing required (Kongara, 2008, Murrell and Johnson, 2006).

Finally, assessment of pain in animals by giving analgesic drugs, then measuring the behavioral and physiological responses is widely used (Livingston, 2010, Livingston and Chambers, 2000).

1.2.3.4 Assessment of pain in sheep

Pain assessment in sheep is often difficult. Sheep have evolved prone to attack by predators, such as wolves (J. Fitzpatrick et al., 2006). Different predators like coyotes (Canada, and US), black-backed jackals (Africa), and dingo (Australia) attack sheep as prey (Ensminger

and Parker, 1986). In New Zealand they are rarely attacked by keas and (more commonly) feral dogs but are still considered free from predators (Shelton, 2004, Linhart *et al.*, 1982). Sheep usually tend to mask the effect of pain by expressing normal behaviour in spite of being in painful conditions (Fitzpatrick *et al.*, 2006), presumably because animals showing signs of injury are more likely to be picked out by predators. Presence of people is also a contributing factor for a sheep's pain hiding (Beausoleil *et al.*, 2005) and they tend to behave normally when people are around. This does not mean that sheep do not experience pain. Hyperalgesia, which is an increased sensitivity to pain (Lorenz, 2011), has been observed both in sheep with chronic mastitis (Dolan *et al.*, 2000) and in lame sheep (Ley *et al.*, 1995). Various pain related studies in sheep have been conducted in the past (Molony and Kent, 1997, Waterman *et al.*, 1988, Nolan *et al.*, 1987a, Nolan *et al.*, 1987b). However, sheep pain has not gained as much attention as companion animal pain (Short, 2003). Therefore pain assessment and management in sheep is necessary to prevent welfare compromise and lowered production.

Measurement of physiological variables such as plasma cortisol levels have been used as a method to assess sheep pain (Mellor *et al.*, 2000, Mellor and Stafford, 2000). Mellor and Stafford, observed that the different methods of castration and tailing in lambs elevate cortisol levels to different concentrations which may indicate the distress and pain induced by that method. Similarly, Graham *et al.* (1997) studied different behavioural responses as well as cortisol responses in lambs after docking with different methods and obtained results where the maximal increase in cortisol level was observed after rubber ring application for docking compared to other methods such as docking iron and rubber ring with Burdizzo. A corresponding increase in active behaviour was also observed. These results were supported by (Grant, 2004), when he studied behavioural responses to all routine husbandry practices such as ear tagging, docking, castration, mulesing etc. with different methods amongst which all the methods involving rubber ring for castration and docking exhibited elevated behavioural responses. These behavioural responses included vocalisation, restlessness, the number of times a lamb stood up and lay down, tail wagging, kicking or foot stamping, rolling, jumping, licking or biting the wound site, hyperventilating, trembling, stretching and easing quarters etc. (Grant, 2004). Therefore, these behavioral responses can be considered helpful for assessment of pain in sheep.

Neurophysiological techniques such as EEG recording has been investigated as a method to study pain in sheep. EEG recordings of changes in the brain activity of sheep that were subjected to a painful stimulus demonstrated that the response in the brain to pain was similar to that of humans (Ong *et al.*, 1997). Johnson *et al.* (2009) recorded EEG readings during rubber ring castration of lambs over first 7-10 days of their post-natal life. They found significant changes in EEG indices of nociception and opined that mechanisms that suppress responses of the foetus to noxious stimulation may still be active in the first few days after birth. This method though has a disadvantage. The recording of responses to the noxious stimulation is only practical under general anaesthesia. As anaesthesia blunts the responses to noxious stimulation the need for general anaesthesia is an obvious limitation of this technique (Johnson *et al.*, 2009).

Similarly, behavioural responses by using pain scales as described earlier such as NRS and, VAS etc. have also been used to assess the sheep pain due to lameness (Welsh *et al.*, 1993, Ley *et al.*, 1989) and also after castration to study the efficacy of carprofen (Steiner *et al.*, 2003).

Other behavioural responses such as changes in nociceptive threshold responses (thermal, mechanical and electrical) have also been investigated in sheep to evaluate the analgesic efficacy various drugs or to investigate sensitisation of peripheral nociceptors following chronic pain by many researchers (Chambers *et al.*, 1995, Main *et al.*, 1995, Ley *et al.*, 1989, Nolan *et al.*, 1988, Nolan *et al.*, 1987a, Nolan *et al.*, 1987b). The analgesic action of alpha 2-adrenoceptor agonists and opioids, measured using a thermal and a mechanical test were shown in a study by Nolan *et al.,* (1987), Waterman *et al.,* (1988) showed that intrathecal injections of the alpha 2-adrenoceptor agonists, xylazine and clonidine, into the cervical region of the spinal cord of conscious unrestrained sheep produced a dose-dependent analgesia of the forelimbs as measured using a mechanical pressure device.

Along with change in physiological parameters and behavioural response/changes; nociceptive testing is a significant and sensitive tool to assess analgesia in sheep.

1.2.3.4.1 Mechanical nociceptive testing

Several devices for delivering noxious mechanical stimuli have been developed for the use in sheep (Haerdi-Landerer *et al.*, 2003, Chambers *et al.*, 1995, Main *et al.*, 1995, Welsh and Nolan, 1995, Ley *et al.*, 1989, Nolan *et al.*, 1988, Nolan *et al.*, 1987a, Nolan *et al.*, 1987b).

Waterman *et al.* (1988) conducted an analgesic study where the sheep were placed in a small pen in a quiet room and the mechanical pressure testing device was attached to one forelimb in the region of the lower end of the radius. The device produced a gradually increasing pressure through a small pin pressing against a bony area of the leg. This pressure was recorded on a dial in units which were converted to Newtons (N). The response of the animal to the stimulus was indicated by lifting of the leg, and the pressure at this point was recorded.

1.2.3.4.2 Thermal nociceptive threshold testing

Main *et al.* (1995), Ley *et al.* (1989) measured thermal thresholds in sheep using a clip incorporating a heater, a thermocouple and an inertia cut-off device which was attached to the pinna of the ear as described by (Nolan *et al.*, 1987a). A gradual increase in the temperature of the device elicited a well-defined response, either an ear flick or head shake which operated the cut-out and also recorded the threshold temperature.

The assessment of pain is necessary to guide treatment with analgesic drugs.

1.2.4 Analgesics

Analgesics are drugs that eliminate pain. However, most of these drugs act on the sensory and emotional components of pain to control it without affecting consciousness. Some of these drugs alter the threshold of pain by acting as anti-hyperalgesic and they mostly reduce the pain rather than completely eliminating it, though this can be dose dependent (Nolan, 2000).

Analgesics are classified as (Riviere and Papich, 2013, Singh, 2011, Hewitt, 2000)

1. Opioids
2. Nonsteroidal anti-inflammatory drugs (NSAIDs)
3. Alpha-2 adrenoceptor agonists

4. N-methyl-D-aspartate (NMDA) receptor antagonists
5. others

Analgesics are used by veterinarians to provide analgesia in many painful conditions such as post-operative or post trauma pain, musculoskeletal pain, soft tissue inflammation etc. in companion animals such as dogs and cats; however among large animals only horses and cattle are routinely given analgesics (Flecknell, 2008). According to (Riviere and Papich, 2013), since 1998 use of analgesics in veterinary market has been increased. The main limitations for widespread use of these analgesics in farm animals is their high cost and lack of accurate pain assessment to guide the dosing regimen (Hewson *et al.*, 2007).

In animals such as dogs and cats opioids class of analgesics is used to manage pain (Robertson and Taylor, 2004). However, CNS associated side effects such as sedation, euphoria, dysphoria, and excitement have been reported in small animals (Papich, 2000). In case of farm animals like cattle and sheep, opioids induced side effects such as light sedation, vocalization and restlessness have been observed (Bassert and Thomas, 2014) and cheap drugs like morphine are not effective in ruminants and at the same time they can cause residues in food animals (Chambers *et al.*, 2002). Also, with alpha-2 adrenoceptor agonists sedation and ataxia are common side effects in large animals (Bassert and Thomas, 2014, Chambers *et al.*, 2002). Local anaesthetics are short acting though, they are cheap and some of the metabolites are being considered as carcinogenic in Europe (Chambers *et al.*, 2002). NSAIDs on the other hand primarily act on the peripheral sites of pain instead on CNS to reduce associated CNS related side effects and also have zero or minimal withholding time for milk (Chambers *et al.*, 2002, Papich, 2000). Thus, high costs, CNS related side effects and short acting nature of the non-NSAIDs makes them disadvantageous while, making NSAIDs the most effective class of analgesics for ruminants (Chambers *et al.*, 2002).

1.2.5 NSAIDs

NSAIDs have been used to treat various inflammatory conditions along with fever, musculoskeletal pain and arthritis since ancient times. The first recorded use was in the Assyrian era (4000 B.C.) and then Sumerian era (3000 to 1900 B.C.) in the form of willow

tree bark and leaves (Mahdi *et al.*, 2006, Mackowiak, 2000, Homer and Sylla, 1996). Egyptian Ebers Papyrus (1534 B.C.) mentions use of willow as antipyretic and anti-inflammatory (Fuster and Sweeny, 2011) and Hippocrates, the Greek physician, in the fourth century (B.C.) also prescribed willow leaves and bark to treat pain, fever, inflammation and to treat pain during childbirth in women (Seaman, 2011, Vlot *et al.*, 2009). Discorides used willow bark to treat the patients with rheumatism (Calixto *et al.*, 2000). Other physicians such as Celsus, Gallen and Pliny the Elder have considered willow for rheumatism treatment (Vane, 2000a). The first modern use of willow was around 1763 when Sir Edmond Stone submitted a letter to the Royal Society of London on the use of willow to treat fever and pain (Vane, 2000a, Stone, 1763).

The glycoside salicin in willow is the main compound of interest (Vlachojannis *et al.*, 2011). It is metabolised to salicylic acid and other salicylates to produce anti-inflammatory, anti-pyretic and analgesic effects. The first synthetic NSAID, sodium salicylate, was discovered in the early 19th century, followed by acetyl ester of salicylic acid (aspirin) in 1898 by Felix Hoffman of the Bayer Pharmaceutical company (Riviere and Papich, 2013). Aspirin gained popularity over the years for treatment of rheumatism. However, the mechanism of action of aspirin as well as other NSAIDs was not known until Sir John Vane described it first in 1971 (Vane, 1971). After this discovery, many NSAIDs were invented.

1.2.5.1 Mechanism of action of NSAIDs

NSAIDs act by inhibiting the cyclooxygenase (COX) enzyme which is required for the synthesis of prostanoids, i.e. prostaglandins and thromboxanes (TXA_2) (Vane, 2000b). These prostanoids have functions in mediating pain pathways during inflammatory conditions as well as some vital roles in other physiological processes (Zarghi and Arfaei, 2011). Prostanoid synthesis is initiated with the release of fatty acids from the cell membrane phospholipid by tissue damage and conversion by phospholipase A_2 to arachidonic acid (AA). The precursor AA is acted upon by COX which has two main isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) and also by some other enzymes such as lipoxygenase (LOX) and its isoforms to form various lipid mediators (formed from group of oxygenated C_{20}fatty acids) collectively referred as eicosanoids (Riviere and Papich, 2013, William L. Smith *et al.*, 2000). The action of COX enzymes

produces prostaglandin G_2 (PGG_2) which is unstable and converted to prostaglandin H_2 (PGH_2) by peroxidase; further synthesis of other prostaglandins (PGD_2, PGE_2, PGI_2, PGF_2 and TXA_2) is also accomplished by catalytic synthase enzymes (Figure:1.3) (Rao and Knaus, 2008). Prostaglandins are found in inflammatory exudates as their synthesis is increased in response to tissue damage (Davies *et al.*, 1984). Therefore, NSAIDs act on the COX enzymes to prevent the synthesis of prostanoids (figure 1.4) and ultimately produce analgesic effects. However, the knowledge about functions of prostaglandins, COX enzymes and selective actions of NSAIDs on COX is required to understand the mechanism of action of NSAIDs in detail.

1.2.5.2 Prostaglandins and their functions

The term prostaglandin was first introduced by Euler in 1935, when he discovered this acidic lipid substance in the human seminal plasma due to the assumption that it is secreted by prostate gland (Horton, 1969). Later, many scientists revealed the biosynthesis of prostaglandins from AA. Prostaglandins are found in physiological systems such as gastrointestinal, central nervous system (CNS), endocrine, respiratory, immune system etc. and also in pathological conditions such as inflammation, cancer, cardiovascular disease and hypertension where they exert mainly harmful effects (Hata and Breyer, 2004, Narumiya, 2003). Therefore, they have both constitutive and induced functions.

1.2.5.2.1 PGs in inflammation

PGH_2 is the precursor for the main bioactive prostaglandins, PGD_2, PGE_2, PGI_2 and PGF_2 which are present in most cells; however, their biosynthesis is remarkably increased in response to inflammation, especially in acutely inflamed tissues (Ricciotti and FitzGerald, 2011). Each prostanoid has specific tissues where preferential synthesis takes place e.g. $PGF_{2\alpha}$ in uterus, PGI_2 in endothelium etc. (Ricciotti and FitzGerald, 2011, Breyer *et al.*, 2001).

The synthesis of prostaglandins depends upon COX enzymes which have two main isoforms, COX-1 and COX-2. COX-1 is considered to produce the PGs which have constitutive function, while COX-2 is induced by inflammatory processes (Brzozowski *et al.*, 2001). COX-1 preferentially links with thromboxane synthase, PGF synthase, and PGE

(cytosol) synthase, while COX-2 prefers PGI and the PGE (microsomal) synthase (Smyth *et al.*, 2009).

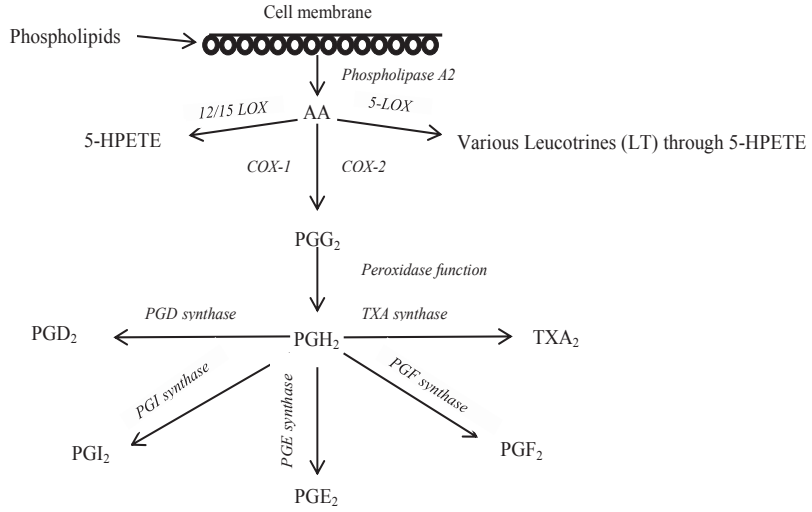

Fig 1.3: *Synthesis of prostaglandins, edited from (Rao and Knaus, 2008)*

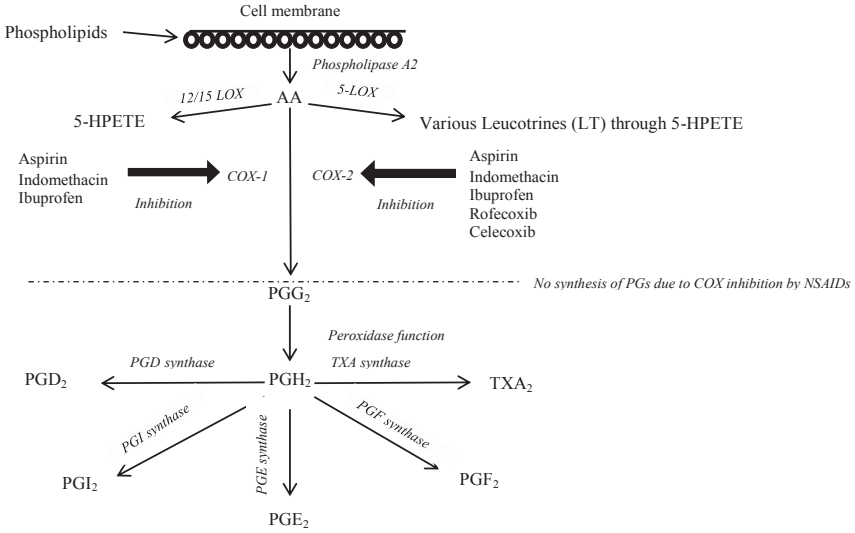

Fig 1.4: *Mechanism of action of NSAIDs, edited from (Rao and Knaus, 2008)*

**5-HPETE (5-hydroperoxyeicosatetraenoic acid)*

PGs bind to specific rhodopsin-like-7-transmembrane-spanning G protein-coupled receptors (Ricciotti and FitzGerald, 2011). There are eight prostanoid receptors, E prostanoid receptor (EP) 1, EP2, EP3 and EP4 which bind PGE; D prostanoid receptor (DP1); F prostanoid receptor (FP); I prostanoid receptor (IP); and thromboxane receptor (TP) (Breyer *et al.*, 2001).

1.2.5.2.2 PGE_2

PGE_2 is a COX-1 and 2 derived PG exhibited in many animal species and is widely involved in biological processes such as immunity, gastrointestinal integrity, fertility and blood pressure; however, impairment in its synthesis is followed by series of pathological conditions such as chronic inflammation, Alzheimer's disease, or tumorigenesis (Legler *et al.*, 2010). PGE_2 is involved in all classical processes of inflammation such as redness, swelling and pain which makes the role of PGE_2 prominent in inflammation (Ricciotti and FitzGerald, 2011). EP1 receptors are involved in the typical sign of inflammation, hyperalgesia, which occurs through peripheral as well as central activation (Moriyama *et al.*, 2005). EP2 and EP4 are involved in collagen induced arthritis where development of swelling is due to these receptors (Honda *et al.*, 2006). Similarly, EP2 and EP3 are observed in carrageenan induced oedema and pleurisy (Yuhki *et al.*, 2004).

1.2.5.2.3 PGD_2

PGD_2 is widely involved in the various systems of the body such as central nervous system where it plays role in induction of sleep, regulation of body temperature and hormonal release (Nagata and Hirai, 2003, Kobayashi and Narumiya, 2002) and other systems such as the vascular and immune systems where it has specific roles. In the vascular system it inhibits the aggregation of platelets and in the immune system it is secreted by mast cells after activation with antigen in allergic conditions such as asthma (Nagata and Hirai, 2003, Kobayashi and Narumiya, 2002).

1.2.5.2.4 PGI_2

PGI_2 or prostacyclin, is a prostaglandin that affects many body systems. It has two main functions as inhibition of platelet aggregation and it acts as vasodialator (Kelton and Blajchman, 1980). This eicosanoid has an important role in the cardiovascular system

through its receptor IP and along with vasodilation it is an inhibitor of platelet aggregation, leukocyte adhesion, and vascular smooth muscle cells proliferation (Kawabe *et al.*, 2010). Apart from its protective role, this PG is present in inflammatory exudates in arthritis (Ricciotti and FitzGerald, 2011).

1.2.5.2.5 PGF2α

This PG has a role in various activities of the reproductive system such as in luteolysis (in ruminants including sheep is most important), uterine smooth muscle contraction, and instigation of parturition; apart from this, it is also involved in the renal function, myocardial function and pain (allodynia) (Ricciotti and FitzGerald, 2011, Kunori *et al.*, 2009, Eguchi *et al.*, 1992, Silvia *et al.*, 1991). $PGF_{2\alpha}$ is present in acute and chronic inflammatory exudates in conditions such as arthritis, obesity, diabetes etc. (Higdon and Frei, 2003). In humans $PGF_{2\alpha}$ has been reported to cause bronchoconstriction, especially asthamatic people are more prone to this action; however response differs on individual basis (Pasargiklian *et al.*, 1976).

1.2.5.2.6 TXA_2

TXA_2 is largely a COX-1 derivative (Ricciotti and FitzGerald, 2011). It has mixed role as pro- and anti-inflammatory mediator as it is evident in the asthma (Tilley *et al.*, 2001). It is involved in platelet aggregation (Gryglewski *et al.*, 1978). It is also a potent vasoconstrictor and therefore has potential risk in induction of cardiovascular disorders (Cheng *et al.*, 2002).

1.2.5.3 Cyclooxygenase (COX) enzymes

Cyclooxygenase (COX) is the enzyme required to catalyse the process of prostaglandins synthesis. It has two main isoforms COX-1 and COX-2 and these enzymes are also known as prostaglandin endoperoxide H synthases (PGHS) (Smith *et al.*, 1996). The enzyme was first discovered from sheep seminal vesicles when Sir John Vane described the mechanism of aspirin inhibiting the enzyme COX and ultimately preventing the synthesis of prostanoids (Vane, 1971). After about 20 years, COX-2 was discovered in early 90's with about 60% similar amino acid sequencing as that of COX-1 with a different expression pattern and biology (Smith *et al.*, 1996). Recently, third isoform called COX-3 has also been discovered

which is considered as the variant of COX-1 (Chandrasekharan *et al.*, 2002) and may only be present in dogs.

1.2.5.3.1 COX-1

COX-1 is a constitutive enzyme which is present in almost every tissue and responsible for the synthesis of prostaglandins that are important in many vital physiological functions (Talley *et al.*, 2000). PGE_2, $PGF_{2\alpha}$ and TXA_2 are predominantly COX-1 derived and they play important physiological functions (Ricciotti and FitzGerald, 2011). Classic NSAIDs such as aspirin and indomethacin inhibit COX-1 and prevent synthesis of prostaglandins required for protective functions such as maintenance of integrity of gastrointestinal mucosa, reproductive functions related to $PGF_{2\alpha}$ (Willoughby *et al.*, 2000, Mitchell *et al.*, 1993).

1.2.5.3.2 COX-2

The discovery of COX-2 enzyme led to the development of COX selective NSAIDs. COX-2 is an isomer of COX-1 with a slight difference in its amino acid sequencing (COX-1 has 576 amino acids as opposed to COX-2 with 581 amino acids) (Rouzer and Marnett, 2009). COX-2 is not present in all tissues normally but has dramatically increased levels after exposure to cytokines (IL-1, TNFα), growth factors, bacterial toxins etc.(Riviere and Papich, 2013, Dubois *et al.*, 1998). COX-2 has wide range of functions which includes both constitutive physiological and pathological processes. In the reproductive system of mice COX-2 is involved in the ovulation, fertilization, and implantation as well as during the completion of pregnancy (Lim *et al.*, 1997). It is also constitutively present in monocytes, macrophages, endothelial cells, spinal cord, brain and ciliary body of the eye etc. (Riviere and Papich, 2013). However, in the brain, it is involved in the neurodegenerative disorders (Alzheimer's disease) and also synthesises PGs which induce fever (Teismann *et al.*, 2003). It has also a significant role in certain cancers (Riviere, 2009). The presence of COX-2 in the cartilage and synovial fluid in osteoarthritis and rheumatoid arthritis shows its role in inflammatory and painful conditions which can be considered due to its action at peripheral as well as central sites (Dubois *et al.*, 1998). Due to the participation of COX-2 in these non-constitutive functions, COX-2 selective NSAIDs have been developed so that they can specifically inhibit COX-2 without disrupting the COX-1 functions (DeWitt, 1999). Coxibs

are a new class of COX-2 selective NSAIDs which includes deracoxib, mavacoxib, robenacoxib, firocoxib for veterinary use (Riviere and Papich, 2013). There are several *in vitro* test systems available for testing the selectivity of COX-2 inhibitors. These tests are classed into three main groups as purified/recombinant enzymes, cultures of intact cells and human whole blood assay (Giuliano and Warner, 1999).

1.2.5.3.3 COX-3

Simmons *et al.* (1999) and Willoughby *et al.* (2000) proposed a third isoform of this enzyme family, COX-3, which might represent a new therapeutic target. However, Chandrasekharan *et al.* (2002) discovered COX-3 was derived from the COX-1 gene but retained intron 1 in mRNA. COX-3 is expressed in canine cerebral cortex and in lesser amounts in other tissues analysed. In human, COX-3 mRNA is expressed as an approximately 5.2-kb transcript and is most abundant in cerebral cortex and heart. Intron 1 is conserved in length and in sequence in mammalian COX-1 genes (Botting and Ayoub, 2005, Chandrasekharan *et al.*, 2002). COX-3 is expressed efficiently in insect cells as membrane-bound proteins (Chandrasekharan *et al.*, 2002). COX-3 possesses glycosylation-dependent cyclooxygenase activity (Warner and Mitchell, 2002). Comparison of canine COX-3 activity with murine COX-1 and -2 demonstrates that this enzyme is selectively inhibited by drugs such as paracetamol, phenacetin, antipyrine and dipyrone. Thus, inhibition of COX-3 could represent a primary central mechanism by which these drugs decrease pain and possibly fever (Warner and Mitchell, 2002, Chandrasekharan *et al.*, 2002).

1.2.5.4 Additional possible mechanisms of action of NSAIDs

It has been established that some NSAIDs act not only on COX-1 and COX-2, but also inhibit the nuclear transcription factor κB that is essential for cytokine gene expression during inflammation (Vaish and Sanyal, 2011, Lawrence, 2009). Inhibition of NFκB, related transcription factors, or cytokines themselves, could be considered a potential treatment for acute and chronic inflammatory pain (Carr and Goudas, 1999). Another possible mechanism of action could be the inhibition of 5-LO (5-lipoxygenase) which ultimately inhibits leukotrienes synthesis; tepoxalin is an example of a dual inhibitor i.e. COX and 5-LO (Kirchner *et al.*, 1997). Inhibition of NFκB which controls the expression of COX-2 and

cyclin-1; also inhibition of TNF (tumour necrosis factor) by most of the NSAIDs such as aspirin, ibuprofen, sulindac, phenylbutazone, naproxen, indomethacin, diclofenac, celecoxib has been demonstrated and should be considered as an additional mechanism of action of NSAIDs (Takada *et al.*, 2004). Similarly, various other mechanisms of action of NSAIDs described are inhibition of action of eicosanoids on their receptors (Funk, 2001), stimulation of nuclear receptor peroxisome proliferator-activated receptor-gamma (PPAR-γ) (Fahmi *et al.*, 2002, Jiang *et al.*, 1998)inhibition of bradykinin (Engelhardt *et al.*, 1995), modulation of release of pro-inflammatory cytokines (e.g. IL (interleukin) -1, IL-6, TNF-α), increased intracellular breakdown of ATP to adenosine, inhibition of neutrophil activation and ultimately preventing release of oxygen radicals (superoxide, hydroxyl) as well as lysosomal and non-lysosomal enzymes (Riviere and Papich, 2013), modulation of synthesis of nitric oxide (Bergh and Budsberg, 2005, Wallace *et al.*, 1994) etc. Further, a spinal mechanism of action of NSAIDs has been reported in different studies where intra-thecal administration of NSAIDs inhibits behavioural hyperalgesia produced by the action SP and NMDA (by formation of PGs) in the spinal cord (McCormack, 1994a, Malmberg and Yaksh, 1994).

1.2.5.5 Therapeutic uses of NSAIDs

NSAIDs are generally used as anti-inflammatory, anti-pyretic and analgesic drugs. Their use during perioperative conditions, in acute injury e.g. lameness, accidental or sports injuries (usually in equines and sometimes in dogs), musculoskeletal conditions is common (Lascelles *et al.*, 1997, Welsh and Nolan, 1995).

NSAIDs are also used in animals to treat chronic pain, specifically arthritis in horses and dogs (Goodrich and Nixon, 2006, Fung and Kirschenbaum, 1999). This means they are likely to be useful in other animals such as sheep suffering lameness.

NSAIDs act as antithrombotic agents as they inhibit blood clotting by blocking the formation of TXA_2 by COX-1 (Riviere and Papich, 2013) especially aspirin which irreversibly inhibits COX-1 in platelets and therefore is used in cats to treat aortic embolism (Smith *et al.*, 2003).

Similarly, the use of NSAIDs in oncology has also been revealed as some promising results were observed in controlling the growth of neoplastic cells in rats and dogs (Bergh and

Budsberg, 2005) and also been used routinely in some cancers such as colon and rectal cancers in people (Rayburn *et al.*, 2009).

1.2.5.6 Side effects of NSAIDs

The most common side effects of NSAIDs are gastrointestinal irritation and ulcer in monogastric animals and humans (Beck *et al.*, 2000, Bjarnason *et al.*, 1993). Rarely renal failure (especially with COX-inhibitors) has also been observed in animals such as dogs and in humans (Lomas and Grauer, 2015, Whelton and Hamilton, 1991). Apart from these, some other very rare adverse effects are also reported in humans which are similar for all NSAIDs. Central nervous system associated symptoms such as headaches, tinnitus and dizziness. Cardiovascular symptoms include fluid retention, hypertension, oedema and rarely, congestive heart failure. Gastrointestinal symptoms involve abdominal pain, dysplasia, nausea, vomiting, ulcers and bleeding. Other side effects such as thrombocytopenia, neutropenia, abnormal liver enzymes, asthma, skin rashes (pruritis) and renal insufficiency. (Katzung *et al.*, 2004).

In ruminants such as cattle only reduction in fertility i.e. irregular oestrous cycles, reduction in pregnancy rates and reduced formation of corpus leutium have been reported (Stahringer *et al.*, 1999). Only one report of gastrointestinal impairment (abomasal ulceration) due to NSAID (ibuprofen) in ruminants (calves) is evident as far as our knowledge till date (Walsh *et al.*, 2016) .

1.2.5.7 Classification of NSAIDs

All NSAIDs have potentially similar properties. Chemically, all are weak acids and have similar pharmacological actions i.e. anti-inflammatory, anti-pyretic and analgesic properties, and also clinical uses (Riviere and Papich, 2013). However, they can be classified considering different criteria such as chemical properties, clinical uses, COX enzyme selectivity etc. (Conaghan, 2012). Therefore, researchers are attempting to improve NSAIDs classification. Frölich (1997), have classified NSAIDs on their COX selectivity and other criterion nonetheless, Griswold *et al.* (1997) disagreed with this classification to some extent, though he agrees the necessity of re-classification of NSAIDs.

According to chemical properties, classically NSAIDs are described as two weak acid groups namely, carboxylic acids (R-COOH) and enolic acids (R-COH) (Nolan, 2000). Further classification of these acid groups' compounds is shown below (table 1.3) as described by Riviere and Papich (2013) for veterinary use. In addition to these, another group of NSAIDs is COXIBs on the basis of their COX selectivity i.e. the ability/preference of the NSAID to inhibit COX-1 or COX-2 or both and is expressed as the ratio of the COX-2 IC_{50} to the COX-1 IC_{50}, so that the more COX-2-selective an agent the smaller is the ratio expressed (Hawkey, 1999); (IC50 is the half maximal inhibitory concentration. It is a measure of the effectiveness of a substance/drug in inhibiting a specific biological or biochemical function; here, inhibition of enzymes COX-1 and/or COX-2). Some NSAIDs inhibit COX-1 enzyme, some inhibit specifically COX-2 enzyme while some are non-selective. The existing drugs which selectively inhibit COX-2 enzyme are meloxicam, nimesulide, etodolac and nabumatone; however, other specifically designed drugs have been emerged as a drug development programme and these drugs preferentially select COX-2 (Hawkey, 1999). These drugs are efficient in reducing gastric ulceration and irritation due to their selectivity to COX-2 in animals such as rats and in humans (Silverstein *et al.*, 2000, Hawkey, 1999). Examples of these drugs are coxibs (specially, rofecoxib and celecoxib).

Again, classification by Conaghan (2012) on the basis of COX selectivity as well as chemical properties and pharmacological uses has been considered as below.

1.2.5.7.1 Selective COX-2 inhibitors (COXIBs)

These drugs have activity against COX-2 enzymes and have been helpful in providing major improvement in pain therapy (Sinatra, 2002). These drugs exhibit no inhibition or partial inhibition of COX-1 enzyme. The major advantage due to these drugs is improved GI tract safety. The examples of COXIBs used in the veterinary medicines are fircoxib, deracoxib, mavacoxib and robenacoxib (Riviere and Papich, 2013).

1.2.5.7.2 COX inhibiting nitric oxide donors (CINODs)

These are nitroesters of older non-selective COX inhibitors such as aspirin, indomethacin and phynylbutazone. These drugs with ester linkage are hydrolysed when administered in vivo so that parent NSAID is generated along with the vasodilator nitric oxide (NO). NO is

supposed to enhance potency of the drug and gastric tolerance (Wallace *et al.*, 1994). However, no CINOD has been introduced in veterinary medicine (Riviere and Papich, 2013).

Table 1.3: *Classification of NSAIDs on the basis chemical properties*[ab]

Carboxylic acids	**Enolic acids**	**COXIBs**
Salicylates • Sodium salicylate • Acetylsalicylic acid (aspirin)	Oxicams • Meloxicam • Piroxicam • Tenoxicam	COX-2 inhibitors • Celecoxib • Rofecoxib • Firocoxib • Mavacoxib • Deracoxib • Robenacoxib • Cimicoxib
Indoleacetic acid • Etodolac	Pyrazolones • Phenylbutazone • Oxyphenbutazone • Isopyrin • Dipyrone	
Indolines • Indomethacin		
Thiophenacetic acid • Diclofenac • Eltenac		
2-Arylpropionic acids • Carprofen • Ketoprofen • Vedaprofen • Flurbiprofen • Ibuprofen • Naproxen		
Anthranilic acids • Flunixin • Meclofenamic acid • Tolfenamic acid • Mefenamic acid		
Quinolines • Cincophen		

[a]*As described by (Riviere and Papich, 2013),* [b]*edited from (Wright, 2002)*

1.2.5.7.3 Dual COX/ 5-LOX inhibitors

This class of compounds is dual inhibitor of COX as well as 5-LOX. Earlier drugs demonstrated low potency. Recently, second-generation dual inhibitors have been developed. Tepoxalin and licofelone are two compounds which are used in veterinary

medicine, mainly in dogs. Licofelone has also shown to inhibit immune inflammation in an asthma model in sheep (Laufer, 2001). These drugs have also demonstrated improved GI tract and renal safety profiles (Riviere and Papich, 2013).

Salicylates were the subject of this study and are therefore described in detail.

1.2.6 Salicylates

Salicylates are one of the most ancient drugs and their use was widespread in medieval times and described in various pharmacopeia (Mahdi *et al.*, 2006, Needs and Brooks, 1985). In 1829, Leroux isolated salicin as an active ingredient from willow bark. In 1838, salicylic acid was discovered (Hedner and Everts, 1998) and its first therapeutic use was in 1860, when Kolbe synthesised it. It was used as an external antiseptic, antipyretic, anti-rheumatic and anti-gout agent. In 1875, sodium salicylate was introduced as an antipyretic (Calixto *et al.*, 2000) and methyl salicylate was obtained from meadowsweet. The use of sodium salicylate, methyl salicylate and phenyl salicylate became common during 1860s and many forms of salicylates are currently available (Needs and Brooks, 2012, Hedner and Everts, 1998).

1.2.6.1 Salicin

Salicin (figure 1.5) is the water soluble glucoside with molecular formula $C_{13}H_{18}O_7$ and molecular weight 286.26 Da. It is also known as 2-(Hydroxymethyl) phenyl β-d-(glucopyranoside) (Gopaul *et al.*, 2010). Salicin is metabolised mainly to salicylic acid which is believed to be the active form. Apart from salicylic acid, salicyluric acid and gentisic acid are other metabolites of salicin (Schmid *et al.*, 2001a).

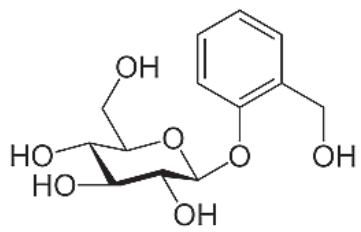

Fig 1.5: *Structure of salicin molecule*

1.2.6.2 Sodium salicylate

Sodium salicylate (figure1.6) (molecular formula: $C_7H_5NaO_3$, molecular weight: 160.11Da) is the sodium salt of salicylic acid also called as 2 hydroxybenzoic acid monosodium salt. The aqueous solution of this salt is weakly acidic (pH 5-6) (WHO, 2006). When administered in animals or humans; sodium salicylate converts to salicylic acid by the process of hydrolysis (Amann and Peskar, 2002, Amann *et al.*, 2001). Sodium salicylate yields approximately 86 % salicylic acid.

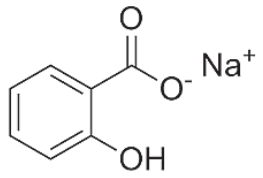

Fig 1.6: *Structure of sodium salicylate molecule*

1.2.6.3 Salicylic acid

Salicylic acid is the form which exerts therapeutic effects such as antipyretic, analgesic and anti-inflammatory at different concentrations (Vane and Botting, 1998). All the salicylates are hydrolysed to salicylic acid in-vivo. Salicylic acid has molecular formula, $C_7H_6O_7$ (Figure 1.7) and molecular weight 138.12 Da. It is a weak acid with pKa 3.01 and is also known as 2-hydroxybenzoic acid. It belongs to a large group of plant phenolic compounds (Hayat *et al.*, 2013). It is also used topically in the treatment of skin diseases such as psoriasis, acne apart from its use as analgesic and the mechanism of action in these treatments is completely different from that of its action as analgesic (Lebwohl, 1999, Jacquet *et al.*, 1988).

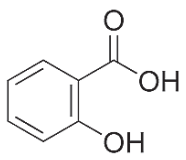

Fig 1.7: *Structure of salicylic acid molecule*

1.2.6.4 Aspirin/acetylsalicylic acid

Aspirin (molecular formula: $C_9H_8O_4$, molecular weight: 180.157 Da, pKa 3.49) (figure 1.8) is a weak acid. It was first synthesised by Felix Hoffman at Bayer pharmaceuticals to treat his father's rheumatism. Later, as research director of Bayer, he introduced this drug to the world in 1899 (Vane and Botting, 1998). It became a popular drug over the decades and is still used commonly to treat pain, fever etc. However, the adverse effects caused by long term use have reduced its use in painful conditions and its use as anti-platelet agent has become more popular. Similarly, aspirin has shown promising results in prevention of several types of cancers such as colorectal cancer, angina, transient ischemic attacks and thrombosis when taken at smaller doses in long term (Katzung *et al.*, 2004, Sandler *et al.*, 2003).

Aspirin itself produces an anti-platelet effect; however, it is rapidly metabolised to acetic acid and salicylate by tissue and blood esterases and therefore, as such has a half-life of about 15 minutes in vivo (Hsu, 2013, Katzung *et al.*, 2004).

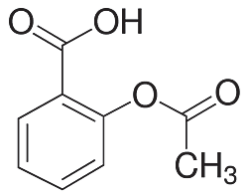

Fig 1.8: *Structure of aspirin molecule*

1.2.6.5 Pharmacological actions of salicylates

1.2.6.5.1 Anti-inflammatory activity

Salicylates are believed to exert their pharmacological/therapeutic effects by inhibiting the activity of COX enzymes. However, their potency is a matter of discussion (Amann and Peskar, 2002). According to some views, salicylates exhibit a similar mechanism of action as aspirin. Aspirin mainly acetylates the essential serine at the active site of COX in platelets irreversibly and therefore, inhibits the synthesis of prostanoids. Aspirin is rapidly

deacetylated to yield salicylates which are therefore probably mediate the anti-inflammatory activities. This is evident from several in-vivo studies (Amann and Peskar, 2002, Higgs *et al.*, 1987). Nonetheless, an in-vivo study (Chiabrando *et al.*, 1989) on the inhibitory effects of salicylates on the prostaglandin inhibition demonstrates that all the inhibitory effects of aspirin are not mediated by salicylates. Most studies show weak inhibitory activity of salicylate against prostaglandin biosynthesis when introduced in intact cells. However, interestingly, some show moderately preferential COX-1 inhibition by salicylates (Mitchell *et al.*, 1993) while others show selectivity for COX-2. The explanation for variable potencies of salicylate in prostaglandin synthesis inhibition could be the concentration of arachidonic acid present. Also, proteins available in the cell may bind to the salicylates and arachidonic acid which also alters their potency (Amann and Peskar, 2002, Amann *et al.*, 2001, Mitchell *et al.*, 1997).

Salicylates tend to accumulate in inflammatory exudates in higher concentrations than that of the plasma, which makes plasma concentration a poor measure of pharmacological effect (Lees, 2003, Amann and Peskar, 2002, McCormack, 1994a, McCormack, 1994b).

However, the role of salicylates in extracellular signaling pathways as mentioned in the other possible mechanisms of action of NSAIDs is evident. Almost all the possible mechanisms including inhibition of nuclear transcription factor κB, TNF, cytokines, modulation of release of pro-inflammatory cytokines, entrapping of free hydroxyl radicals are shown by salicylates, especially sodium salicylate (Funk, 2001, Cronstein *et al.*, 1999, Colantoni *et al.*, 1998, Epstein *et al.*, 1997, Van Jaarsveld *et al.*, 1994), except the activation of PPAR-γ (Lehmann *et al.*, 1997).

Salicylates do not acetylate COX-1 in the gastric mucosa irreversibly and therefore they do not cause gastric ulceration to the same extent as aspirin (Amann and Peskar, 2002). On the other hand, salicylates do not encourage the formation of lipoxins (15-epi-lipoxins), while aspirin promotes the formation of lipoxins when COX-2 is acetylated with aspirin (Paul-Clark *et al.*, 2004, Serhan, 1997). Lipoxins exhibit anti-inflammatory activities (Machado *et al.*, 2006).

1.2.6.5.2 Analgesic activity

The analgesia produced by salicylates is mild. The analgesic effect is probably mediated both peripherally and centrally (Bannwarth *et al.*, 1995, Houser and Paré, 1973).

1.2.6.5.3 Antipyretic activity

The antipyretic activity of salicylates is mediated by blockade of PGE production by pyrogens in the hypothalamus. However, when administered at toxic doses, they can act as pyretic agents due to dehydration produced by salicylate intoxication (Brunton *et al.*, 2011, Cranston *et al.*, 1970).

1.2.6.6 Absorption, distribution, biotransformation and excretion of salicylates

Salicylate absorption after oral administration is rapid, partially from the stomach and generally from the upper small intestine in humans and in monogastric animals. Salicylates are weak acids and are less ionised at lower pH and readily absorbed by passive diffusion in the non-dissociated salicylic acid form (Needs and Brooks, 1985, Hollister and Levy, 1965). In ruminants' the drug administered in solid form usually absorbed well from the rumen if it's a weak acid; however, when administered in liquid form it is initially received by reticulum and may pass through reticulo-omasal orifice to reduce ruminal degradation and providing more drug for intestinal absorption; this applies to all NSAIDs including salicylates (Riviere and Papich, 2013).

The absorption of salicylates in general depends upon the site of absorption (for instance, rectal absorption is lower than intestinal, absorption of salicylic acid from intact skin is very rapid), presence of food in the stomach as it may bind to the digesta, pH of the site etc. (Brunton *et al.*, 2011).

Distribution of salicylates occurs throughout the body tissues and transcellular fluids depending on the pH. Salicylates are readily distributed across choroid plexus and can also cross the placental barrier. Salicylates bind to plasma proteins (80-90 %), especially albumin (Brunton *et al.*, 2011). Therefore, volume of distribution of salicylates is low (0.5 to 0.7 L/kg). This leads to affinity for inflammatory exudates that are rich in proteins. However, at higher doses of salicylate, the binding sites of plasma proteins are saturated and consequently, a high volume of distribution is seen due to free salicylates in the plasma.

Thus the volume of distribution of the salicylates depends upon the albumin available for binding and also on the presence of other compounds competing for the protein binding sites (Brunton *et al.*, 2011, Needs and Brooks, 1985).

The metabolism of salicylates in humans occurs primarily in hepatic endoplasmic reticulum and mitochondria, although it occurs in many other tissues too. The metabolites of salicylate are produced after its conjugation with different compounds. Salicyluric acid is the chief metabolite as a result of conjugation with glycine; phenolic glucoronide and acyl glucoronide are also important conjugates. A minor fraction is also oxidised to gentisic acid and to gentisuric acid. 2, 3 dihydroxybenzoic acid and 2, 3, 5-trihydroxybenzoic acids are also generated (Brunton *et al.*, 2011, Needs and Brooks, 1985, Levy, 1981).

The excretion of salicylates occurs through urine as salicylic acid (10 %), salicyluric acid (75 %), salicylic phenolic glucuronide (10%), acid glucoronides (5%) and gentisic acid (less than 1 %). However, the elimination of salicylate depends on the dose and species as well as the pH of the urine. Alkaline urine enhances excretion of salicylates while acidic urine diminishes it to lower than normal (Riviere and Papich, 2013, Brunton *et al.*, 2011).

1.2.6.7 Therapeutic effects and pharmacokinetic parameters of salicylates in different species

Salicylates are mostly used in humans for the treatment of inflammatory painful conditions such as rheumatoid arthritis, apart from fever, headache and other painful conditions (Preston *et al.*, 1989, Boardman and Hart, 1967). Moderate analgesic and antipyretic effects of salicylates are produced when plasma salicylate concentration is less than 60μg/mL and the dose of salicylate for analgesia and antipyresis ranges from 325 to 650 mg orally every four hours in adult humans. However, their action is diverse in different animal species due to differences in the pharmacokinetics, which are affected by various factors (for instance, extent of plasma protein binding, bioavailability and pH of the urine affecting the elimination from the body) (Needs and Brooks, 2012, Katzung *et al.*, 2004).

Clinically, pharmacokinetics of the salicylates differs widely between animal species and extrapolating from one species to other is not recommended. Davis and Westfall (1972), have reported salicylate half-life values in several animals which display a wide range of variation: goat 0.8 hrs, horse 1 hr, pig 5.9 hrs, dog 8.6 hrs and cat 37.6 hrs. (All other

pharmacokinetic parameters observed by different researchers in different animal species are discussed in the following chapter, pharmacokinetics of sodium salicylate in sheep). Similarly, in calves administration of intravenous sodium salicylate prior to castration resulted in a half-life of 0.63hrs and lowering of plasma cortisol level which indicates analgesic efficacy (Coetzee *et al.*, 2007a). In cattle, the therapeutic plasma concentration of salicylate for anti-inflammatory effect is reported to be 30µg/mL (Gingerich *et al.*, 1975). At this plasma concentration of salicylate in post-partum cattle, Farney *et al.* (2013) have shown blockade of inflammation. Therefore, in each species, it is important to design a separate pharmacokinetic study to assess the disposition of this drug.

The basics of pharmacokinetics in general are discussed further.

1.2.7 Pharmacokinetics

Pharmacokinetics involves the study of absorption, distribution, metabolism and excretion (ADME) from the body after the dosage form of the drug is administered (Smith *et al.*, 2012) (Figure 1.9). Metabolism and excretion together considered as elimination while the whole process through distribution to elimination is usually termed as drug disposition (Rosenbaum, 2012). Drug disposition is a multifaceted process which varies from individual to individual with factors such as age, gender, genetic make-up, species or breed of animals (Riviere, 2009). The ADME are discussed in further detail below:

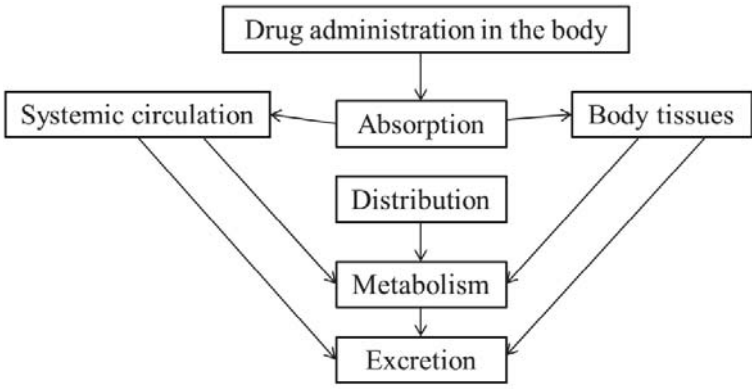

Fig 1.9: *Pharmacokinetics -Disposition of the drug in the body after administration, edited from: (Katzung et al., 2004)*

1.2.7.1 Absorption

Absorption of the drug is its movement from the site of administration to blood or systemic circulation (Riviere, 2009). Absorption of the drug depends upon the route of drug administration and the form of the drug administered. Drugs administered intravenously are absorbed directly in to the circulatory system while drugs administered through extravascular routes take a longer time to get into circulation. Similarly, the absorption of a liquid formulation of the drug is quick as it does not require any dissolution; however, tablets or capsules are solid and require disintegration and dissolution before absorption. Dissolution is partly dependent on the dissociation constant of the drug. Orally administered drug is mostly absorbed by GI tract epithelium to reach into the portal circulation; however, the drug which is extensively metabolised in the liver may not reach the systemic circulation in high enough concentration to produce therapeutic effects.

In general, absorption of the drug depends upon various elements such as formulation, particle size, physicochemical properties (e.g. pH, lipophilicity) of the drug, route of administration, solubility of the drug, species of the animal, systemic environment of the animal, pathological and physiological condition etc. (Riviere and Papich, 2013, Brunton *et al.*, 2011)

Absorption is directly related to the bioavailability of the drug which is the fraction of the drug which reaches the systemic circulation and the clinician is usually concerned about this. Therefore, bioavailability is also affected by the same factors as absorption (Brunton *et al.*, 2011).

1.2.7.2 Distribution

After absorption or administration into the circulation, the drug is distributed in the different body fluids such as plasma, interstitial fluid, intracellular fluid etc. to reach the tissues of the different organs. The distribution of the drug depends upon several physiological factors of the body as well physiochemical properties of the drug. The physiological factors include cardiac output, regional blood flow, capillary permeability, tissue volume etc. while, physiochemical properties of the drug involves its molecular weight, pK_a and lipid solubility (Brunton *et al.*, 2011, Riviere, 2009).

Most drugs are distributed in the first instance to the highly perfused organs such as heart, liver, kidney, brain etc.; then they are delivered to skin, fat and most of the viscera slowly (Riviere, 2009). The lipophilic drugs such as cyclosporin, paclitaxel etc. are highly distributed (Fahr *et al.*, 2005).

1.2.7.3 Metabolism

Drug metabolism or biotransformation is a series of enzymatic or chemical reactions through which the drug is changed to produce its therapeutic effects or terminate biological activity respectively. Metabolites are usually more polar (hydrolphilic) (Brunton *et al.*, 2011). These reactions are classified as phase I and phase II reactions occurring primarily in hepatocytes. Phase I reactions involve simple biotransformation processes such as hydrolysis, oxidation, reduction where the parent drug is mostly converted to a more polar metabolite by introduction or exposure of a functional group (-OH, -NH etc.). The resultant metabolite is either more active than the parent compound or if sufficiently polar, then it is readily excreted by the kidney. Phase I reactions are catalysed by isoforms of the enzyme family cytochrome P450 (CYP450). Phase II reactions are usually conjugation reactions and most phase I metabolites undergo these reactions to become more polar (Gibson and Skett, 2001).

Examples of phase I reactions where pro-drug is acted upon by CYP450 enzyme isoforms are nabumetone, an NSAID which is converted to its active form 6-methoxy-2-naphthylacetic acid to produce its analgesic effects (by inhibition of COX-2) with reduced gastrointestinal irritation. Another example is losartan, an extremely selective, competitive angiotensin II receptor type 1 antagonist and pharmacologically active itself. However, it is oxidized by cytochrome P450 to its 5-carboxylic acid derivative. This metabolite is known as EXP3174, which is 10–40 times more potent than that of losartan (Montellano, 2013).

Phase II conjugation reactions forms covalent linkage of functional group of the parent compound or phase I metabolite with glucuronic acid, amino acids, acetate, glutathione or sulphate to form highly polar inactive compounds which are rapidly excreted through urine and faeces. Morphine is the exceptional example where its active conjugate 6-glucoronide metabolite is a more potent analgesic than its parent drug (Brunton *et al.*, 2011).

Though enzymes involved in the metabolism of drugs are primarily located in the liver, other organs such as GI tract, kidneys and lungs can be involved in metabolic activity as they have significant metabolic capacity. For instance, a significant amount of the orally administered dose of the drug may be inactivated metabolically in the GI tract or in liver before reaching systemic circulation. This metabolism is known as first-pass metabolism and it minimises the oral availability of highly metabolised drugs such as morphine (Brunton *et al.*, 2011).

Hence, metabolism or biotransformation plays an important role in altering the activity of the drug to either terminate or potentiate its effect.

1.2.7.4 Excretion

Excretion of the drug is the process of its elimination from the body either in the unchanged form or after its conversion to metabolites. The kidney is the most important organ for excretion of drugs and their metabolites. Three distinct processes are involved during excretion of the drugs: glomerular filtration, active tubular secretion and passive tubular reabsorption. Alteration in renal function affects all the three processes. Excretion depends upon the glomerular filtration rate and the extent of plasma binding. Only unbound drug is filtered through the kidney. Many other factors affects the renal excretion such as ionisation of the metabolite, active carrier mediated tubular secretion in the proximal renal tubule, transporters (multi-drug-resistance-associated protein type 2 i.e. MRP2) localised in the apical brush-border membrane responsible for secretion of conjugated metabolites, blood pressure etc.

In the proximal and distal tubules, the process of passive reabsorption of the unionised weak acids and bases is carried out. Tubular cells are less permeable to ionised forms of weak electrolytes; therefore, passive absorption of such electrolytes depends upon the pH of the urine. When tubular urine is made alkaline, weak acids are ionised and rapidly excreted. For instance, salicylic acid excretion is augmented after alkalisation of urine (Brunton *et al.*, 2011).

Apart from kidney, other organs such as lungs are important for elimination of some drugs such as anaesthetic gases. Some compounds are also excreted through the faeces as they are primarily unabsorbed post oral administration or they are metabolites (especially

glucuronides) excreted in the bile or secreted in the intestinal lumen without reabsorption (Brunton *et al.*, 2011). Some metabolites are reabsorbed through the intestinal lumen and this progression is known as the enterohepatic recycling which extends the half-life of the drug due to its prolonged presence in the circulation (Roberts *et al.*, 2002).

Factors such as lipophilicity of the drug, ionisation, polarity and molecular weight affect the excretion of the drug. Other routes through which drugs are eliminated are skin (sweat), saliva, tears, hair, breast milk and meat (in case animal is slaughtered and used as food) etc. (Brunton *et al.*, 2011, Katzung *et al.*, 2004).

1.2.7.5 Clinically important pharmacokinetic parameters

The principles of pharmacokinetics are useful to understand the disposition of drugs in general. However, clinically, pharmacokinetics of a particular drug of choice can be quantified and its dosing regimen can be established by calculating pharmacokinetic parameters which apply to the general population. There are four basic pharmacokinetic parameters: clearance (Cl), volume of distribution (V_d), half-life ($T_{1/2}$) and bioavailability (F) (Benet and Zia-Amirhosseini, 1995).

Apart from these parameters area under curve (AUC) is an important parameter for pharmacokinetic and pharmacodynamics analyses.

1.2.7.5.1 AUC

It is the total area under the curve that describes the measured concentration of drug in the systemic circulation over time (Brunton *et al.*, 2011) (figure:1.10).

There are several formulae for calculating AUC. However, basic formula for AUC is as follows:

$$AUC_1 = \frac{C_0 + C_1}{2} X\ (t_2 - t_1)$$

Where, C_0 = plasma/serum drug concentration at time t_1

C_1 = plasma/serum drug concentration at time t_2

$$AUC_{0-t} = AUC\ 1 + AUC2 + - - - - - - - - - - - + AUClast$$

$$AUC_{last\ -\infty} = \frac{C_{last}}{K}$$

Where, *K = slope/elimination constant for last 4 or 5 concentrations*

C_{last} = plasma/serum drug concentration at last time point

AUC is an essential parameter to calculate clearance as well as the bioavailability of the drug.

1.2.7.5.2 Clearance

Clearance of the drug is the most significant parameter when a drug dosing regimen is being designed (Brunton *et al.*, 2011). Clearance is the volume of plasma which is completely free of drug per unit of time (Brunton *et al.*, 2011, Urso *et al.*, 2002). However, to be more precise, the clearance should be defined as the ratio of two terms i.e. the rate of drug elimination and the corresponding plasma drug concentration (Toutain and Bousquet-Melou, 2004a). Therefore, plasma clearance can be expressed in the units of volume, time and body weight (mL/hr/Kg) as:

$$Plasma\ clearance = \frac{Total\ (body)\ rate\ of\ drug\ elimination}{Plasma\ concentration}$$

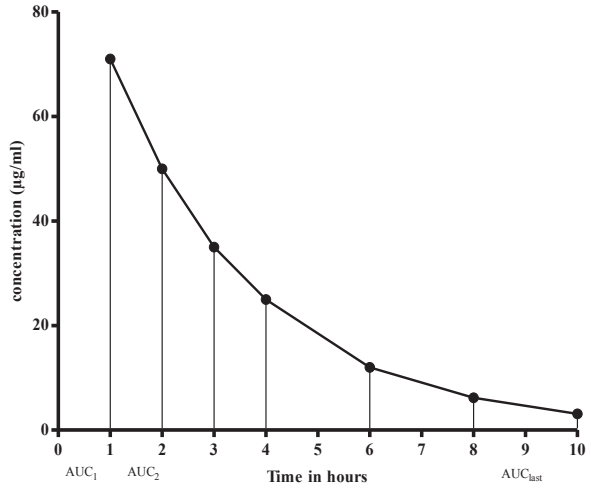

Fig 1.10: *Area under curve showing the measured concentration of the drug during given time*

This equation is accurate for the drugs following first order kinetics, where constant fraction of drug in the body is eliminated per unit time; therefore, this is dose independent reaction and most of the drugs follow first order kinetics. However, for the drugs that follows zero order kinetics, where the constant amount of drug in the body is eliminated per unit time indicating dose dependence. Therefore, for the drugs following zero order kinetics, the clearance can be derived in the units of volume/time as:

$$Clearance = \frac{V_m}{(K_m + C)}$$

Where, V_m= *the maximal rate of elimination,*

K_m = *the concentration at which half the maximal rate of elimination is reached (mass/volume)*

C = concentration of the drug in the plasma

Clearance can be constitutively represented as additive function due to elimination of the drug from different organs, such as kidney, liver and others. Therefore systemic clearance is given as:

$$Cl = Cl_{hepatic} + Cl_{renal} + Cl_{other}$$

Where, *Cl = clearance of the drug from body/total systemic clearance*

$Cl_{hepatic}$ = *clearance of a drug from liver*

Cl_{renal} = *clearance of a drug from kidney*

Cl_{other} = *clearance from GI, skin, lung etc.*

In general, systemic clearance of the drug following first order kinetics is calculated using bioavailability and the concentration of the drug in the plasma at steady state which is given by AUC as described above and therefore systemic clearance is derived as:

$$Cl = \frac{F.Dose}{AUC}$$

Where, *F is the bioavailability*.

The drugs administered intravenously have 100% bioavailability hence, in such cases clearance of the drug is calculated as:

$$Cl = \frac{Dose}{AUC}$$

1.2.7.5.3 Volume of distribution/ apparent volume of distribution

Volume of distribution of drug is an apparent or theoretical volume which would be required to contain the amount of drug in the body at the same concentration as in the plasma; or, mathematically, the ratio of the amount of drug in body at time't' and the drug plasma concentration at that time (Toutain and Bousquet-Melou, 2004b, Benet and Galeazzi, 1979). The volume of distribution is significant parameter when considering drug disposition and also while calculating the loading dose to achieve targeted therapeutic plasma concentration of the drug. It is expressed in volume/mass units i.e. mL/kg or L/kg.

$$V_d = \frac{Dose}{C_0}$$

Where, V_d = *volume of distribution*

C_0 = *concentration of drug in the plasma at time zero*

1.2.7.5.4 Half-life

Half-life of a drug is the time required to decrease the plasma concentration to half (Brunton *et al.*, 2011). This is a hybrid parameter and dependent on the other two basic independent pharmacokinetic parameters volume of distribution (V_d) and clearance (Cl). Therefore, half-life ($T_{1/2}$) is expressed in the units of time as hours (hr) or minutes.

$$T_{1/2} = 0.693 \times V_d/Cl$$

Or

$$T_{1/2} = 0.693 \times Kel$$

Where, *0.693= log of 2*

Kel = elimination rate constant of a drug

Half-life is used to calculate the dosing interval to maintain the therapeutic concentration of the drug in the body when multiple dosing is required. In general, half-life predicts an

accumulation of the drug in the body and ultimately the time required to achieve steady state equilibrium (Toutain and Bousquet-Melou, 2004b).

1.2.7.5.5 Bioavailability

Bioavailability (F) essentially reaches the systemic circulation to produce therapeutic effects (Toutain and Bousquet-Melou, 2004a). It is usually expressed in percentage (%). The bioavailability of a drug is 100% or F= 1 when administered intravenously as the entire drug is "absorbed" directly in the systemic circulation. In the case of extra-vascular routes, such as oral, sub-cutaneous, intramuscular etc., bioavailability depends upon the rate of drug absorption compared to elimination. The extravascular route of drug administration is usually associated with the escape of some fraction of drug during gastrointestinal tract (GI) metabolism or absorption in case of oral administration. Therefore, to calculate the bioavailability through these routes, comparative or relative bioavailability to the intravenous dose is more useful.

The absolute bioavailability for extravascular routes can be calculated as:

$$F(\%) = \frac{AUC_{route}}{AUC_{iv}} \times \frac{Dose_{iv}}{Dose_{route}}$$

Bioavailability varies widely from 0 to 1. Therefore, for drugs with lower bioavailability, drug dose required is larger to produce therapeutic effects (Brunton *et al.*, 2011).

1.2.7.5.6 Mean residence time

Mean residence time (MRT) of a drug is the mean time or period for which drug was present in the body. It can be defined as the mean time for the intact drug molecules to transit through the body and involves all kinetic processes, including in vivo release from the dosage form, absorption into the body, and all disposition processes (Riegelman and Collier, 1980).

It is calculated from the AUC and AUMC (area under the moment curve) as:

$$MRT = \frac{AUMC}{AUC}$$

1.2.7.6 Compartmental and non-compartmental pharmacokinetics

Pharmacokinetic analyses can be simplified by representing drug distribution within the body by a compartment in which drug concentrations are considered uniform. Clinical application of pharmacokinetics usually requires simple calculations (Atkinson *et al.*, 2012).

The concentration of drug in the plasma after its administration in the body at different time points i.e. plasma concentration-time profile, is the main measurement from which the pharmacokinetics of the drug can be derived using standard pharmacokinetic equations (Baggot, 2008). In the plasma, depending upon the characteristic of the drug it can bind to the plasma proteins and therefore, bound and free drug concentrations are available for analysis. However, free drug concentration is biologically active and it is more useful to calculate the pharmacokinetics from this (Smith *et al.*, 2006).

1.2.7.6.1 Compartmental pharmacokinetics

One compartment model of pharmacokinetics assumes a body as single compartment in to which the drug is distributed uniformly (Figure 1.11).

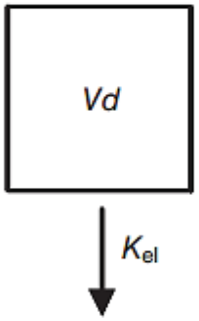

Fig 1.11: *One-compartment-open pharmacokinetic model (Riviere, 2011a)*

The elimination of the drug is regulated by a constant - elimination rate constant K_{el}. Therefore, the observed concentration of the drug in the plasma i.e. C_p would be a function of volume of distribution (V_d) that would vary after administration of the dose (D) with respect to K_{el} (Riviere and Papich, 2013). The equation of single compartmental model when a semilogarithmic plot of concentration versus time is plotted after intravenous drug administration (Figure 1.12) is represented as:

$$C_p = C_p 0 e^{-Kelt}$$

Where, *Cp0 = the plasma concentration extrapolated to time 0 after intravenous drug administration*

t = time at which C_p is observed

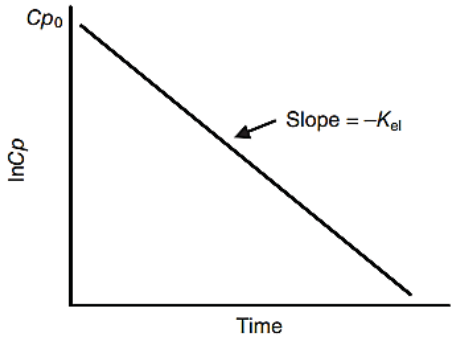

Fig 1.12: *Semilogarithmic concentration-time profile for a one-compartment drug with slope -K_{el} and intercept C_p0 (Riviere, 2011a)*

The V_d in this model is calculated as:

$$V_d = \frac{D}{C_p\,0}$$

The clearance is interpreted as:

$$Cl = V_d \times K_{el}$$

Half-life calculation for one compartmental model is given as:

$$T_{1/2} = \frac{0.693 K_{el}}{Cl}$$

AUC for this model can be calculated as:

$$AUC = \frac{C_p 0}{K_{el}}$$

When absorption is involved in one compartment model calculations, i.e. when the drug is administered by extravascular route, then absorption constant (K_a) must be taken into

consideration as the drug in this case is not administered directly into the circulation and its absorption from the site of administration is required. (Figure 1.13). However, the process of elimination remains unchanged as in case of intravenous route (Riviere and Papich, 2013).

Fig 1.13: *One-compartment-open pharmacokinetic model with first order absorption (Riviere, 2011a)*

Therefore, in this model, the concentration 'C' at time zero becomes more complex function of two exponential terms when semilogarithmic plot (Figure 1.14) of concentration versus time is plotted and the equation is given as:

$$C = \frac{K_a\, FD}{V_d(K_a - K_{el})}\,[e^{-K_{el}t} - e^{-K_a t}]$$

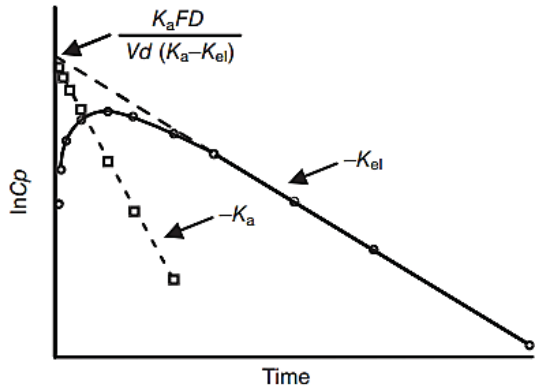

Fig 1.14: *Semilogarithmic plot of concentration versus time for one compartment open model with first order absorption with two slopes -K_a and -K_{el} (Riviere, 2011a)*

Therefore, C is governed by the fraction of drug absorbed and available in systemic circulation from where it will be then eliminated. Hence, in this case K_a must be greater than

K_{el}. As the K_a approaches zero, the elimination phase is continued and the exponential term of absorption phase would be eliminated. In case, if K_{el} is greater than K_a, then the equation is would be reversed and K_{el} would be replaced as K_a. This phenomenon is also called 'flip-flop' kinetics (Riviere and Papich, 2013). However, for any extravascular route, the drug disposition study would not be as reliable as intravenous route, due the limitation factors such as absorption and systemic availability on which the other routes are dependent and complete bioavailability (F=1) cannot be assumed except by the intravenous route (Riviere, 2011a).

Two compartment models assume there are central and peripheral compartments (Figure 1.15). Many drugs do not follow a simple one compartment model due to non-linearity of the concentration-time profile. In this case, biologically, the drug is distributed in different regions of the body at different rates and these regions are considered as central and peripheral compartments on the basis where drug is instantaneously distributed. It is assumed that the drug is first distributed in the central compartment (central compartment is composed of the blood plasma and extracellular fluids of the vital organs such as liver, kidney, heart, lungs etc.) and from here it is distributed slowly to the peripheral compartment (remaining body parts) with a distribution rate constant usually called K_{12} and then re-distributed back to the central compartment with another constant called K_{21}. Elimination occurs from the central compartment at a constant rate (K_{10}) which is equivalent to K_{el} in a one compartment model (Riviere, 2011a, Gabrielsson and Weiner, 2001). The constants K_{12} and K_{21} are considered slower than K_{10}. Therefore, in a two compartment model, the concentration versus time profile is an outcome of two pharmacokinetic processes (Figure1. 16): distribution phase (α) and elimination phase (β). The equation of two compartment model is given as:

$$Cp = A.e^{-\alpha t} + B.e^{-\beta t}$$

Where, *A = intercept for distribution phase α*

B = intercept for elimination phase β

The intercepts A and B are also called as macro constants which can be derived from K_{12}, K_{21} and K_{10} also known as micro or inter-compartmental constants (Gabrielsson and Weiner, 2001).

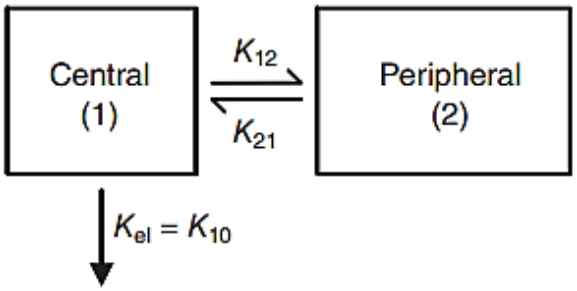

Fig 1.15: *Two-compartment-open pharmacokinetic model (Riviere, 2011a)*

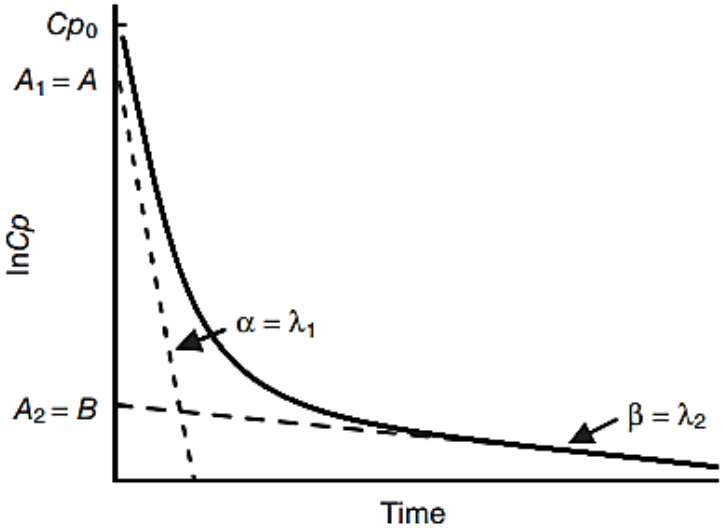

Fig 1.16: *Semilogarithmic graph of concentration versus time for two compartment open mode, describing distribution phase(α)and elimination phase(β)with their respective intercepts A and B (Riviere, 2011a)*

Two compartment model exhibits three volumes of distribution as volume of distribution at central compartment (V_c), volume of distribution at peripheral compartment (V_p) and total volume of distribution (V_t). However, volume of distribution at steady state (V_{ss}) is the component which is broken down as central and peripheral volume of distribution can be estimated irrespective of the method used for the calculation (Riviere, 2011a).

$$V_c = \frac{Dose}{A + B}$$

Where, $A+B = C_p0$

$$V_{ss} = V_c \left(\frac{K_{12} + K_{21}}{K_{21}}\right)$$

$$V_p = V_{ss} - V_c$$

$$V_t = V_c + V_p$$

The clearance can be derived as normal function of central volume of distribution and K_{el} or K_{10}.

$$Cl = \frac{V_c}{K_{el}}$$

In two compartment model, there are two half-lives; half-life of distribution ($T_{1/2\alpha}$ or $T_{1/2D}$) and half-life of elimination ($T_{1/2\beta}$ or $T_{1/2E}$).

$$T_{1/2D} = \frac{0.693}{\alpha}$$

$$T_{1/2E} = \frac{0.693}{\beta}$$

When extravascular dosing is used in a two compartment model, the equation becomes the more complex (Figure 1.17). In this case, along with inter-compartmental constants (K_{12}, K_{21}) and elimination constant K_{10} or K_{el}, additional first order absorption constant (K_{01}) is also considered.

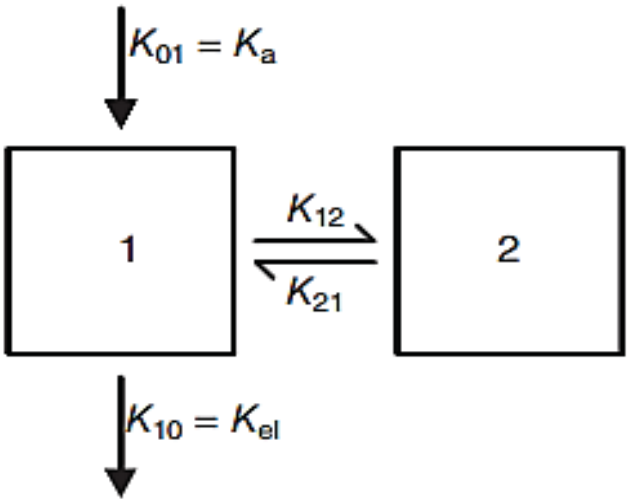

Fig 1.17: *Generalised two compartment model for extravascular dose of drug with absorption constant (K_{01})(Riviere, 2011a)*

$$C_p = (k_{01}.D/V_c).[A'_1\, e^{-\lambda_1 t} + A'_2\, e^{-\lambda_2 t} - A'_3\, e^{-k_{01} t}]$$

In this equation, λ_1, λ_2 and K_{01} are slopes of the three phases as shown in the figure 1.18, A^n are different than those derived in intravenous two compartment model (Riviere, 2011a, Gabrielsson and Weiner, 2001). They are derived as:

$$A'_1 = (k_{21} - \lambda_1) \Big/ [(k_{01} - \lambda_1).(\lambda_2 - \lambda_1)]$$

$$A'_2 = (k_{21} - \lambda_2) \Big/ [(k_{01} - \lambda_2).(\lambda_1 - \lambda_2)]$$

$$A'_3 = (k_{21} - k_{01}) \Big/ [(\lambda_1 - k_{01}).(k_{01} - \lambda_2)]$$

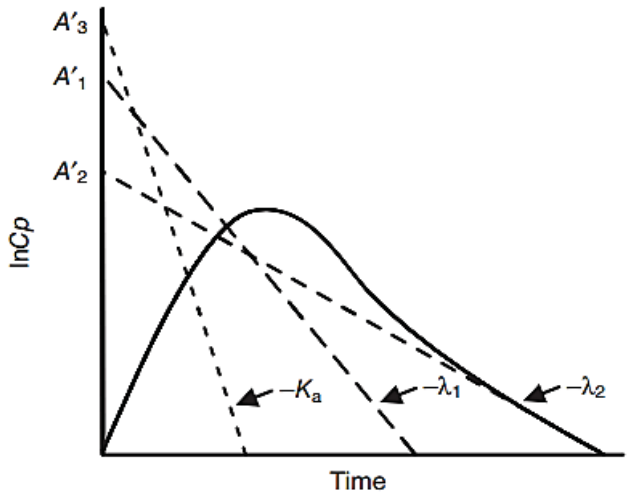

Fig 1.18: *Semilogarithmic plot of concentration versus time for two compartment model after extravascular administration of drug (Riviere, 2011a)*

1.2.7.6.2 Non-compartmental pharmacokinetics

Non-compartmental pharmacokinetic analysis (NCA) has been adopted as the preferred method since 1979 when it was developed and applied in radiation decay analysis (Gabrielsson and Weiner, 2012, Riviere, 2011b). The main advantage of this method is that no assumption is required as in compartmental analysis. The calculations are based on the statistical moment theory by using plasma drug concentration versus time (CT) profile. This is more stochastic methodology. In NCA, basic pharmacokinetic parameters can be estimated using primary CT data and considerably simpler calculations are involved to derive them. For instance, MRT_{iv} (after intravenous administration of drug) can be calculated by using AUMC (area under first moment curve) and AUC (MRT_{iv} = AUMC/AUC) where, AUMC is a CT-T profile (Riviere, 2011b).

The half-life of a drug becomes a function of MRT and is derived as:

$$T_{1/2} = 0.693(MRT) = {}^{0.693(V_{ss})}/_{Cl}$$

The clearance is calculated by general approach as:

$$Cl = \frac{D_{iv}}{AUC}$$

Volume of distribution can be estimated from basic AUC and AUMC parameters or also as a function of clearance.

$$V_{ss} = \frac{D_{iv}\, AUMC}{AUC^2}$$

$$V_d = Cl(MRT)$$

The NCA following extravascular routes requires the consideration of mean absorption time (MAT) to calculate the absorption constant K_a. MAT can be calculated from the MRT_{iv} parameter (Riviere, 2011b).

$$MAT = MRT_{route} - MRT_{iv}$$

$$K_a = {}^{1}\!/\!_{(MRT - {}^{1}\!/\!_{K_{el}})}$$

1.2.7.6.3 Advantages and disadvantages of compartmental versus non-compartmental pharmacokinetic analysis

Compartmental analysis of drug pharmacokinetics has been a traditional approach and is mathematically functional, however, the assumption of compartments in these analyses is not always realistic and therefore some parameters estimated by these methods can be ambiguous (Gillespie, 1991). The relatively simple approach of non-compartmental analysis assumes no difference in linear and non-linear models and therefore no specific demarcation of these models is possible when analysed non-compartmentally (Cobelli and Toffolo, 1984). In non-compartmental models there are no specific assumptions as in compartmental models and also curve fitting is not required. Therefore, the calculations are simpler. Similarly, the basic information or parameters (such as clearance, volume of distribution, AUC, AUMC etc.) required by clinicians can be derived without assumptions or limiting factors with non-compartmental analysis (Cutler, 1978) which makes its use more popular among clinicians (Riviere, 2011b). Compartmental analyses are more complex and derive more accurate estimations if the assumption of the compartments is physiologically real and the non-linear models can also be systematically calculated (Cobelli and Toffolo, 1984).

Both compartmental and non-compartmental approaches for pharmacokinetic analysis of drug involve their own merits and demerits. Therefore, to achieve more accurate and reliable results, application of both models for pharmacokinetic analysis should always be preferred.

1.2.7.6.4 Applications of pharmacokinetics

Pharmacokinetics plays an important role in the discovery of new drug and evaluation of drug therapy. If a drug cannot get to its site of action, it cannot work. Clinical application of pharmacokinetics besides its application in research has enormously advanced the field of pharmacology.

Apart from pharmacokinetics, pharmacodynamics is the other important element of pharmacology to evaluate the effect of the drug on the body.

1.2.8 PK-PD modelling

PK-PD (pharmacokinetic-pharmacodynamic) modelling is based on the dose response relationship over time and its application involves the identification of the effect of the drug in vivo under physiologic and pathologic conditions, determining dosing regimen and dosage form of the drug to achieve the concentration to produce the desired effect (Pérez-Urizar *et al.*, 2000). Figure 1.19 depicts the concept of PK PD modelling.

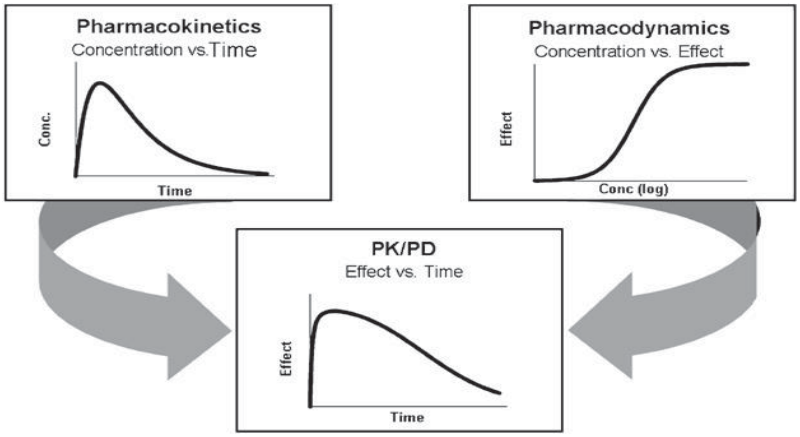

Fig 1.19: *PK-PD modelling concept from: (Mehrotra et al., 2006)*

PK-PD models are developed on the basis of drug concentration and effect relationship according to the pattern and extent of the effect produced in proportion to the drug concentration.

1.2.8.1 Linear model

This model considers direct proportionality of the drug concentration to drug effect and quantification of drug effect is possible which can be expressed as:

$$E = S.C + E_0$$

Where, *E = intensity of effect,*

C = drug concentration,

S = slope of the line,

E_0 = value of the effect when no drug is present

E_0 can be dropped from the equation if there is no effect in the absence of drug (Pérez-Urizar *et al.*, 2000).

This model is not appropriate when the drug concentration is to low or too high as the linearity follows the direct proportionality between drug concentration and drug effect only in the medium range of drug concentration (Schwinghammer and Kroboth, 1988).

1.2.8.2 Log linear model

Log linear model has been adapted from the linear model and is similar to it except the logarithmic use and also use of the constant term. The use of logarithm allows to create a linear concentration-effect curve. It can be represented as:

$$E = S.Log\ C + I$$

Where, I = imperic constant which has no physiologic value.

This model is useful for higher concentration range of drug effect up to about 80% possible effect. However, at the zero concentration of the drug, the model fails to evaluate the effect (Schwinghammer and Kroboth, 1988).

1.2.8.3 The E_{max} model

This is the most widely used model for many drugs over wide range of concentrations and is also the simplest model. This model is applicable for the drugs where the effect of the drug produced is directly proportional to the drug concentration in the body and maximum possible effect can be calculated with this model as:

$$E = \frac{E_{max}\ .\ C}{EC_{50} + C}$$

Where, *C = concentration of the drug,*

E = effect produced by the concentration C,

E_{max} = *maximum possible response that can be attributed to the drug,*

EC_{50} = *drug concentration that can produce 50 % of the maximum possible effect.*

This model can be converted to calculate the inhibition percentage when, the effect of the drug is inhibitory (Schwinghammer and Kroboth, 1988).

Another models such as Sigmoid E_{max} model and inhibitory are also used with some modified patterns of drug effect with respect to the concentration (Pérez-Urizar *et al.*, 2000).

PK-PD modelling in the analysis of drug efficacy or in the development of new drug can be useful. NSAIDs usually bind to plasma proteins and hence, free or unbound plasma concentrations of NSAIDs are lower than the total concentration (Riviere and Papich, 2013). However, during inflammatory conditions, NSAIDs are accumulated gradually in the inflammatory exudate and sometimes even exceed their concentrations in the plasma (Scherkl *et al.*, 1996). Though plasma concentrations are often indicators of drug effects, for NSAIDs this is not true. Where plasma concentrations do not represent the drug concentrations at the site of action (inflammatory exudates), the pharmacodynamic effect may be measured by calculating their IC50 from the concentration of prostaglandins or concentration of drug in the inflammatory exudates (Lees *et al.*, 2004). However, this is technically difficult.

As discussed in the introduction, it is essential to explore new analgesic drugs for sheep. Therefore, in this study, an effective, abundantly available, cheap and natural analgesic in the form of willow leaves has been investigated.

1.2.9 Willow

Willow (*Salix spp*) is a genus of tree of over 300 species, and belongs to the family *Salicacae* (Newsholme, 1992). Along with poplar (*Populus spp*) (which also contains salicin) willow has been distributed in many countries (over 90) of the world like United States, Canada, United Kingdom, Russia, France etc. (Ball *et al.*, 2005). It is a plant of sub-tropical and tropical zones and originally from the northern hemisphere (Kuzovkina and Quigley, 2005, Argus, 1997).

Willow is a versatile plant having many valuable properties and contributes to the treatment of many problems such as prevention of soil erosion, phytoremediation and carbon sequestration. It is a suitable wood for shelterbelts, timber and generation of electricity (Kuzovkina and Quigley, 2005, Heller *et al.*, 2004, Greger and Landberg, 1999). Non-wood products of willow include fodder and shelter for livestock, birds, wild habitat (Smith *et al.*, 1978). It is also appropriate for fencing, to make wood items such as baskets, fish traps, bats and many other ornamental things (Scott and Huskisson, 1976, Warren-Wren, 1965). Besides all these attributes, willow has medicinal properties.

Willows are easily propagated vegetatively. They are popular because of high growth and early adaptation to new habitats (Verwijst, 2001). Therefore willow has a significant role to play in today's world.

1.2.9.1 Willow in New Zealand

Willow is not native to New Zealand and was introduced in 1840s by early European settlers (Phillips and Daly, 2008, Charlton *et al.*, 2007). Willow was utilized in New Zealand primarily for the control of soil erosion by planting on the banks of rivers and streams by farmers, councils etc. However, over the years its usefulness was expanded and now it has been used as a fodder for livestock such as cattle, goats and sheep during drought periods (Olsen and Charlton, 2003, Wilkinson, 1999, McCabe and Barry, 1988).

Over 60 willow species were imported in 1934 to Christchurch botanical gardens from the Kew and since then over 100 clones have been developed in New Zealand (Charlton *et al.*, 2007, Hussain, 2007). To identify the species of the willow is difficult for many reasons (Karp *et al.*, 2011) and many hybrids are seen. Most commonly in the New Zealand, willows are classified as tree willows, osier willows and shrub willows (McIvor, 2013, Stace, 1998, Douglas *et al.*, 1996).

Various species/crosses of different species/clones which are popular and commonly used for different purposes in New Zealand are as follows (Charlton *et al.*, 2007) (figure 1.20:a, b, c, d, e).

1. Tree willows: *Salix matsudana, S. babylonica, S.alba, S.matsudana×alba*

Fig 1.20 (a) *S. babylonica**

Fig 1.20 (b) *S. matsudana×alba***

Fig 1.20 (c) S. Alba^

2. Osier willows: *S. purpurea* 'Kinuyanagi', *S. viminalis* 'Gigantia'

Fig 1.20 (d) *S. purpurea*^

Fig 1.20 *(e) S. viminalis*^^

Sources: **Trees planet: http://treesplanet.blogspot.co.nz/, **The New Zealand Poplar and willow research trust http://www.poplarandwwillow.org.nz, ^ Google (copyright-free images), ^^Valerie J.:http://gardeners-word.blogspot.co.uk/*

3. Shrub willows: *S. glaucophylloides,* grey willow, pussy willow

These willows have different cultivars and most of them are used to prevent soil erosion. Cultivars of *S.matsudana×alba* such as Moutere, Aokautere, Tangoio are most popular as well as drought resistant clones. Tangoio is especially utilized as a fodder for livestock with optimum nutritive value and palatability (Kemp *et al.*, 2001, Oppong *et al.*, 1996, McCabe and Barry, 1988).

Willows used in hill country and river engineering are usually tree species, in particular clones of *S. matsudana* and hybrids of S. *matsudana× alba*, such as 'Tangoio' and 'Moutere' (Harman, 2004).

Willows in New Zealand shed a large quantity of leaves in autumn and early winter i.e. April to June. Precise time and duration of leaf-fall depends on cultivar of willow and weather (personal observation).

1.2.9.2 Willow in livestock as fodder

Willow has high feeding value for livestock (Kemp *et al.*, 2001, Oppong *et al.*, 1996). It has been well accepted by livestock including sheep, cattle, goats and deer (Kovalchik and Elmore, 1992, McCabe and Barry, 1988, Seip and Bunnell, 1985). Cattle will eat trimmings up to 10 to 25mm and sheep up to 5mm in diameter. Both cattle and sheep will strip off and eat the bark (Oppong *et al.*, 1996, McCabe and Barry, 1988). Willow leaves are also high in zinc and magnesium, which are important animal health minerals.

1.2.9.3 Willow as fodder in sheep

There is an extensive literature on willow leaves as fodder in sheep, which supports the fact that willow leaves' feeding is adequate for maintenance. In 1983, Dann and Axelsen conducted an experiment during a drought where they fed a sole diet of willow leaves and twigs to four merino hoggets and four crossbred lambs and they found 1.9 and 1.8 kg weight gain over 6 weeks without any ill effects of the diet (Dann and Axelsen, 1986).

The preliminary study conducted by Oppong *et al.* (1996) concludes that willow cultivar Tangoio is a potential maintenance feed for sheep during summer drought and early autumn. Similarly Kinuyanagi willow can be used as a supplementary drought fodder due to its high edible forage yield (Oppong *et al.*, 1996). It has also been proposed that willow and poplar trees provide forage for livestock of moderate nutritive value (Kemp *et al.*, 2001). Feeding of willow leaves or trimmings to ewes during mating resulted in reduction of live weight loss and increase in reproductive rates (McWilliam *et al.*, 2005). Also for mating ewes in dry summer/autumn, full access to willow fodder blocks is a beneficial supplement in NZ (Pitta *et al.*, 2007). Willow fodder block grazing can also be helpful in supportable farming systems for hoggets as it maintains growth rates, increases reproductive rates as well as reduces dag formation in parasitized hoggets (Musonda *et al.*, 2009).

Ramírez-Restrepo *et al.* (2010) conducted a study to analyse the effect of willow feeding on methane production and blood composition in ewe hoggets. They observed that the willow leaves as fodder were accepted and preferred by the ewe hoggets (Ramírez-Restrepo *et al.*, 2010).

Therefore, willow leaves/bark can be used as fodder, is readily eaten, has no obvious adverse effects and is a potential maintenance ration for sheep and other livestock.

1.2.9.4 Medicinal history of willow

The medicinal history of willow has already been described in the section NSAIDs.

The species *S. alba* L., *S. fragilis* L. *and S. purpurea* L. are most commonly used for medicinal purposes (Setty and Sigal, 2005). The active component of *Salix spp*. is considered to be salicin. However, studies have revealed that willow has other salicin derivatives (phenolic glycosides), such as salicortin, fragilin and tremulacin 23-26% as total salicin derivatives and additionally flavonoids, condensed tannins and polyphenols (Calixto *et al.*, 2000, Trease and Evans, 1978). Distinctive flavonoids are glycosides of naringenin, isosalipurpuroside or eriodictyol which had been given less attention initially (Bonaterra *et al.*, 2009, Nahrstedt *et al.*, 2007).

1.2.9.5 Variation in salicin content and other phenolic glycosides in willow

Salicin content in the willow varies a with age, season, location, species (Rank, 1991), (Chapter 5), (Kenstaviciene *et al.*, 2008). Kenstaviciene et al., in 2008 observed variation in salicin content in different species (from 0.04% in *S. viminalis* "Americana" to 12.06% in *S. acutifolia*). Rank, in 1991 in addition to species variation, observed that the salicin content not in all but in some species declines over summer in the USA, (Nevada and central and eastern California). On the contrary, in New Zealand when we conducted a study over the year for detection of salicin content in willow, we observed that most of the willows we studied had high salicin content over the summer (November to March) in NZ i.e. when plants were stressed.

Variation in phenolic glycosides has also been observed with differences in the sex of the plant. Some studies showed that male plants have lower salicin content and phenolic

glycosides than female plants (Elmqvist *et al.*, 1991, Palo, 1984); whereas Nichols-Orians *et al.* (1993) observed no variation in salicin content as well as phenolic glycosides content due to sex difference but they found that genetic clonal differences in willow causes variation in the phenolic glycosides.

1.2.9.6 Pharmacological properties and products of willow

Willow bark and leaves extracts are used to formulate many pharmaceutical products and dietary supplements such as an analgesic, antipyretic, antiphlogistic and weight loss enhancement remedies (Pobłocka-Olech *et al.*, 2010, Schmid *et al.*, 2001b) in different forms such as tablets, capsules, liquid formulations and powder for pain relief (Biegert *et al.*, 2004, Boullata *et al.*, 2003). The use of willow products is very common in European countries for treatment of headache, back ache, fever and to treat arthritis (Biegert *et al.*, 2004).

Apart from antipyretic, anti-inflammatory properties, willow bark and leaf extracts has shown anti-cancer, anti-oxidant and platelet aggregation effects.

Tremulacin is one of the derivatives of salicin which has anti-inflammatory effect (Yang *et al.*, 1994). Chronic inflammation can progress to cancer in many situations and in such cases anti-inflammatory/cancer activity in willow bark and leaf extract has been shown from its polyphenol and flavonoid components rather that salicin (Nahrstedt *et al.*, 2007).

Clinical studies were conducted where effect of young willow (*Salix safsaf salicacae*) leaf extract on leukemic cells of 18 patients in Egypt was observed and majority of blasts of acute myeloid leukemia were killed by leaf extracts (El-Shemy *et al.*, 2003). In 2007, same extract was used against human carcinoma cells in vivo and in vitro. In vivo Ehrlich Ascites Carcinoma Cells were injected into the intra-peritoneal cavity of mice and willow extract was fed through stomach tube. In vivo tumour growth was reduced and death was delayed. While, in vitro the willow extract killed about 70-80 % of abnormal cells. The targeted cell death in this case was occurred by apoptosis due to DNA fragmentation within treated cells. In conclusion, metabolites within the willow extract may act as tumour inhibitors that promote apoptosis, cause DNA damage, and affect cell membranes and/or denature proteins (El-Shemy *et al.*, 2007).

Clinical trial was conducted by Mills et al., in 1996 to observe the effect of willow bark extract in patients suffering from osteoarthritis resulted in lesser pain in patients (Mills *et al.*, 1996).

Chrubasik et al., in 2000 conducted a study in 210 patients with chronic low back pain where they were given high dose of willow bark (240 mg salicin) or low dose of willow bark extract (120 mg salicin) or placebo. This was a four week blinded study. At the end of four weeks higher number of patients from high dose willow bark treatment group were pain free as compared to other two groups (Chrubasik *et al.*, 2000). This study provides evidence that willow bark extract may be a useful treatment for low back pain.

A study reported by Krivoy et al., shows lesser inhibition of platelets by *Salicis cortex* extract when compared to acetylsalicylic acid (aspirin) (Krivoy *et al.*, 2001).

1.2.9.6.1 Side effects and toxicity

There are very few reports of side effects of willow bark. Vlachojannis et al., have collected and cited many studies of treatment with willow bark extract in human and they have even argued over the restriction imposed by the EMA (European Medicines Authority) limiting use of willow bark to 4 weeks (Vlachojannis *et al.*, 2014) because, in their opinion, the use of willow bark for longer duration than four weeks does not have potential risk. They have also found few reports of adverse effects due to willow bark. In 2003, a patient had severe anaphylactic reaction with history of allergy to salicylates (Boullata *et al.*, 2003). Another patient with deficiency of glucose-6-phosphate dehydrogenase developed haemolysis after taking willow herbal preparation (Baker and Thomas, 1987). One more study where 436 patients with different musculoskeletal pains were treated with willow bark extract has suggested that use of willow bark extract in long term pain might be effective as the overall adverse events during this study were low (up to 3 % in osteoarthritis and/or fibromyalgia) (Uehleke *et al.*, 2013, Vlachojannis *et al.*, 2014). However, willow does contain some gastro protective substances (Vlachojannis *et al.*, 2014, Pobłocka-Olech *et al.*, 2010, Pobłocka-Olech and Krauze-Baranowska, 2008) while of some species e.g. *S. daphnoides, S. pupurea* contain carcinogenic compounds such as pyrocatechol (Pobłocka-Olech *et al.*, 2010). Apart

from these few cases, there is no information or evidence of side effects of willow bark available.

There are no reports of any side effects due to consumption of willow in animals.

There are no reports of any toxicity in humans or animals due to willow.

1.2.9.6.2 Common popular willows

Common Name	**Scientific Name**
Golden Willow	*Salix alba*
White Willow	*Salix alba*
Silver Willow	*Salix alba*
Napoleon's Willow	*Salix babylonica*
Weeping Willow	*Salix babylonica*
Goat Willow	*Salix caprea*
Great Willow	*Salix caprea*
Pussy Willow	*Salix caprea*
Grey Willow	*Salix cinera*
Violet Willow	*Salix daphnoides*
Bitter Willow	*Salix elagnos*
Crack Willow	*Salix fragilis*
Broadleaf Willow	*Salix glaucophylloides*
Downy Willow	*Salix lapponum*
Corkscrew Willow	*Salix matsudana*
Dragon Claw Willow	*Salix matsudana*
Matsudana Willow	*Salix matsudana*

Tortured Willow	*Salix matsudana*
Black Willow	*Salix nigra*
Purple osier	*Salix purpurea*
Silesian Willow	*Salix silesiaca*
Osier	*Salix viminalis*
Golden weeping Willow	*Salix xchrysochroma*
Pussy Willow	*Salix xereichardtii*
Kemp Willow	*Salix xsepulcoalis*

1.3 PURPOSE OF THIS WORK

This thesis set out to answer the questions:

1. Is the salicin derivative salicylic acid analgesic in sheep?
2. Is natural source of salicin i.e. willow leaves have any seasonal variation in salicin concentration in New Zealand?

The work involved a number of laboratory and farm studies described in the following chapters.

2 DEVELOPMENT AND VALIDATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR DETECTION OF SALICYLIC ACID IN SHEEP PLASMA

ABSTRACT

A simple, efficient and robust HPLC method for analysis of salicylic acid in sheep plasma was developed and validated in this study. The aim of this thesis is to investigate the analgesic efficacy of salicylic acid in sheep. Therefore, there was a need to validate a HPLC method before conducting the pharmacokinetics of salicylic acid in sheep study. The various methods published for detection of salicylic acid were not validated for sheep plasma. Also lack of reproducibility was one of major drivers of developing a method specifically for this study. A proper validation protocol recommended by FDA (USA Food and Drug Administration) was followed. The limit of detection (LOD) (0.0625µg) of salicylic acid when compared to other methods showed the high sensitivity of the method. The relative standard deviation (RSD) for intra-day and inter-day variability (Mean±SD) were 1.45±1.03 and 1.58±1.26% respectively. Linearity of the method for salicylic acid was 0.9996. Overall RSD (Mean±SD) for recovery was 1.32±0.39%. Sheep plasma samples were successfully analysed with this method for salicylic acid as aimed. The HPLC method developed here is simple, reproducible and may be adapted to any species.

2.1 INTRODUCTION

There are numerous HPLC techniques developed and reported in the literature for analysis of salicylic acid (SA). These techniques have been developed for the detection of salicylic acid in biological fluids such as plasma, serum, urine, tissue exudate and other matrices. Rumble et al. (1981) developed an HPLC method to analyse aspirin and its metabolites including SA in human plasma and urine. Although they introduced very simple method of preparation for plasma samples, they did not mention the lower limit of detection (LOD) for either analyte. The lowest SA standard concentration mentioned in Rumble and co-workers'

study was 0.5µg/mL. (Reidl, 1983) developed a HPLC technique for detection of aspirin, SA and other metabolites in the plasma, urine and tissue homogenates of rabbit and human. The LOD for this method was 0.2µg/mL. Also, the column used in this method was LiChrosorb RP-18 (15 cm X 4 mm I.D., packed with 5 µm) and was thermostatic at 45ºC in a water bath. The working backpressure of the pumps of HPLC system was 27.5Mpa and the flow rate of the mobile phase (methanol: water; 40:60) was 1.5mL/minute during the analysis. This method had lower retention time of 5.5 minutes for the SA at the detector wavelength of 280nm. Similarly, Fogel et al. (1984), O'Kruk et al. (1984), Mays et al. (1984) developed other HPLC techniques using UV detectors with different columns and HPLC systems. The LOD of SA reported by these authors was in the range of 0.2 to 1µg/mL suggesting possibility of further improvement in sensitivity of the method. Moreover, the extensive course of sample preparation described by these authors is time consuming. The pre-processing of blood samples for storage immediately post-collection from animals/humans was required for the unstable compounds such as aspirin in the study reported by (Rumble *et al.*, 1981) as it is hydrolysed quickly (Shaw and Tsai, 2012); on the contrary, for our study pre-processing of sample was not required due to the compound to be analysed (SA) being stable. In the present analysis, only SA assayed. Other HPLC techniques described by Mallikaarjun et al. (1989), Jian-Hua and Smith (1996), Pirola et al.(1998), Sawyer and Kumar (2003), Pirker et al. (2004), Croubels et al. (2005), Chaudhary et al.(2010), Shaw and Tsai (2012) include simultaneous determination of aspirin and SA. These methods are not sensitive enough for a pharmacokinetic study in sheep. The reported LOD in these methods was 0.2µg/mL, only. In our study, we will be using a lower dose of SA which may need a very sensitive method for analysis. These methods also were not reproducible in our laboratory, which limits their use. Likewise, none of these methods was developed specifically for sheep plasma which contributed to the problems. Therefore, in the present study attempts were made to develop and validate a HPLC technique to analyse SA. This method of analysis will further be used in the pharmacokinetic study of SA in sheep after administration of sodium salt of SA (sodium salicylate) in the chapter 3, pharmacokinetics of sodium salicylate (NaS) in sheep.

This chapter is structured in two parts. First part includes the preliminary work carried out and the selection of final method. Second part describes the validation study.

2.2 [A] PRELIMINARY WORK AND DEVELOPMENT OF HPLC TECHNIQUE FOR DETERMINATION OF SALICYLIC ACID IN SHEEP PLASMA

2.2.1 HPLC system

The HPLC system consisted of two LC-20 AD pumps, SIL- 20 AC HT auto-injector, SPD-M20A diode array detector (DAD), CTO-20A column oven, DGU-20 A3 degasser (Shimadzu Japan). All the chromatographs were analysed in the software LC Solutions (Shimadzu, Japan). The DAD was set at 230nm wave length for SA detection throughout the experimental procedure.

2.2.2 Reagents and solutions

All reagents used in the experiment were HPLC grade. Acetonitrile methanol and acetic acid (glacial, 100% anhydrous) were purchased from Merck, KGaA; 64271 Darmastadt, Germany. Orthophosphoric acid (OPA) was purchased from BDH Limited, Poole, England. Milli-Q water (Milli-q PFplus system, Millipore Cooperation, USA) was used during the experiment.

The stock solution was prepared by dissolving 0.5 g of NaS (Laboratory grade reagent, Fisher Scientific UK) in Milli-q water (1000µg/mL concentration). Salicylic acid (SA) standards at different concentrations ranging from 100µg/mL to 0.03125µg/mL were prepared by serial dilutions of the stock solution with the mobile phase.

2.2.3 Column selection

Three different columns were used during preliminary experiments. Initially a Phenomenex, C18A (Luna® 5 µm C18 100 Å, LC Column 150 x 4.6 mm) reversed phase (RP) column was used. It was then replaced by Phenomenex, Kinetex (C18, 100 µm, 150 x 4.6 mm) RP column. The third column used during this developmental process was Synergi Hydro® (C18, RP 4µ, 80 Å, LC Column 150 x 4.6 mm) column. The column selection was based on peak height, resolution and column back pressure and stability.

2.2.4 Column temperature

The column temperature was initially set at 32ºC. It was then increased to 35ºC and finally to 40ºC during the analysis.

2.2.5 Mobile phase composition

Initially mobile phase was composed of water: acetonitrile: acetic acid (71:28:1). It was further altered with respect to analytical column. In the new mobile phase, acetic acid was replaced by OPA. Therefore, this mobile phase was composed of water: acetonitrile: OPA (71:28:1).

2.2.6 Mobile phase flow rate

Mobile phase was initially run at flow rate of 1mL/minute. It was gradually reduced during experimental conditions to 0.8mL/minute and further to 0.6mL/minute. However, 0.8mL/minute flow rate of mobile phase was retained as a working flow rate.

2.2.7 Sample preparation methods

Two types of sample preparation methods were tried during the developmental procedure. Initially a liquid-liquid extraction (LLE) method was used. It was followed by solid phase extraction (SPE).

2.2.7.1 Liquid-liquid extraction

During this process, 300µL of the drug-free sheep plasma was spiked with a known amount of SA standard (500µL). This (300µL plasma + 500 µL of known SA standard) was considered as a sample. This sample was diluted with 5 mL of 10% acetic acid in acetonitrile followed by vortex mixing for 20 seconds and the centrifugation at 3000g for 20 minutes. Supernatant was separated and dried under compressed air at 30ºC. Dried sample was reconstituted with 200µL of mobile phase and again centrifuged (3000g) for 10 minutes. The volume of 50µL of this sample was injected in the HPLC machine for analysis. Further variations to this method are listed in table 2.1. These variations include, a change in volume of plasma, volume of spiked standard, concentration and pH of acetonitrile used for precipitation of plasma proteins.

2.2.7.2 Solid phase extraction

2.2.7.2.1 Method 1

Phenomenex strata-X 3ml, 60mg SPE cartridge was used for solid phase extraction. The plasma sample was prepared by using 500µL drug-free sheep plasma and 500µL of SA standard of known concentration. This sample was vortex mixed for 30 seconds. The SPE cartridge was activated with 1 mL of 100% methanol followed by equilibration with 1 mL of Milli-Q water. The vortex mixed sample was then loaded on to the SPE cartridge followed by washing with 50% methanol. Ten minutes drying period was allowed under full vacuum and the elution was collected in a test tube as a sample in 1 mL of 100% methanol. This eluate was dried under a gentle stream of compressed air at 50ºC and reconstituted with 500µL of mobile phase followed by 30 seconds of vortex mixing. The reconstituted sample was then transferred to an Eppendorf vial for centrifugation at 14000g for 10 minutes. 50µL of this final sample was injected in the HPLC machine for analysis.

2.2.7.2.2 Method 2

The plasma sample used in this method was pre-processed with LLE. Initially 1 mL drug-free sheep plasma was spiked with 500µL of known concentration of SA and vortex mixed together. This mixture was diluted by adding 5mL of 10% acetic acid in acetonitrile and was centrifuged at 3000 g for 20 minutes. The supernatant was separated and 1 mL of this supernatant was used as a sample for SPE. Remaining procedure for SPE was followed as described in method 1 with only exception of volume of mobile phase used to reconstitute the dried plasma sample which was reduced to 400µL in this method.

We also tried many variations in the SPE method by changing the volume of plasma, time spent on vortex mixing and change in the drying temperature. All these modifications are listed in tables 2.2 and 2.3.

2.2.8 Results

2.2.8.1 Chromatographic conditions

The initial C18A (Luna® 5 µm C18 100 Å, LC Column 150 x 4.6 mm) reversed phase (RP) column was used in all LLE methods. Although, in the method 10 (table 2.1) drug recovery

was optimum; the backpressure of the column was high - above 15MPa. High backpressure caused the HPLC system to stop frequently. The retention time for SA with this column was 12.8±0.9 minutes.

The Luna column was replaced by Phenomenex Kinetex (C18, 100 µm, 150 x 4.6 mm) RP column. Although retention time for SA with this column was significantly less at 5.0±0.6 minutes, the higher column backpressure than the Luna column made us change to another HPLC column.

Synergi Hydro® (C18, RP 4µ, 80 Å, LC Column 150 x 4.6 mm) column resulted in stable column backpressure and optimum drug recovery with the SPE sample preparation method. The retention time of the SA with this column was 9.4±0.5 minutes. Overall performance of Synergi Hydro column was superior to the other two columns. The chromatographs of same SA concentrations in three columns are shown in figure 2.1.

A column temperature of 32ºC exhibited higher column backpressure and increased retention time as compare to 35ºC. Further increase in the column temperature to 40ºC lowered the backpressure of column and retention time of SA without affecting drug recovery, and peak resolution.

The mobile phase flow rate of 1mL/minute generated more than 10MPa column back pressure. A reduction of flow rate to 0.8mL/minute lowered the column backpressure to 6-7MPa without any effect on peak resolution. However, a further reduction of mobile phase flow rate to 0.6mL/minute resulted in reduction in peak efficiency. Chromatographs in figure 2.2 shows the prolonged retention time and altered peak efficiency with reduced flow rate of mobile phase. Hence, the final flow rate of the mobile phase was set at 0.8ml/minute.

2.2.8.2 Sample preparation methods

2.2.8.2.1 Liquid-liquid extraction

The original LLE method (method 1, table 2.1) resulted in enormously high recoveries of the drug. These recoveries were inappropriate as they were more than 1000% and were unacceptable. Therefore, further LLE methods were developed. An inconsistency in drug recoveries ranging from 65 to 300% was obtained with Methods 2 and 3.

Table 2.1: Modifications in LLE phase method

Method No.	Sample (µL)	Acetic Acid	Vortex mixing	Centrifugation	Supernatant	Drying	Reconstitution (mobile phase) + centrifugation	Injection volume
1.	300+500µL standard	5mL (10 %)	20 sec	3000 rpm, 20 mins	Yes	32 ºC	200 µL	50µL
2.	300+500µL standard	5mL (10 %)	20 sec	3000 rpm, 20 mins	Yes	32 ºC	300 µL	50µL
3.	300+500µL standard	5mL (10 %)	20 sec	3000 rpm, 20 mins	Yes	32 ºC	400 µL	50µL
4.	300+500µL standard	5mL (10 %)	20 sec	3000 rpm, 20 mins	Yes	32 ºC	500 µL	50µL
5.	500+500µL standard	5mL (10 %)	20 sec	3000 rpm, 20 mins	Yes	32 ºC	500 µL	50µL
6.	500+500µL standard	2 mL (100%)	20 sec	3000 rpm, 20 mins	Yes	32 ºC	500 µL	50µL
7.	1000+ 500µL, standard	5mL (10% acetic acid in acetonitrile)	30 sec	3000 rpm, 20 mins	Yes	32 ºC	400µL	50µL
8.	1000+ 500µL, standard	5mL (10% acetic acid in acetonitrile)	30 sec	3000 rpm, 20 mins	Yes	32 ºC	200µL	50µL
9.	1000+ 500µL, standard	5mL (15% acetic acid in acetonitrile)	30 sec	3000 rpm, 20 mins	Yes	32 ºC	200µL	50µL
10.	500+500µL standard	5mL (15 % acetic acid in acetonitrile)	30 sec	3000 rpm, 20 mins	Yes	32 ºC	500 µL	50µL

Table 2.2: Modifications in SPE method

Method No.	**1**	**2**	**3**	**4**	**5**
Sample	500µL plasma	1mL plasma*	1mL plasma*	1mL plasma**	1mL plasma**
Dilution	500µL standard	-	-	-	-
Vortex mixing	30 seconds	-	-	-	-
SPE cartridge activation	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% methanol
SPE cartridge equilibration	1mL, Milli-q water	1mL, Milli-q water	1mL, Milli-q water	1mL, Milli-q water	1mL, Milli-q water
SPE cartridge Sample load	1mL plasma + standard	1mL plasma	1mL plasma	1mL plasma	1mL plasma
SPE cartridge washing	1mL 20% methanol	1mL 20% methanol	1mL 20% methanol	1mL 20% methanol	1mL 20% methanol
Drying under full vacuum	10 minutes	10 minutes	10 minutes	10 minutes	10 minutes
Elution	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% methanol
Drying	50 ºC, gentle air pressure	50 ºC, gentle air pressure	50 ºC, gentle air pressure	50 ºC, gentle air pressure	50 ºC, gentle air pressure
Reconstitution	500µL mobile phase	400µL mobile phase	200µL mobile phase	400µL mobile phase	200µL mobile phase
Injection volume	50µL	50µL	50µL	50µL	50µL
Column temperature	40 ºC	40 ºC	40 ºC	40 ºC	40 ºC
Flow rate	0.8mL/min	0.8mL/min	0.8mL/min	0.8mL/min	0.8mL/min

***** Processed with LLE to get final 1 mL concentration for SPE (1mL plasma+500µL standard vortex mixed, followed by mixing with 5 mL of 10% acetic acid in acetonitrile and centrifuged at 3000 rpm for 20 minutes. Separated the supernatant and 1mL of this was used as sample for SPE)

****** Processed with LLE to get final 1 mL concentration for SPE (1mL plasma+500µL standard vortex mixed, followed by mixing with 5 mL of 15% acetic acid in acetonitrile and centrifuged at 3000 rpm for 20 minutes. Separated the supernatant and 1mL of this was used as sample for SPE)

Table 2.3: Further modifications in SPE method

Method No.	6	7	8	9	10
Sample	500µL Plasma	500µL Plasma	500µL Plasma	500µL Plasma	500µL Plasma
Dilution	500µL Standard	500µL Standard	500µL Standard	500µL Standard	500µL Standard
Vortex mixing	30 seconds	30 seconds	30 seconds	30 seconds	2 minutes
SPE cartridge activation	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% methanol
SPE cartridge equilibration	1mL, Milli-q water	1mL, Milli-q water	1mL, Milli-q water	1mL, Milli-q water	1mL, Milli-q water
SPE cartridge Sample load	1mL (plasma + standard)	1mL (plasma + standard)	1mL (plasma + standard)	1mL (plasma + standard)	1mL (plasma + standard)
SPE cartridge washing 1	1mL 50% methanol	1mL 50% methanol	1mL 20% methanol	1mL 20% methanol	1mL 20% methanol
SPE cartridge washing 2	-	1mL 50% methanol	-	-	-
Drying under full vacuum	10 minutes	10 minutes	10 minutes	10 minutes	10 minutes
Elution	1mL, 100% methanol	1mL, 100% methanol	1mL, 100% Acetonitrile	Washing 1 (20%methanol)	1mL, 100% methanol
Drying	50 ºC, gentle air pressure	50 ºC, gentle air pressure	50 ºC, gentle air pressure	50 ºC, gentle air pressure	40 ºC, gentle air pressure
Reconstitution	300µL mobile phase	300µL mobile phase	300µL mobile phase	300µL mobile phase	300µL mobile phase
Injection volume	50µL	50µL	50µL	50µL	50µL
Column temperature	40 ºC	40 ºC	40 ºC	40 ºC	40 ºC
Flow rate	0.8mL/min	0.8mL/min	0.8mL/min	0.8mL/min	0.8mL/min

These methods were not considered further. The methods 4, 5 and 6 yielded low drug recoveries - less than 50% and were also unacceptable. Methods 7, 8 and 9 could not produce prominent peaks. Table 2.4 shows findings after all the modifications incorporated in LLE.

Finally, method 10 with LLE worked with good recoveries and improved peaks in terms of shape. However, the column backpressure was elevated and the HPLC pumps cut out during analysis

2.2.8.2.2 Solid phase extraction

Solid phase extraction of the sample as described in method 1 (table 2.2) resulted in inadequately low drug recoveries up to 8% (table 2.5). Similarly, other SPE methods 2, 3, 4 and 5 carried out with the samples pre-processed by LLE also produced lower recoveries up to 19% (table 2.5).

Sample preparation with SPE in methods 6, 7 and 8 also resulted in inconsistent drug recoveries and ranged from 21 to 61% (table 2.5). Method 9 produced very poor recoveries up to about 2%.

However, method 10 with further minor modifications was able to produce ideal drug recoveries (up to 103%) and simultaneously, stable pressure was maintained. Therefore, this method of sample preparation was considered further for validation study.

Table 2.4: Findings after incorporation of different modifications in LLE as described in table 2.1

Method No.	Findings
1 -	Recoveries of SA more than 1000%.
2 -	68 to 70% recoveries for higher standards (110 and 10 µg)and 117 to 300% recoveries for lower standards (62.5 and 31.25ng) of SA
3 -	Similar to method 2. All-over recoveries of SA ranged from 65 to 245%
4 -	Lower recoveries of SA ranging from 10-20%
5 -	Lower recoveries of SA (22-25%)
6 -	Lower recoveries of SA (15-25%)
7 -	No prominent peaks
8 -	No prominent peaks
9 -	No prominent peaks
10 -	78-95% recovery of SA

Table 2.5: Findings after incorporation of different modifications in SPE as described in table 2.2 and 2.3

Method No.	Findings
1 -	Very low recoveries of SA 6-8%
2 -	Very low recoveries about 8-10%
3 -	About 7% recoveries for all SA standards tested
4 -	7-16% recoveries of SA
5 -	6-19% recoveries of SA
6 -	42-61% recoveries of SA
7 -	21-33% recoveries of SA
8 -	38-50% recoveries of SA
9 -	1.5- 2.05% recoveries of SA
10 -	92-103% recoveries of SA

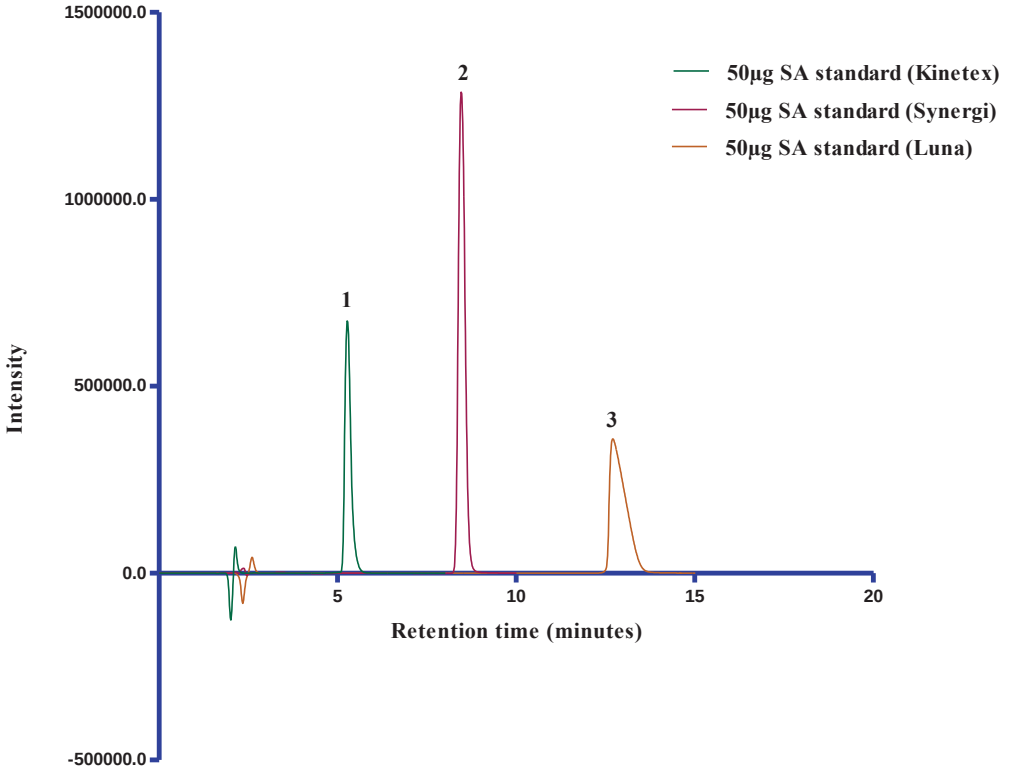

***Fig 2.1**: Chromatographs of 50µg SA standard in the mobile phase. Peak 1 represents SA extraction with Phenomenex Kinetex C18 column; although, the retention time is lesser (5.0±0.6 minutes), peak resolution is not ideal. Peak 2 represents SA extraction with Synergi Hydro C18 column; the peak resolution is superior to other two columns and retention time (9.4±0.5 minute) is increased as opposed to Kinetex column. Peak 3 represents SA extraction with Luna C18 column with poor peak resolution and higher retention time (12.8±0.9) minutes*

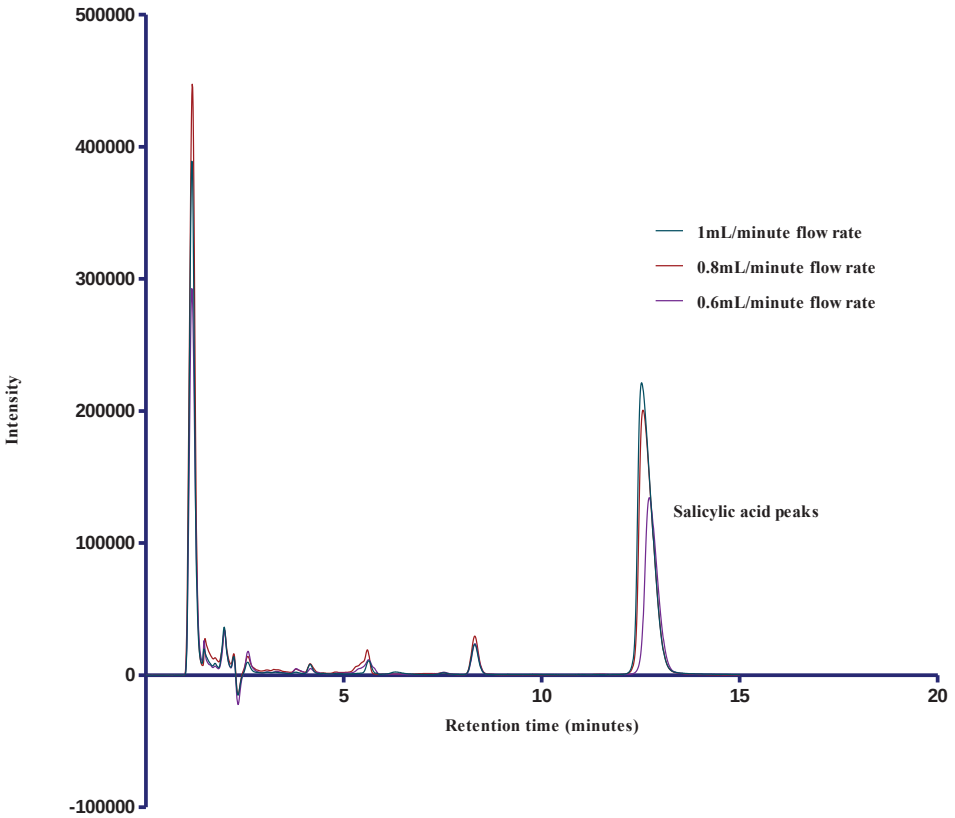

Fig 2.2: *Chromatographs of 10µg SA standard in blank plasma at different flow rates of mobile phase. The peak efficiency with lowest flow rate 0.6mL/minute is lower (3596.74 N). Peak efficiency with flow rate 0.8ml/minute and 1mL/minute is 17874.67N and 17882.24N respectively which are not significantly variable*

**Peak efficiency was calculated with the formula N (peak efficiency) = t_r (retention time)/$W_{0.5}$ (width of the peak's half height)*

2.2.9 Discussion

A simple and robust method for analysis of SA in sheep plasma was developed. The HPLC methods published so far for SA were irreproducible. Thus for this study, it was necessary to develop and validate a sensitive and reproducible method for SA analysis in sheep plasma samples.

During the development of this HPLC method a variety of chromatographic conditions were considered and performance checks were conducted to assure optimum peak efficiency, comparable retention time and recovery of our analyte of interest.

The column selection was prioritised as it is important to select the ideal column for isolation of analyte (Gilroy *et al.*, 2003, Cruz *et al.*, 1997). As described in the results, three different analytical columns (LunaC10 RP, Kinetex C18 RP and Synergi Hydro C18 RP) were used and the Synergi Hydro, C18, RP had optimum performance and was selected for the final method. We first tested Luna C18 column, based on the published literature and also because it is the most commonly used column in drug analysis. We experienced exceptionally high back pressure with the Luna C18 column. Thus, we decided to test Kinetex C18 due to its new core-shell technology. In other columns the particles of packaging material are completely porous silica particles providing a porous surface. In contrast, core-shell technology in Kinetex provides non-porous surface to provide better and faster separation of the analyte (Fekete *et al.*, 2012). The Kinetex C18 also gave high back pressure. The possible reasons for high backpressure could be the bonding of stationary phase in the column and the impurities in the final sample which might deposit on the non-porous surface of column packing material to cause higher backpressure. On the other hand, the Synergi Hydro column had bonded phase endcapped with a proprietary polar group which provides optimum retention of both hydrophobic and polar compounds through polar interactions, hydrogen bonding or electrostatic interactions. This improved peak efficiency and produced stable column backpressure. Thus, this column gave higher peak resolution and efficiency along with lower back pressure compared to other HPLC columns tested in this experiment.

The column temperature also plays an important role in elution of an analyte (Vanhoenacker and Sandra, 2007). It is inversely proportional to the column pressure and retention time.

Increase in column temperature decreases column pressure and retention time of the analyte and vice-versa (Poppe *et al.*, 1981). Initially column temperature was maintained at 32ºC, it was then increased to 35ºC and finally to 40ºC which resulted in lower retention time as well as lower column pressure. Also, peak efficiency and drug recoveries were unaffected due to change in temperature. Hence, the final column temperature was set at 40ºC.

Mobile phase used in this experiment did not require additional buffer solution to maintain the pH. Moreover, gradient flow rate of the mobile phase as reported by other researchers was not necessary for this method. Acetic acid in the mobile phase was replaced with OPA. The peak resolution and reproducibility were improved with OPA as the SA is well dissociated with this stronger acid (Heyrman and Henry, 1999). In addition, OPA yielded stable pressure in association with Synergi Hydro-RP column compared to acetic acid. Similar improvements in peak resolution and pressure stability were observed by (Long and Henderson Jr, 2009) after replacement of acid component of mobile phase with alternate acid for pH maintenance.

The mobile phase components described by (Fogel *et al.*, 1984) are similar to our final mobile phase components. However, their ratio (76:24:0.5) differs from the one which we used in our method (71:28:1). A similar isocratic flow rate of different mobile phase composition without phosphate buffer was described by (Sawyer and Kumar, 2003) for separation of SA and aspirin in the effervescent tablets.

Mobile phase flow rate was set to 0.8mL/minute as lower back pressure was maintained without compromising the peak efficiency as opposed to high back pressure with 1mL/minute (figure 2.1). Generally, high flow rate of mobile phase results in high column pressure and vice-versa (Graham and Rogers, 1980).

Recovery of the drug in the first method was abnormally high (more than 1000%), which could be due to the dilution of the blank plasma with SA. A 300µL of drug free plasma sample was spiked with 500µL of SA. After drying under gentle stream of air, it was reconstituted 200µL of mobile phase. Thus, the higher recoveries were due to dilution error.

In other LLE methods, modifications in the method of sample preparation were carried out to achieve high recoveries and a cleaner sample. The various volumes at different

percentages of acetic acid were used to enhance plasma protein precipitation to improve isolation of analyte. Also, vortex mixing time was increased from 20 seconds to 30 seconds for better mixing of plasma with acetic acid to ensure protein precipitation. Similarly, to obtain higher recoveries, either plasma sample volumes or mobile phase volumes (for re-constitution) or both were altered. However, due to loss of drug (either protein binding or loss during separation in LLE) the recoveries in these methods were inadequate. Though method 10 with LLE worked well to achieve ideal recoveries of SA; the increase in column back pressure limited the use of this method. In this case, back pressure might have increased due to the deposition of proteins or impurities in the plasma sample on to the column surface.

The SPE method was introduced to improve recoveries and clean-up of the plasma samples. While using this method, initially few plasma samples were pre-processed with LLE followed by SPE to assess the recovery. However, the drug loss was obvious and would have occurred due to pre-LLE processing which resulted in poor recoveries. Another probable cause for the loss of the drug could be extensive (twice) washing during the SPE clean-up procedure. Therefore, to prevent excessive loss of drug during washing, further change was incorporated where the sample was washed with 20% methanol rather than 50% methanol. Also, for elution of sample, methanol (100%) was replaced by acetonitrile (100%) to see if recovery improved. However, the findings were not satisfactory. Methanol has been reported to have highest strength to elute (Norberg-King, 2005). Due to inconstant and low recoveries, the possibility of loss of drug during the washing procedure was presumed. This loss was confirmed by using the post-washing (with 20% methanol) waste solution as elute and processed further to prepare final sample. The recoveries obtained due to this modification were even lower. This showed that there was no significant loss of drug during the washing process. Finally, to ensure proper mixing of plasma and the drug, the samples were vortex mixed for 2 minutes instead of 30 seconds. Another concern about the loss of drug at high temperature (50ºC) during drying was considered and the temperature for drying was reduced to 40ºC as this is the commonly used temperature in routine sample preparation methods (Vaudry and Biggio, 2011). These two variations confirmed the importance of mixing of samples and critical temperature to consider for drying the sample to prevent the loss of drug from it. Finally, ideal recoveries of the drug were obtained with this last method (10) of SPE.

2.3 [B] VALIDATION OF HPLC TECHNIQUE FOR DETECTION OF SALICYLIC ACID IN SHEEP PLASMA

2.3.1 Material and methods

2.3.1.1 Reagents and solutions

Acetonitrile HPLC grade was purchased from Merck, KGaA; 64271 Darmastadt, Germany and Orthophosphoric acid from BDH Limited, Poole, England. Sodium salicylate (Laboratory grade reagent) was purchased from Fisher Scientific UK. The stock solution of NaS was prepared in Milli Q water (Milli-q PFplus system, Millipore Cooperation, USA) by dissolving 0.1g NaS in 100mL. The working standard solutions were prepared in mobile phase fresh daily from the stock solution.

2.3.1.2 Sample preparation and analysis

2.3.1.2.1 Solid phase extraction

All samples and standards (spiked in blank the plasma) were prepared for analysis by using solid phase extraction (SPE) method with Phenomenex strata-X 3ml, 60mg SPE cartridge (Phenomenex, Auckland, New Zealand). A 500µL of plasma was spiked with either 500 µL of a known concentration of NaS standard or Milli-Q water (in case of plasma sample obtained from the test sheep administered with NaS). The sample was vortex mixed for 2 minutes. The cartridge was activated by 1 mL (100 %) methanol followed by equilibration with 1 mL of Milli-Q water. 1 mL sample was loaded followed by a single washing with 20% methanol. The cartridge was then dried for 10 minutes under full vacuum. The elution was carried out with 1 mL of 100% methanol, collected in a glass test tube. This eluent was dried under the gentle stream of compressed air at 40ºC and reconstituted with 300µL of mobile phase. The sample was vortex mixed for 30 seconds and then centrifuged at 14000 rpm for 10 minutes. The injection volume was 50 µL. Each sample was injected twice in the HPLC machine

2.3.1.3 Chromatographic conditions

The mobile phase was prepared by mixing water, acetonitrile and orthophosphoric acid at 71:28:1 proportion. The pH of mobile phase was 2.54. Mobile phase was filtered through

0.2µm membrane filter. Analysis of the samples was carried out under isocratic conditions. The flow rate was 0.8mL/minute. A Synergi Hydro® (C18, RP 4µ, 80 Å, LC Column 150 x 4.6 mm) column was used for sample separation. The oven temperature was set at 40ºC. All the chromatograms were analysed at 230nm wavelength.

2.3.1.4 Selectivity/Specificity

The selectivity of the method was detected by analysing the drug-free plasma sample from 10 different sheep, processed with the same method.

2.3.1.5 Accuracy/Recovery

Accuracy of the method was determined by spiking three different concentrations of the standards of SA (from the stock solution) in the blank plasma samples. Recovery of the SA was obtained by comparing the peak areas of spiked plasma samples to that of the pure standards of the SA using linear regression model for estimation of the true recovery.

2.3.1.6 Precision

Precision of the HPLC method was checked by intra- and inter-day variation. Three concentrations of SA (50, 0.5 and 0.25 µg/mL) spiked with the blank sheep plasma were analysed for three consecutive days. The samples for each concentration were prepared every day and were run in three different batches each day in triplicate for intra-day variation. This was repeated for three different days to check inter-day variation.

2.3.1.7 Linearity

Five different concentrations of SA ranging from 50 to 0.125µg/ml were spiked with blank plasma (three sets; set 1, set 2, set 3) and their linearity was assessed by plotting the graph of peak areas against the concentrations. Linear regression model in the statistical software GraphPad Prism 5 was used to assess the linearity.

2.3.1.8 Limit of quantification

A range of low concentrations of SA (0.0625, 0.125, 0.250, 0.50 µg/mL) were analysed to detect the quantifiable limits.

2.3.1.9 Standards

Six different concentrations of NaS standards, 50µg/mL, 5µg/mL, 0.5µg/mL, 0.25µg/mL, 0.125µg/mL and 0.0625µg/mL were prepared from stock solution in mobile phase (diluting the stock solution with mobile phase to get desired standard concentration) to make a calibration curve.

2.3.2 Chromatographic analysis of sheep plasma samples (pilot study)

This pilot study was approved by Massey University Animal Ethics Committee. Two sheep were administered an intravenous dose of NaS at 50mg/kg body weight in right jugular vein. Blood samples were collected at 0, 0.25, 0.5, 1, 2, 4 and 6 hours in heparinised vacutainers from the left jugular vein. The samples were immediately placed on ice and were centrifuged at 7000g for 15 minutes. The plasma was separated and stored at -70^{0}C until the day of analysis.

2.3.3 Results

The method detected the presence of SA in sheep plasma sample at the specified retention time (9.4±0.5 minutes). Figure 2.3 shows three chromatographs, the first chromatograph of a blank plasma sample demonstrates the absence of a peak at the retention time for SA. The second chromatograph of plasma sample spiked with known (50µg) SA exhibits the SA peak; whereas, SA standard (50µg) in mobile phase also shows the peak at the same retention time. Recovery for the tested (50µg, 0.5µg, 0.25µg) concentrations ranged between 97.67±0.004% (for 0.25µg/mL) to 102.67±0.01% (for 0.5g/mL). Relative standard deviation (RSD %) for recoveries was ranging between 0.87 to 1.51% (table 2.6). The formula used to calculate RSD is as below:

$$RSD\ (\%) = \frac{Standard\ Deviation\ (SD)}{Mean} \times 100$$

Table 2.7 and 2.8 shows the intra- and inter-day variation respectively. The maximum RSD % for intra- and inter-day variation was 3.38 and 2.73% respectively for the concentration 0.5µg/mL of NaS. Mean r^2 (correlation coefficient) of linearity was 0.9996± 0.00056. Table 2.9 shows the slope, Y intercept and r^2 values for three different sets of SA concentrations. Figures 2.4 and 2.5 show the peaks for different lower concentrations of SA in mobile phase

and spiked in blank plasma. The limit of quantification based on the signal to noise ratio of 10 in this method for SA was 0.0625µg (figures 2.4 and 2.5). The calibration/standard curve (Figure 2.6) was prepared using liner regression model (r^2= 0.9999) in software GraphPad Prism (Prism 5 for windows, version 5.01, August, 2007, GraphPad Software, Inc. CA, USA). Figure 2.7 shows the concentration time curve for SA after HPLC analysis of sheep plasma sample collected for pilot study. The maximum concentration (C_{max}) observed during this study was 18.02µg at 15 minutes (T_{max}) after drug administration while the minimum concentration was observed (1.17µg) at 2 hours.

Table 2.6: Recovery of SA after spiking in the blank plasma

SA(µg/mL) spiked	Mean (n=9) SA (µg/mL) recovered	Recovery (%)	SD (n=9)	RSD (%)
50	49.68	99.35	0.43	0.87
0.50	0.52	102.67	0.01	1.58
0.25	0.25	97.67	0.004	1.51

Table 2.7: Intra-day variation (between batches) of SA standards spiked in blank plasma

Intra-day variation						
Day 1						
SA (µg/mL)	**Batch 1 AUC**	**Batch 2 AUC**	**Batch 3 AUC**	**Mean AUC**	**SD**	**%RSD**
50	17391069	17161620	17139974	17230888	139142	**0.81**
0.5	186428	188462.70	196570.30	190487	5366	**2.82**
0.25	92528.33	93838.67	95898	94088.33	1698	**1.81**
Day 2						
SA (µg/mL)	**Batch 1 AUC**	**Batch 2 AUC**	**Batch 3 AUC**	**Mean AUC**	**SD**	**%RSD**
50	17107325	17151203	17215729	17158086	54529	**0.32**
0.5	182108.70	180526.70	183519.30	182051.60	1497	**0.82**
0.25	92888.67	92537.67	94937.67	93454.67	1296	**1.39**
Day 3						
SA (µg/mL)	**Batch 1 AUC**	**Batch 2 AUC**	**Batch 3 AUC**	**Mean AUC**	**SD**	**%RSD**
50	17326608	17009368	17177841	17171273	158722	**0.92**
0.5	175659	180970	187883.70	181504.20	6130	**3.38**
0.25	90166.33	91258.67	91483.33	90969.44	705	**0.78**

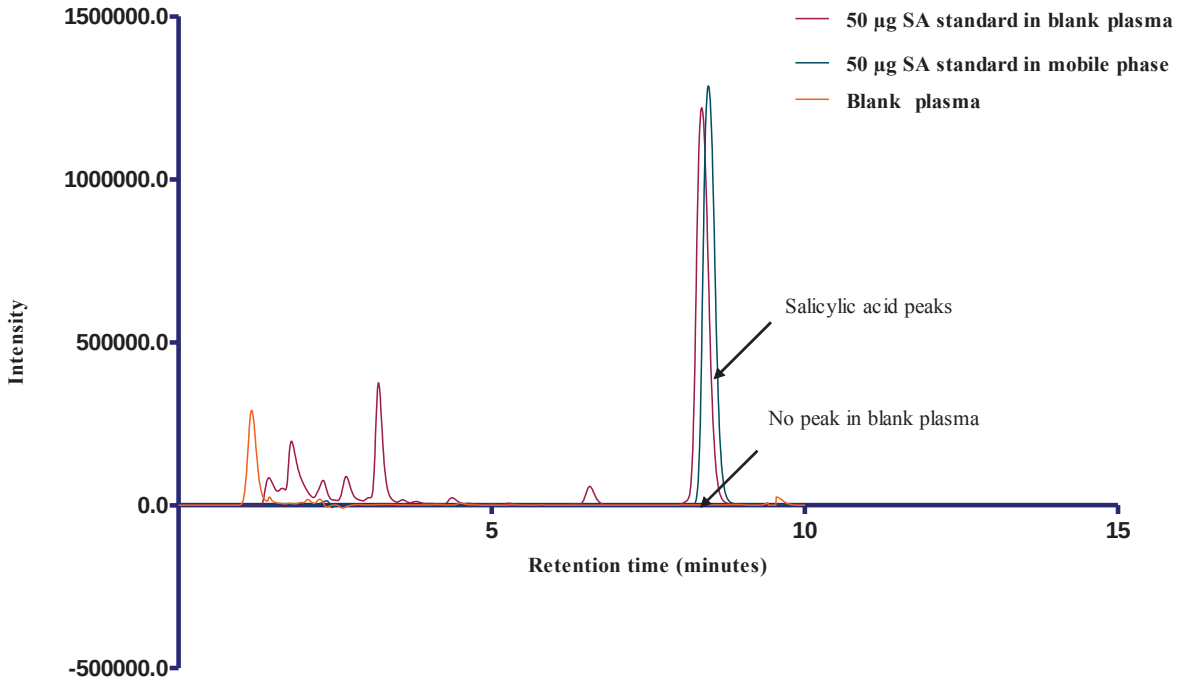

***Fig 2.3**: Chromatographs showing absence of SA (SA) peak in the blank plasma and presence of SA peaks in mobile phase and SA spiked blank plasma sample*

Table 2.8: Inter-day variation (between days) of SA standards spiked in blank plasma

Inter day variation						
	Mean AUC's (n=3)			AUC		
SA (µg/mL)	**Day 1**	**Day 2**	**Day 3**	**Mean**	**SD**	**% RSD**
50	17230888	17158086	17171273	17186749	38790	**0.23**
0.5	190487	182051.6	181504.2	184680.9	5036	**2.73**
0.25	94088.33	93454.67	90969.44	92837.48	1648	**1.78**

Table 2.9: Results of validation of linearity for SA detection method

	Slope	**y- intercept**	**r^2**
Set 1	167090	100830	0.9996
Set 2	167071	107302	0.9996
Set 3	177099	104822	0.9997
Mean	**170420**	**104318**	**0.9996333**
SD	5784.19	3265.30	0.000056
% RSD	**3.39**	**3.13**	**0.0058**

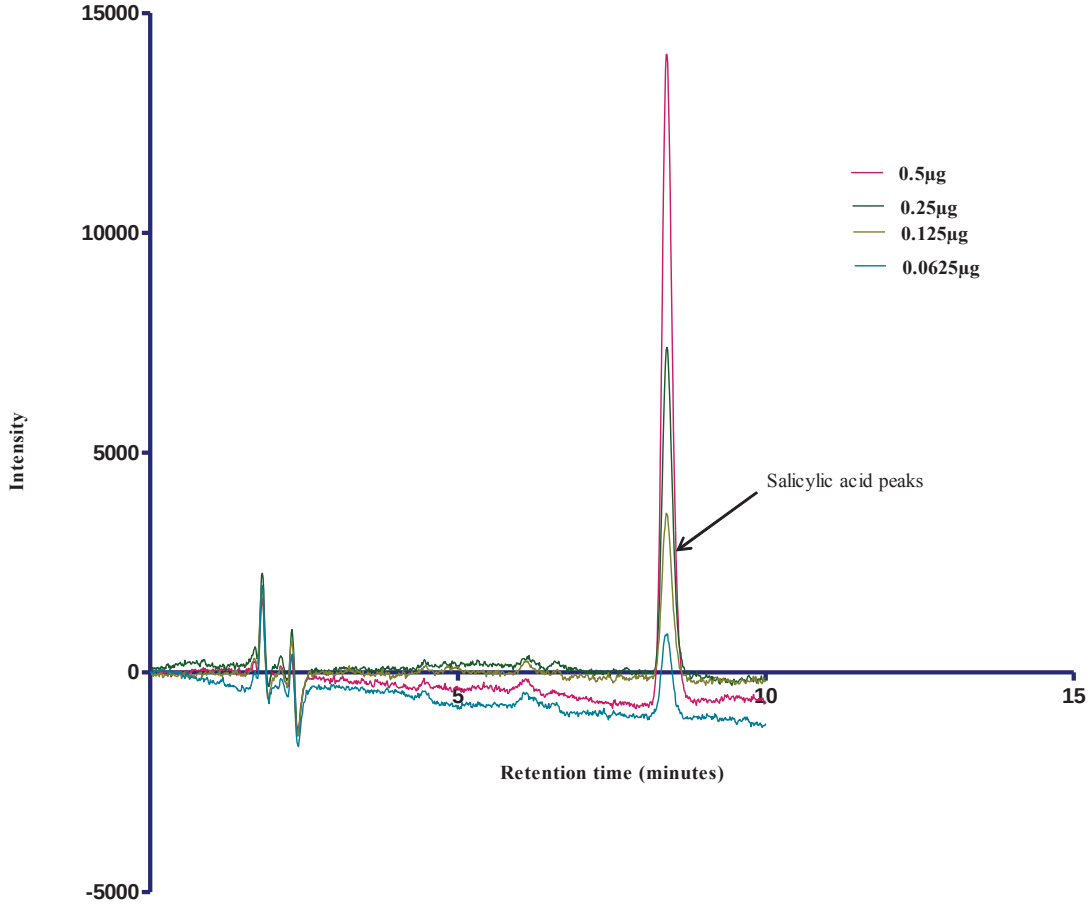

Fig 2.4: *Chromatographs showing lower concentrations of SA in mobile phase*

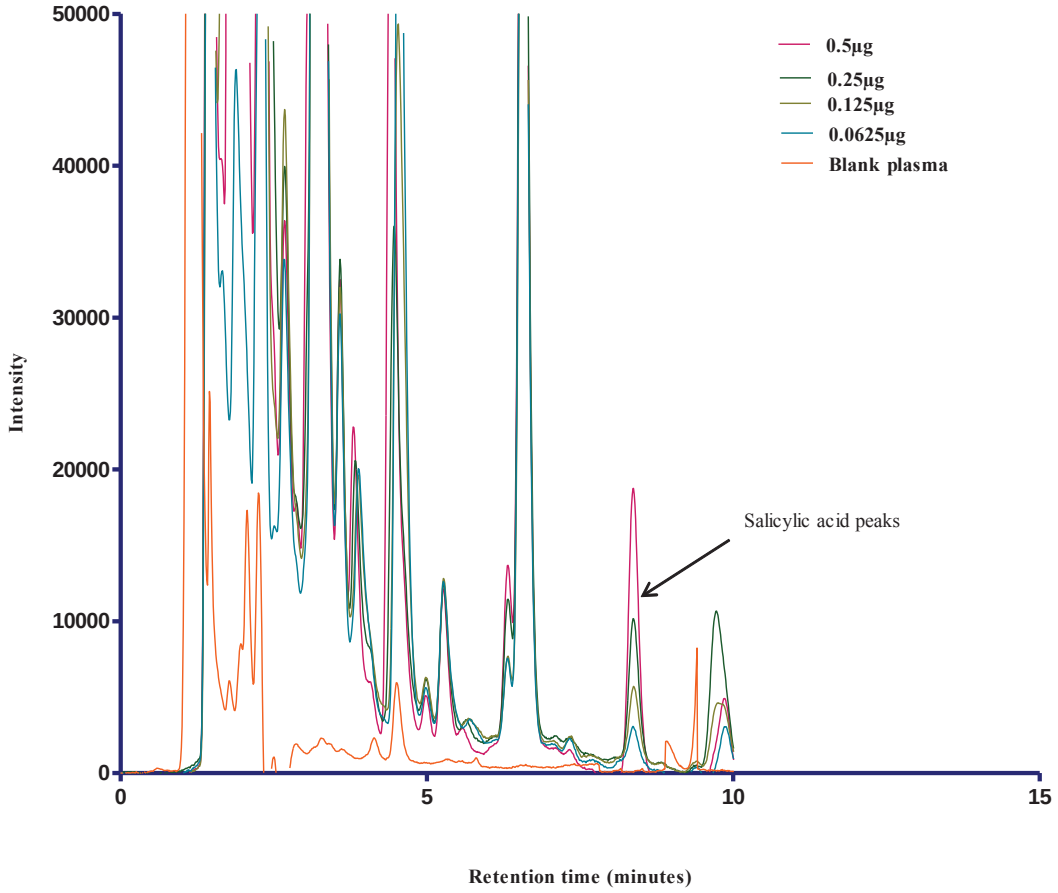

Fig 2.5: *Chromatographs showing lower concentrations of SA spiked in blank plasma and a blank plasma sample*

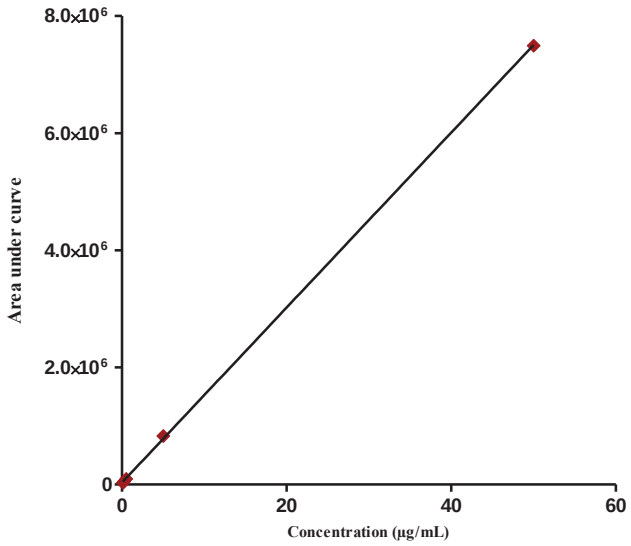

Fig 2.6: *Calibration/standard curve for SA*

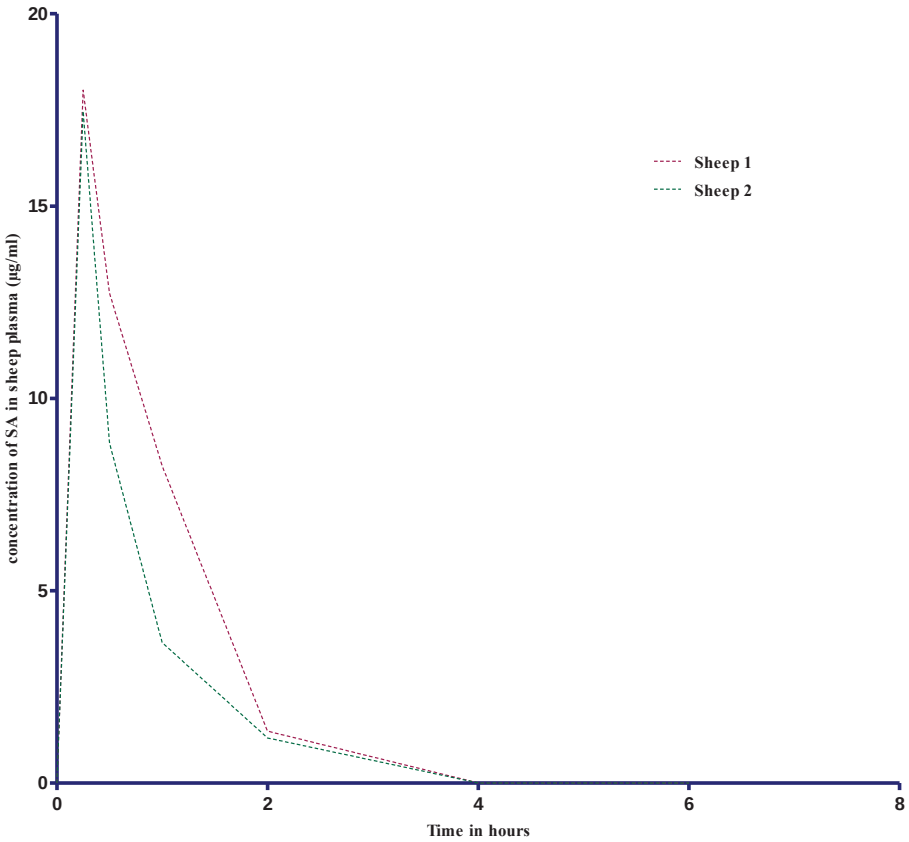

Fig 2.7: *Concentration-time curve of the SA in sheep plasma determined by the validated HPLC method*

2.3.4 Discussion

The validation study of the parameters such as selectivity, accuracy, precision and linearity showed that the % RSD for each parameter well within the limits defined by (FDA, 2007).

The sensitivity of our method is better than that reported in published literature. The limit of detection of SA described by other workers ranges from 5µg to 0.1µg (Sawyer and Kumar, 2003, Pirola *et al.*, 1998, Jian-Hua and Smith, 1996, O'Kruk *et al.*, 1984, Mays *et al.*, 1984). Our method can quantify as low as 0.0625µg of SA in sheep plasma. This sensitivity could be attributed to the SPE method used to prepare plasma sample which isolates SA by improving clean-up of sample and yielding more drug so that smaller quantities can be detected.

Maximum % RSD for intra-day variation in our HPLC method for SA analysis was lower (3.38%) than Chaudhary *et al.* (2010) (7.71%) and comparable to Mallikaarjun *et al.* (1989) (2.78%). Similarly, inter-day variation for our method was less (2.78%) than other studies Mallikaarjun *et al.* (1989) (8.90%), O'Kruk *et al.* (1984) (4.20%), and Chaudhary *et al.* (2010) (5.51%).

Various published HPLC methods for SA analysis mentioned above were not reproducible in our lab due to differences in instrumentation and our requirement for analysis of SA in sheep plasma. No method has been published to analyse SA in sheep plasma, thus validation of this method is significant. However the expensive SPE process for sample preparation could be a limiting factor of our method. Though SPE cartridges are expensive, they provide maximum recovery of the drug resulting in high sensitivity without any interfering peaks. Also, a Diode Array Detector is less sensitive than Mass Spectrometry (MS) for most of the analytes (Kopec *et al.*, 2013), but is a commonly used detector in pharmacology laboratories due to its cheaper cost. Hence, by using MS, a more sensitive and precise method for SA analysis in sheep plasma may be developed.

The merits of our method include fewer reagents and solutions which makes it simpler. No pre-processing prior to storage of plasma samples is required.

Moreover, SA separation in the plasma samples of sheep administered NaS was achieved with the validated HPLC method. The concentration-time curve of the two sheep (figure 2.5) for plasma SA shows the utility of this HPLC technique.

2.4 CONCLUSION

In conclusion, a simple, accurate, reproducible, sensitive and robust (pH independent) HPLC method for determination of SA in sheep plasma has been developed. The method has been shown to be applicable in pharmacokinetic studies in sheep.

3 PHARMACOKINETICS OF SODIUM SALICYLATE IN SHEEP

ABSTRACT

This study was conducted to determine the pharmacokinetic parameters of sodium salicylate in sheep after oral and intravenous administration. Six healthy sheep were administered intravenous sodium salicylate at four different dose rates 10, 50, 100 and 200mg/kg body weight and another six were drenched with 100 and 200mg/kg orally. A washout period of one week between each treatment was allowed after both intravenous and oral administration. Blood samples were collected at pre-determined time intervals. Analysis of these samples was carried out with High Performance Liquid Chromatography to measure plasma salicylic acid concentrations. The pharmacokinetic data after intravenous administration of sodium salicylate in sheep fitted best a one compartmental model. The concentration of drug extrapolated to time zero (Cp0) for 10 mg/kg was significantly lower than for 200 and 100mg/kg of sodium salicylate. The elimination rate constant (K_{el}) for the lowest dose rate (10mg/kg) was significantly higher than for 200 and 100mg/kg. The volume of distribution for 200mg/kg was significantly higher than other dose rates. Half-lives for 10 and 50 mg/kg body weight were significantly lower than the half-life of 200mg/kg.

Non-compartmental pharmacokinetic analysis was performed after oral administration. Average C_{max} for both oral dose rates, 100 and 200mg/kg were 4.22±2.33 and 8.27±2.38µg/mL, achieved at 1±0 and 0.67±0.26 hours, respectively. Average bioavailability, clearance, volume of distribution and half-life of sodium salicylate/salicylic acid in sheep were 0.53 (53%) and 0.64 (64%), 4.62±1.38 and 5.79±2.16L/hr/kg, 12.48± 4.02 and 16.45±8.33L/kg and 1.86±0.11 and 1.90±0.35 hours for 100 and 200mg/kg, respectively.

The minimum plasma salicylic acid concentration (16.8µg/mL) required to produce the analgesia in humans was achieved in our study after intravenous administration of 100 and 200mg/kg sodium salicylate in sheep. However, reported minimum plasma salicylic acid

concentration in cattle (25 to 30µg/mL) to produce analgesia was only achieved after intravenous administration of 200mg/kg sodium salicylate to sheep in our study.

3.1 INTRODUCTION

Sodium salicylate (NaS) is a NSAID with anti-pyretic, analgesic and anti-inflammatory properties. It has been used as a source/pro-drug for salicylic acid which is the active ingredient as discussed in chapter one.

The use of NaS as a NSAID in humans is well established. In animals such as cattle, it has been used as an analgesic and anti-inflammatory agent (Coetzee *et al.*, 2007a). In sheep, very few pharmacokinetic studies have been conducted. However, pharmacokinetic parameters are not consistent in animals and they can vary with respect to age, gender, weight, breed, environment and climate (Riviere and Papich, 2013, Nawaz and Nawaz, 1983).

Therefore, the current study was conducted to determine the pharmacokinetic parameters of NaS in typical sheep in New Zealand. Other studies have been conducted in the different parts of the world with different breeds of sheep. For instance, Sulaiman and Kumar (1995) in India administered intravenous as well as an oral single dose of NaS at 100mg/kg body weight in Bannur (local Indian breed) sheep. Blood samples were collected at every 15 minutes after intravenous drug administration until one hour and then at two and four hours; after oral administration of drug, blood samples were collected at every 15 minutes until one hour and then at every one hour up to 8 hours. However, they analysed blood samples for total salicylates. In our pharmacokinetic study, only plasma salicylic acid (SA) (active ingredient) concentration was analysed.

Another pharmacokinetic study of salicylate in the sheep was conducted by Ali (2003) in Saudi Arabia. In this study they assessed total salicylate concentrations in the sheep plasma to analyse pharmacokinetic parameters after intravenous and intramuscular administration of DL-lysine-acetyl salicylate (20mg/kg body weight). This study compared the pharmacokinetics in camel, sheep and goats.

The pharmacokinetics of salicylate or aspirin have also been conducted in cattle, horse, rabbit, goat, camel, cat and avian species such as turkey, ostrich, pigeon, chicken etc. as well as people.

In cattle, aspirin was commonly used to treat respiratory ailments caused by bacteria and viruses (Gingerich *et al.*, 1975). The pharmacokinetics of salicylate was studied by Whittem *et al.* (1996) after administration of DL-lysine-acetyl salicylate intravenously in cattle at dose rate of 26 mg/kg body weight. The half-life of salicylate reported in this study was short of about 30 minutes and volume of distribution was lower (1.2L/kg). Coetzee *et al.* (2007b) studied the pharmacokinetics of NaS in calves at 50 mg/kg body weight and observed similar results as Whittem and co-workers. In another pharmacokinetic study in cattle conducted by Short *et al.* (1990), NaS was administered intravenously as well as orally and its excretion was observed by collecting urine samples. The findings of this study suggested that 54% NaS is excreted in its original form after intravenous administration while, after oral administration, less than 12% NaS was eliminated through urine. Another metabolite, salicyluric acid, (the glycine conjugate) was reported to be a major metabolite excreted by cattle after intravenous and oral administration. In goats, when NaS was administered intravenously and orally, its recovery as NaS in urine was 67.9 and 30.2% respectively and salicyluric acid was also excreted as major metabolite (Short *et al.*, 1990). In humans, the major metabolite of SA eliminated in urine was also salicyluric acid (Needs and Brooks, 1985). The pharmacokinetic studies in cattle and goats could be of relevance to compare with sheep, as all these species are ruminants. However, inter-species pharmacokinetic differences should always be considered.

Inter-species variation in pharmacokinetics of salicylate is evident from the studies reported by various authors (table 3.5). The wide disparity of half-lives (about 26 minutes in goats to 22 hours in cats), and clearances (ranging from 0.04L/hr/kg in rabbits to 5.31L/hr/kg in cats) in different animals can be observed. The significant factors which affect pharmacokinetics of salicylate are the extent of plasma protein binding (salicylates highly bind to plasma proteins, especially, albumin), pH of the urine, bioavailability, and extent of absorption from the site of administration (Needs and Brooks, 2012, Katzung *et al.*, 2004). Also, intra-species (within the species in different breeds) and inter-individual (between individuals of the same breed) variation has been observed by some researchers in cattle (Whittem *et al.*, 1996,

Gingerich *et al.*, 1975). Thus, due to inter- and intra-species variability in the pharmacokinetics of salicylate, a distinct pharmacokinetic study in each species is warranted.

A pharmacokinetic study to assess its absorption, variation in its concentrations in the body over time and its metabolism and excretion is essential before clinical use of any drug (Pea and Furlanut, 2008). Pharmacokinetic parameters such as half-life, clearance, volume of distribution must be estimated to calculate dosing regimen of the drug in individual animal/species.

The ultimate aim of this thesis is to assess the analgesic efficacy of salicin derivative, salicylic acid in sheep. SA shows analgesic effects when it is metabolised from salicin which is naturally present in willow leaves. Therefore, study of the pharmacokinetic parameters of salicylate is essential.

The current experiment was conducted to analyse pharmacokinetic parameters of SA at the different dose rates of NaS given intravenously and orally to sheep.

3.2 MATERIALS AND METHODS

3.2.1 [A] Blood collection

3.2.1.1 Animal preparation and study design

Two different studies were conducted for analysing intravenous and oral pharmacokinetic parameters of NaS in sheep. These studies were approved by Animal Ethics Committee, Massey University, Palmerston North, New Zealand. Both studies were carried out at Large Animal Teaching Unit (LATU), Massey, University, Palmerston North, New Zealand.

Six healthy Romney cross sheep (2 males and 4 females) age 5-6 months with similar weights (mean±SD) 42.25±5.7kg were used for intravenous administration of NaS in a randomised crossover study. Sheep were weighed, clipped in the neck area for easy access to the jugular veins from both the sides. An 18 G catheter was placed in one jugular vein used for blood collection, while the contralateral vein was used for injection of the drug.

For the oral pharmacokinetic study a randomised crossover design was followed. Six healthy Romney cross sheep (3 males and 3 females) aged between 8-10 months and weighing

(mean±SD) 38.66±2.7kg were clipped in the neck area in the first instance (to easily raise jugular vein from both the sides to collect the blood after drug administration).

The power analysis was performed to identify the minimum number of animals (effective sample size) required for both intravenous and oral pharmacokinetic studies and accordingly six animals were used in each study.

3.2.1.2 Drug administration

NaS injection was made by dissolving 50g of NaS (Laboratory grade reagent, Fisher Scientific UK) in 100mL of Milli-Q water. NaS solution was administered intravenously via jugular vein at four different dose rates (dose rates starting from 10mg/kg to 500mg/kg were initially approved from Animal ethics committee, Massey University for pilot study. However, only up to 200mg/kg dose rates were used in this study for animals' safety to avoid any side effects of the drug as no other study has been reported with dose rates of salicylate beyond 100mg/kg body weight in sheep) 200mg/kg dose rate,: 10mg/kg, 50mg/kg, 100mg/kg and 200mg/kg. Each treatment was received by six sheep at one week interval between each treatment.

For oral administration of the drug, the solution was prepared as described for intravenous administration. All six sheep allocated for oral administration of NaS received either 200 or 100mg/kg NaS in a randomised crossover trial. A washout period of one week was allowed between each treatment for each sheep.

3.2.1.3 Sample collection

A blood sample of 3mL was collected at each time in heparinised Vacutainers via the catheter.

After oral administration of NaS, blood was collected from jugular venepuncture with vacutainer needle (20 gauge) in a heparinised Vacutainer.

The time intervals for blood collection were 0, 15, 30 minutes and 1, 2, 4, 6 hours after intravenous as well oral administration of NaS with each dose rate.

Initially during pilot study, one sheep was administerd with 200mg/kg body weight of SA and blood was collected upto 72 hours. However, no plasma SA concentration was detected

in the samples collected at 6 to 72 hours post drug administration. Hence, blood sampling was stopped at 6 hours post drug admnistration in both studies.

After collection of blood samples during both studies, they were centrifuged at 7000g for 15 minutes and plasma was harvested and stored at -70^{0}C till the day of analysis (analysis of samples was performed within three weeks after blood collection).

3.2.2 [B] HPLC analysis of plasma samples

All the plasma samples were analysed for SA concentration by the HPLC method as described in chapter two. The chromatographs of sheep plasma sample with SA peaks are shown in figure 3.1.

3.2.3 Pharmacokinetic analyses

The data obtained from HPLC analysis (plasma SA concentrations for each treatment after intravenous and oral administration of NaS) were used to evaluate pharmacokinetics. Non-compartment (NCA) and compartmental pharmacokinetic analyses were carried out in the software SimBiology (MATLAB R, 2014b, Version8.4). The same software was used to analyse AIC (Akaike Information Criterion) and BIC (Bayesian information criterion) values to select the suitability of the model for pharmacokinetic study after intravenous administration of NaS.

3.2.4 Statistical analyses

The Kolmogorov-Smirnov test was performed to check the normality of the data obtained from both intravenous and oral pharmacokinetic analyses.

Normally distributed data for intravenous pharmacokinetics of NaS were then analysed by One-way ANOVA (analysis of variance) with post hoc Tukey's multiple comparison test. Non-normally distributed data were analysed using Kruskal Wallis test with Dunn's multiple comparison test as a post hoc test.

Normally distributed data for oral pharmacokinetics of NaS were analysed for significant differences between the pharmacokinetic parameters of the two dose rates (oral 100 and 200mg/kg NaS) using a paired t test. Non-normally distributed data were analysed using Mann Whitney-U test.

All statistical analyses were carried out in statistical software GraphPad Prism 5(Prism 5 for windows, version 5.01, August, 2007) and were considered significant at $P<0.05$.

3.3 RESULTS

No side effects were observed in any animal throughout both pharmacokinetic studies as animals were monitored continuously for any side effects during experimental period. The age and body weights of the animals in these studies were not significantly different. No significant differences were observed in pharmacokinetic parameters were observed due to differences in the sex of animals.

3.3.1 Pharmacokinetic parameters for SA in sheep after intravenous administration

The maximum plasma SA concentration (C_{max}) in sheep plasma was observed at 15 minute after each treatment as 2.39±1.14µg/mL after 10mg/kg, 17.05±6.65µg/mL after 50mg/kg, 20.82±3.64µg/mL after 100mg/kg and 27.72±6.43µg/mL after 200mg/kg. Plasma concentrations then declined post 15 minutes. The lowest concentrations of SA ranged from 0 to 3.74µg/mL after 200 and 100mg/kg intravenous dose and were detected at six hours. At 50 and 10 mg/kg intravenous dose, SA was detected only till four hours up to 0.03 to 0.4µg/mL. Sheep administered 100 and 200mg/kg body weight of NaS had overall significantly higher ($P<0.05$) AUC (area under concentration-time curve) than 50 and 10mg/kg body weight.

The concentration-time curve and semi-log concentration-time curve for all treatments (10, 50, 100, 200mg) of intravenous NaS in sheep are shown in figure 3.2 (a, b).

Pharmacokinetic parameters of NaS after intravenous administration in sheep at different dose rates are represented below as non-compartment (table 3.1), one compartment (table 3.2) models of pharmacokinetics after statistical analysis.

Analysis of calculated pharmacokinetic parameters in the software SimBiology (MATLAB) for AIC and BIC values, showed the suitability of the pharmacokinetic model. Linear one compartmental model provided the best fit for the data obtained after intravenous administration.

3.3.2 Pharmacokinetic parameters for SA in sheep after oral administration

The C_{max} after a single oral dose for 100 and 200mg/kg was 4.22±2.33µg/ml and 8.27±2.38µg/ml respectively. Bioavailability (absolute) of the drug (SA) was calculated by using the standard formula (Agoram *et al.*, 2001, Amidon *et al.*, 1995) after comparing with the AUC's of intravenous bolus of the NaS. However, the animals used in the intravenous and oral pharmacokinetics studies were different. Therefore, overall average bioavailability for respective doses was used for each animal for further pharmacokinetics analysis. Bioavailability for 200mg/kg oral NaS was 0.64 (64%) and for 100mg/kg it was 0.53 (53%).

The concentration-time curve and semi-log concentration-time curve for 100 and 200mg/kg oral NaS in sheep are shown in figure 3.3 (a, b). The non-compartmental pharmacokinetics of sheep with statistical analysis after oral administration of NaS at 100 and 200mg/kg is shown in table 3.3.

None of the pharmacokinetic parameters were significantly different for oral dose rates 100 and 200mg/kg except AUMC. Also, high volumes of distribution were observed as compared to the intravenous study.

Table 3.1: Average non-compartment pharmacokinetic parameters of all dose treatments (single intravenous NaS bolus in sheep) (mean±SD) with statistical significance

Parameters	Units	200 mg	100 mg	50 mg	10 mg
C_{max}	µg/mL	27.72±6.43[a]	20.82±3.64[a]	17.05±6.65[b]	2.39±1.14[c]
T_{max}	Hours	0.25	0.25	0.25	0.25
AUC $_{0-\infty}$	µg.hr/mL	47.11±13.02[a]	25.95±5.05[ac]	14.94±5.39[bc]	1.42±1.00[b]
AUMC$_{0-\infty}$	µg.hr^2/mL	82.50±39.94[a]	28.62±10.14[ac]	12.32±6.48[bc]	1.29±1.98[b]
MRT	Hours	1.67±0.47[a]	1.07±0.20[b]	0.79±0.20[b]	0.66±0.47[b]
F		1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
Cl	L/hr/kg	4.52±1.22[a]	3.99±0.83[a]	3.89±1.93[a]	9.29±4.64[b]
V_d	L/kg	7.19±1.12[a]	4.16±0.32[b]	2.86±0.98[b]	5.07±2.23[b]
$T_{1/2}$	Hours	1.16±0.32[a]	0.74±0.14[b]	0.54±0.14[b]	0.46±0.32[b]

(Differences are considered significant when $P<0.05$)

Table 3.2: Average one/single compartment pharmacokinetic parameters of all dose treatments (single intravenous NaS bolus in sheep) (mean±SD) with statistical significance

Parameters	Units	200 mg	100 mg	50 mg	10 mg
Cp0	µg/mL	30.53±7.02[a]	25.64±2.41[a]	23.37±12.25[ab]	5.22±5.07[b]
Kel	1/hr	0.63±0.26[a]	0.99±0.19[a]	1.46±0.30[ab]	2.76±1.88[b]
V_d	L/kg	6.83±1.45[a]	3.93±0.34[b]	2.62±1.23[b]	3.76±2.85[b]
Cl	L/hr/kg	4.10±1.48	3.89±0.88	3.74±2.03	6.72±3.94
$T_{1/2}$	Hours	1.30±0.62[a]	0.72±0.13[ab]	0.49±0.11[b]	0.52±0.60[b]

(Differences are considered significant when P<0.05)

Table 3.3: Average of all non-compartment pharmacokinetic parameters (single oral dose of NaS in sheep) (mean±SD)

Parameters	Units	200 mg	100 mg
AUC 0-∞	µg.hr/mL	24.45±7.57	12.26±3.39
AUMC0-∞	µg.hr.hr/mL	65.16±16.57[a]	32.70±8.19[b]
C_{max}	µg/mL	8.27±2.38	4.22±2.33
T_{max}	Hours	0.67±0.26	1±0.00
MRT	Hours	2.75±0.51	2.69±0.15
F		0.64±0.00	0.53±0.00
Cl	L/hr/kg	5.79±2.16	4.62±1.38
V_{ss}	L/kg	16.45±8.33	12.48±4.02
$T_{1/2}$	Hours	1.90±0.35	1.86±0.11
MAT	Hours	1.07±0.66	1.61±0.26
K_a	1/hr	0.64±0.17	0.63±0.11

(Differences are considered significant at P<0.05)

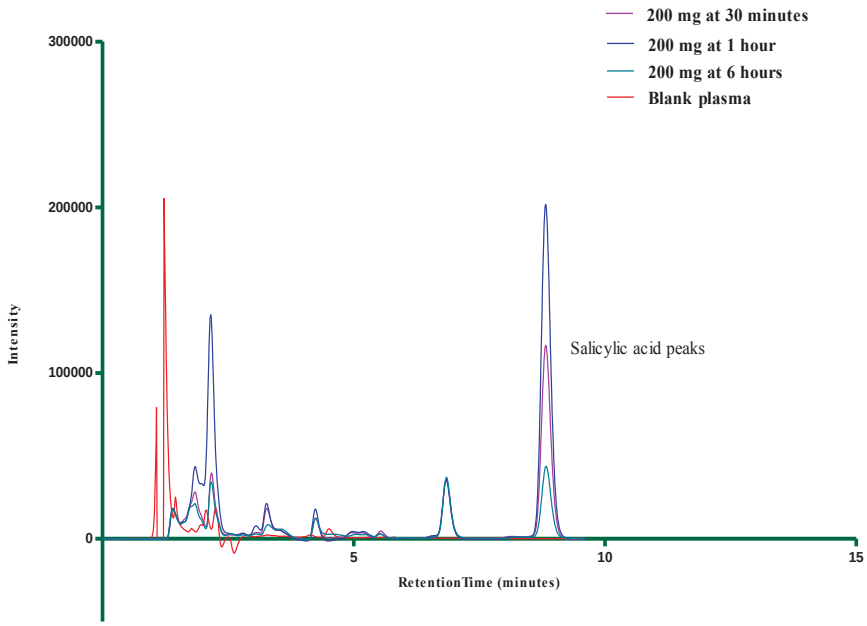

Fig 3.1: *Chromatographs showing SA peaks 30 minutes, 1hour (T_{max}) and 6 hours after oral administration of 200mg/kg NaS in sheep; while a drug-free/blank plasma of a sheep has no peak at the retention time of SA*

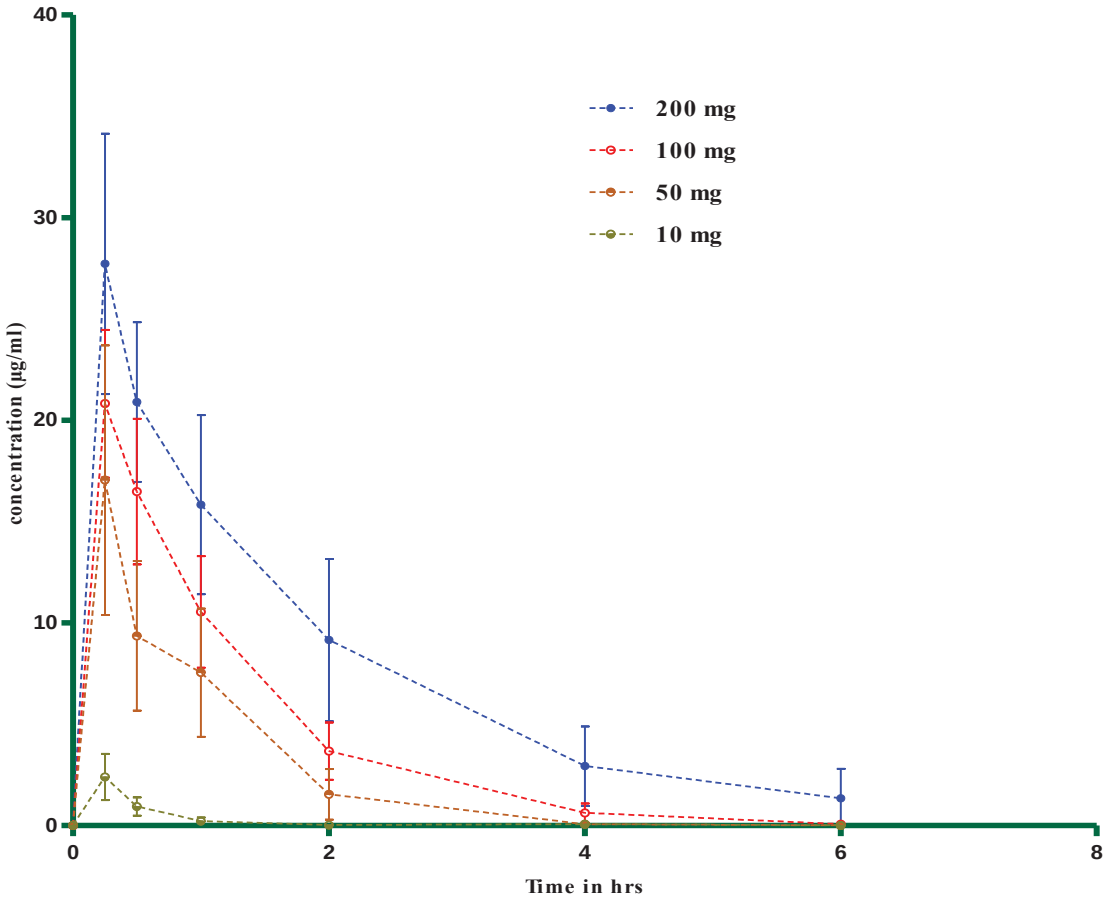

***Fig 3.2a**: Concentration time curve for all treatments (10, 50, 100,200mg/kg body weight) of intravenous NaS in six sheep (mean ± SD)*

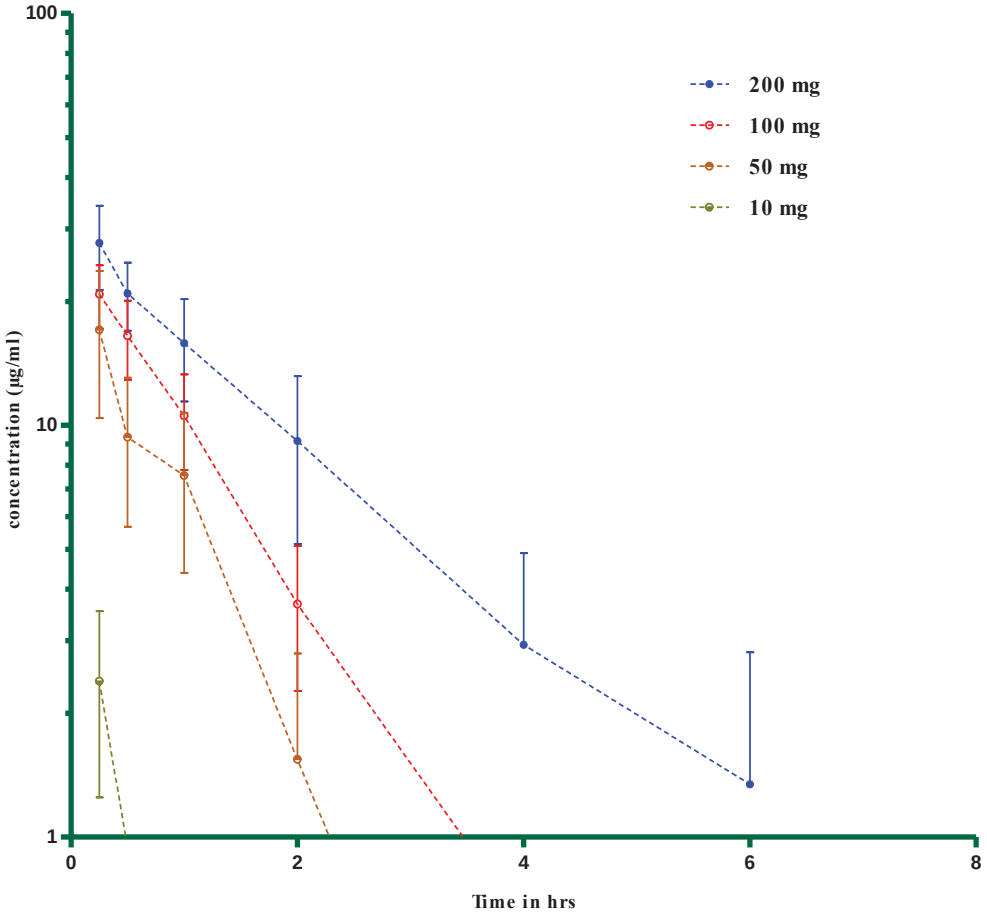

***Fig 3.2b**: Semi-log concentration time curve for all treatments (10, 50, 100, 200mg/kg) of intravenous NaS in six sheep (mean ± SD).*

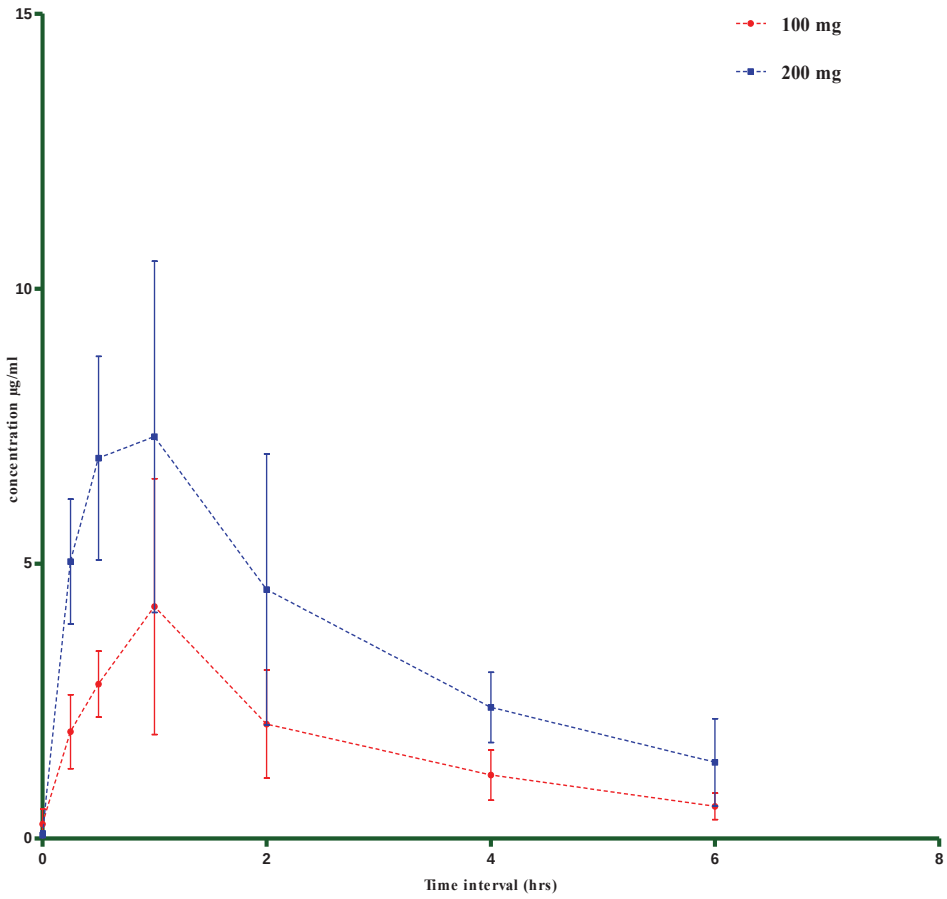

***Fig 3.3a**: Concentration time curve for 100 and 200mg/kg treatments of oral NaS in six sheep (mean ± SD)*

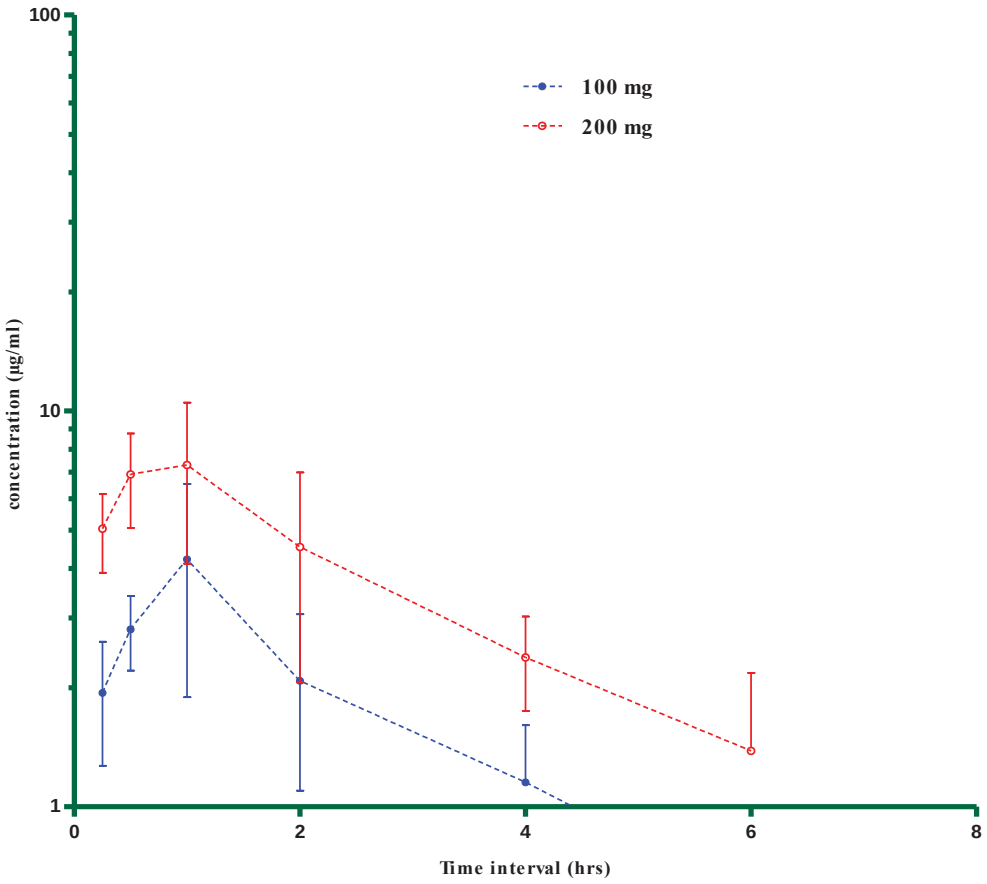

***Fig 3.3b**: Semi-log concentration time curve for 100 and 200mg/kg treatments of oral NaS in six sheep (mean ± SD).*

3.4 DISCUSSION

The pharmacokinetics of SA after intravenous and oral administration of NaS in sheep in our study showed a number of variations. Significant differences between the pharmacokinetic parameters were observed at different dose rates of the single intravenous bolus. The faster clearance at lower dose (10mg/kg) indicates the dose dependent elimination of NaS. NaS usually follows first order kinetics in humans at lower doses (65mg/kg) while at higher doses (100mg/kg) it follows dose dependent kinetics with respect to elimination. Thus, clearances are different at higher doses (Gibson *et al.*, 1975, Levy and Tsuchiya, 1972). It conjugates with glycine to form salicyluric acid while conjugation with glucuronides results in salicyl phenolic glucuronide and acyl salicyl glucuronide (Brunton *et al.*, 2011). It is also hydrolysed to form gentisic acid. When lower doses are administered, SA forms these metabolites at faster rate, while at higher doses, it reaches saturation, especially during conjugation with glucuronide (Needs and Brooks, 1985, Levy, 1981). Therefore, reduced metabolism results in the accumulation of drug in the plasma thus increasing the elimination half-life with reduced clearance. Similar variation in clearance was observed with the single intravenous bolus of anti-neoplastic agent thiotepa (TT) in children and a difference in clearance was observed at different doses of TT (Heideman *et al.*, 1989).

The elimination rate constant (K_{10}) for 10mg/kg was significantly higher than any other treatment groups. In the current study, half-life and mean residence time (MRT) of NaS at 200mg/kg body weight was significantly higher than other three lower doses after intravenous administration. Therefore, the possibility of NaS following Michaelis Menten kinetics (In this kinetics, drug follows first order kinetics i.e. dose independent kinetics at lower dose rates; however, at higher dose rates some metabolites of the drug that are formed gets saturated and follows dose dependent kinetics) at higher doses than 200mg/kg in sheep may be expected as it occurs in people (Levy and Tsuchiya, 1972). This type of kinetics was not observed in the present study. In contrast, a one compartment model fitted best the pharmacokinetic data of all doses of NaS.

The MRT after oral administration of NaS was extended as compared to intravenous dose due to absorption. Half-lives after both oral and intravenous administration of NaS were not

significantly different when respective dose rates of both routes are compared. Hence, rapid elimination of SA is evident after oral administration. This shows the necessity of frequent dosing after oral administration.

The minimum effective plasma concentration (MEC) of SA in humans to produce analgesia is reported to be 16.8µg/mL (Bromm *et al.*, 1991). In the present study, plasma SA concentration was maintained above the human MEC for 30 and 15 minutes after an intravenous dose of NaS at 200 and 100 mg/kg respectively. However, after intravenous doses of 50 and 10mg/kg or a single oral dose of 200 and 100mg/kg NaS could not achieve plasma SA concentrations above the MEC. Thus, these dose rates may not be effective. The plasma SA concentration to produce analgesia in cattle is reported to be 25 to 30µg/mL (Kotschwar *et al.*, 2009, Coetzee *et al.*, 2007a, Gingerich *et al.*, 1975), which was achieved only for 15 minutes after 200mg/kg intravenous dose rate in sheep in our study.

The plasma concentrations of SA achieved during the oral pharmacokinetic study were similar to the study conducted by (Maalouf *et al.*, 2009) in humans, where they administered 162mg aspirin orally and found plasma SA concentrations which were not higher than 10µg/mL. However, the species extrapolation of SA pharmacokinetic parameters is not appropriate (Riviere and Papich, 2013, Levy, 1979). To assess the analgesic efficacy of this drug in sheep, pharmacodynamics associated with the corresponding plasma SA level is essential.

The T_{max} after oral administration at 100 mg/kg observed in our study was similar to one conducted by Sulaiman and Kumar (1995). However, after 200mg/kg NaS oral dose in our study, T_{max} ranged from 0.5 to 1 hour within the six sheep. This shows that individual variation impacts the pharmacokinetic data. The comparison of pharmacokinetic parameters in both studies (Sulaiman and Kumar's study and present study) is represented in table 3.4. The plasma clearance was higher in our studies as compared to Sulaiman and Kumar's study after both intravenous and oral administration of NaS, resulting in shorter half-life in our study compared to theirs. Probably in our study the quantity of available SA was adequate to conjugate with glycine and glucuronide to form their respective salicylate metabolites which can be eliminated readily, resulting in high clearance. The availability of SA for

conjugation in sheep in our study could be due to the different body weights, age of sheep and climatic conditions than that of Sulaiman and Kumar's study.

Bioavailability of NaS after oral administration in our study was higher than previously reported (Sulaiman and Kumar 1995) which could be due the different animals used in both (intravenous and oral) pharmacokinetic experiments. Thus, during computation of pharmacokinetic parameters, the average bioavailability was used instead of bioavailability of each individual sheep. The volume of distribution was also higher in oral pharmacokinetic study which could be due to better absorption though at slower rate of weak acids such as NSAIDs in ruminants in reticulo-rumen (pH 5.5-6.5) (Hardee and Baggo, 1998, Neill and Hickman, 1997, Verbeeck *et al.*, 1983). Thus, higher distribution after oral administration is explicable. Salicylates have low volumes of distribution ranging from 0.1 to 0.2L/kg and they are subject to high individual variation (Rainsford, 2013). The low volume of distribution of salicylates is due to the high plasma protein binding; resulting in lower concentrations of free drug in the plasma. Salicylates are also reported to be highly lipophilic (Rainsford, 2013) due to their weakly acidic nature. This may result in their higher distribution into the body tissues, extracellular fluids and other body fluids that could contribute to the high volumes of distribution as observed in the present study (Remington *et al.*, 2006, Wible, 2005). A higher V_d in our study could also be due to a higher body fat of animals in this study as compared to the study conducted by Sulaiman and Kumar with leaner sheep. Similarly, protein deficiency may also affect the pharmacokinetic parameters (Yue and Varma, 1982). However, the protein parameters were not considered in either study. Overall, apart from C_{max}, other pharmacokinetic parameters of intravenous and oral NaS in sheep differ from another reported study by (Sulaiman and Kumar, 1995) in sheep. These differences could be attributed to the method of pharmacokinetic analysis and calculations, age, breed and weights of the animals, analytical methods used to determine SA or salicylate concentration (In the present study, free/unbound plasma SA concentrations were determined; while, in the other study total serum salicylate concentrations were determined). Also, climatic/environmental differences might well contribute to the varying pharmacokinetic parameters (Modric *et al.*, 1998). It would be valuable to measure the total proteins and lipid profile as well as liver enzymes in plasma.

When compared to other ruminants, the half-life and MRT of NaS in sheep is similar. The metabolism of salicylate may be similar in these species. Table 3.5 shows the comparative pharmacokinetics of NaS in different species.

High inter-animal variation in pharmacokinetic parameters of the present studies supports the wide diversity of pharmacokinetics of NaS as described by (Bope and Kellerman, 2015, Riviere and Papich, 2013, Levy, 1979). Therefore, to extrapolate these parameters from other species to sheep or even from different breeds of the same species is not appropriate.

Table 3.4: Comparison of pharmacokinetic parameters of NaS in the present study with that of the other study conducted by Sulaiman and Kumar (1995) in sheep after intravenous and oral administration

Parameters	Sulaiman and Kumar (1995)	This study	Sulaiman and Kumar (1995)	This study
Dose(mg/kg)	100	100	100	100
Administration	Intravenous	Intravenous	Oral	Oral
Ka (1/hr)	-	-	1.57	0.63
T_{max} (hr)	0.25	0.25	1	1
C_{max}(µg/mL)			71.33	4.22
$T_{1/2}$ (hr)	0.98	0.72	6.18	1.86
V_d (L/kg)	0.342	3.93	2.71	12.48
Cl (L/hr/kg)	0.26	3.89	0.34	4.62
F (%)	-	-	12.82	53

3.5 CONCLUSION

In conclusion, an intravenous dose of 200mg/kg NaS in sheep in our study achieved the MEC for analgesia in cattle (above 25µg/mL). Also, MEC for analgesia in humans (16.8µg/m) was achieved by two intravenous dose rates, 100 and 200mg/kg of NaS in sheep in the present study.

Oral administration of both 100 and 200mg/kg NaS failed to achieve the MEC for analgesia reported for cattle as well as humans in sheep in this study.

Intravenous administration of NaS may provide analgesia in sheep at 100 and 200mg/kg doses. Thus, these two dose rates were considered during our pharmacodynamics study.

Table 3.5: Pharmacokinetic parameters of NaS in different animal species after intravenous administration

Species	**Dose(mg/kg)**	**Form**	**V_d (L/kg)**	**Cl (L/hr/kg)**	**$T_{1/2}$ (hr)**	**Workers**
Sheep	100	SS	0.342	0.26	0.56	(Sulaiman and Kumar, 1995)
Sheep	20	Aspirin	0.202	0.26	0.52	(Ali, 2003)
Goats	20mg	Aspirin	0.21	0.28	0.46	(Ali, 2003)
Calves	50	SS	0.24	0.16	1.23	(Coetzee *et al.*, 2007a)
Camel	20mg/kg	Aspirin	0.20	0.20	0.72	(Ali, 2003)
Rats	10mg/kg	SS	00.39	0.054	15.7	(Yue and Varma, 1982)
Cats	20mg/kg	Aspirin	0.17	5.31	22.26	(Parton *et al.*, 2000)
Rabbit	44mg/kg	SS	0.25	0.04	4.3	(Short *et al.*, 1991)
Chicken	50mg/kg	SS	0.25	0.06	2.9	(Singh, 2011)

4 PHARMACODYNAMICS OF SODIUM SALICYLATE IN SHEEP

ABSTRACT

Pharmacodynamics of salicylic acid in healthy (non-lame) sheep after administration of its sodium salt (sodium salicylate) was investigated in this study. Nociceptive threshold testing was used to evaluate the analgesic efficacy of sodium salicylate. Initially, a randomised, crossover and blinded pilot study was conducted. In this study sodium salicylate was administered to six healthy sheep at four different doses: 10, 50, 100 and 200mg/kg body weight. Saline (0.9%) was also administered to each sheep as a negative control. The mechanical nociceptive threshold testing (using mechanical pressure as a source of nociceptive stimulus) was conducted at specific time intervals up to two hours after administration of each treatment. The results of this study revealed some confounding factors. Thus, the main study design was refined.

The main study was also a randomised, crossover and blinded trial. In this study, two modalities, thermal and mechanical nociceptive threshold testing, were used to analyse the efficacy of sodium salicylate/salicylic acid (SA) in six healthy sheep. Sodium salicylate was administered at two dose rates 100 and 200 mg/kg body weight. Saline (0.9%) 20mL and ketoprofen at 3mg/kg body were used as negative and positive controls respectively during the experiment. Thermal and mechanical nociceptive threshold testing were conducted simultaneously after administration of each treatment at pre-determined time intervals up to two hours. The results of thermal nociceptive threshold testing were inconsistent. However, the findings of mechanical nociceptive threshold testing confirmed the analgesic efficacy of SA in sheep as the post-treatment threshold responses to mechanical nociceptive threshold testing after 100 and 200mg/kg body weight of sodium salicylate and 3mg/kg body weight of ketoprofen were significantly higher than pre-treatment threshold responses. Sheep treated with saline (negative control) did not show any significant difference between pre- and post-treatment thresholds to mechanical nociceptive testing.

4.1 INTRODUCTION

The pharmacokinetic study conducted after single intravenous and oral doses of sodium salicylate (NaS) in sheep has been discussed in chapter 3. Knowledge of the drug's effects in conjunction with optimum pain evaluation methods is essential for investigation of effective analgesic strategies in animals (Viñuela-Fernández *et al.*, 2007). Although sodium salicylate has been in use as an analgesic in farm animals, it is not licensed for use in the sheep. In the literature, studies demonstrating the analgesic efficacy of NaS in sheep have not been reported. Therefore, to explore its utility as an analgesic in sheep with pain, the pharmacodynamics of this drug in healthy animals must be assessed.

Unlike humans, animals lack verbal reporting ability, and are unable to communicate the intensity of pain they experience. Nociceptive threshold testing is one of the effective pain evaluation methods in non-verbal animals. This method of pain assessment has been demonstrated to be valid in animals, as animals show pain related responses to the nociceptive stimulus (Le Bars *et al.*, 2001). These pain responses/behaviours are taken as the end point for the stimulus and thus the threshold. Analgesic effects of a drug are apparent when there is a raised threshold (no response or increased latency of response to the nociceptive stimulus) (Tjølsen and Hole, 1997). However, there are subjective variations in the type of responses (different response to the same type of nociceptive stimulus by individual animal) to the nociceptive stimulus in animals (Bianchi and Panerai, 2002). It is therefore, necessary to identify the response of individual animals prior to the start of experimental procedure.

Many studies have been conducted to assess the efficacy of analgesic drugs by using mechanical (Chambers *et al.*, 1995, Chambers *et al.*, 1994, Waterman *et al.*, 1988) and thermal nociceptive (Ley *et al.*, 1991, Waterman *et al.*, 1991, Ley *et al.*, 1989) threshold testing in sheep. Merits of these testing methods include ease of use and ability to elicit quantifiable responses by animals. In the current study, mechanical and thermal nociceptive thresholds of sheep were tested before and after administration of NaS in order to investigate its analgesic efficacy.

NaS is metabolised to SA to produce its effects after administration in humans and animals. The aim of this thesis was to investigate the analgesic efficacy of SA in sheep. Salicin, the

active ingredient in the willow, is ultimately converted to SA to produce analgesia (Schmid *et al.*, 2001a). Since, reports on NaS (SA) analgesia in sheep are not available; it was decided to assess the effects of NaS on nociceptive thresholds of sheep. The hypothesis was that NaS would raise the nociceptive thresholds of the sheep compared to saline.

This research was conducted as a pilot study followed by the main study with the inclusion of positive and negative control groups.

MATERIALS AND METHODS

4.2 PILOT STUDY

This study was approved by the Animal Ethics Committee, Massey University, Palmerston North, New Zealand and was carried out at the Large Animal Teaching Unit (LATU), Massey University, Palmerston North.

4.2.1 Experimental design and preparation of animals

In this randomised and crossover study, six healthy Romney cross sheep (females) of age 4-6 months, weighing 46.63±6.49kg (mean±SD) were used. The power analysis was performed to identify the minimum effective number of animals/sheep to be used in this experiment. All study animals were kept in a specially arranged pen to reduce the isolation stress. To perform the test procedures each sheep was placed in a restraining metal crate in the pen. One hour was allowed for each sheep to become accustomed to the crate before performing any procedures. Before the start of each experiment, an 18 gauge catheter was placed aseptically into the left jugular vein for test drug administration. Catheters were removed at the end of each experiment.

4.2.2 Description of the mechanical nociceptive device

The device used for testing the mechanical nociceptive thresholds (MNT) of sheep (Figure 4.1: a, b, c) was built in the neuroscience laboratory, Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University by Associate Professor Paul Chambers, Professor Craig Johnson and Mr. Neil Ward (Senior technician). The design of the device was based on the original device built by (Chambers *et al.*, 1994). Several structural modifications were considered and implemented while rebuilding this device to enhance the

performance and accuracy of the device designed by Chambers et al. (1994). A blunt pin of about 2 mm diameter was used to apply pressure against the metacarpal (antebrachium) region of sheep's forelimb. A light weight, circular plastic box was used to house the pin fixed to the light circular metal piston, and a small balloon placed behind the piston to act as a rolling diaphragm. This plastic box, also called a leg unit, was attached to the sheep's leg with the Velcro strap. The balloon in the plastic box was attached to an air compressor via syringe and extension tube. It was inflated by compressed air controlled by the solenoid valve. When inflated, the balloon forced the piston to move forward which in turn drove the pin against the sheep's leg with a force proportional to the pressure in the system to elicit a withdrawal response. A hand-held switch was used to open and close the solenoid to control the force applied to the sheep's leg. In response to this stimulus, the sheep lifted their leg. As soon as sheep responded, the switch opened the solenoid, the pressure in the system dropped to ambient and the stimulus stopped. The pressure required to elicit the response was measured by the pressure transducer driven by a standard Wheatstone bridge arrangement connected to a computer via a PowerLab unit (4/SP, AD Instruments, Dunedin, New Zealand). Ultimately, the pressure was converted to Newtons (N) to record the force applied by the pin to the sheep's leg. For this purpose, a calibration process was carried out.

The device was calibrated daily by using an electronic balance (METTLER PM 4600 DeltaRage®) to convert the pressure in the device into force in N (see below). A cut-off point of 20 N force prevented tissue damage if any sheep did not respond to the stimulus. The force at which the sheep showed a behavioural response was recorded using the Lab-Chart software (For Windows 8, 32-bit and 64-bit editions, 7.3.7) (Lab chart is specifically designed software for working with the data obtained from life sciences experiments. It can detect different types of signals including noxious stimulus responses, electroencephalographic responses, muscle reflexes etc.) in the computer via the PowerLab (4/SP, AD Instruments, Dunedin, New Zealand) (Converts complex analogue to digital data which is decipherable in computer) connected to the device. Further details of each component of the threshold testing device are described in the appendix (2) attached to this thesis.

Merits of this device include convenience and instant reproducibility of the thresholds, and ease of use. Also, the device is robust and easy to fix if any repair occurs. However, the big assembly comprising of power-lab, j-rack, compressor and computer is one limitation along with the necessity of daily calibration.

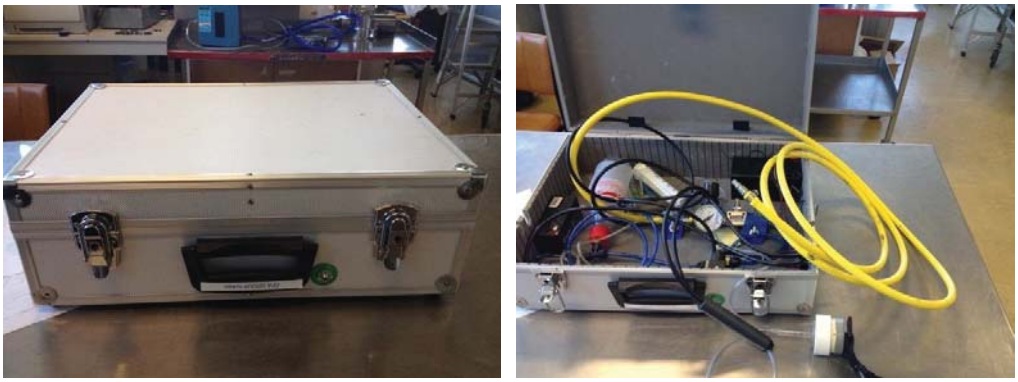

Fig 4.1a: *MNT device used to stimulate nociceptive stimulus in sheep during the study*

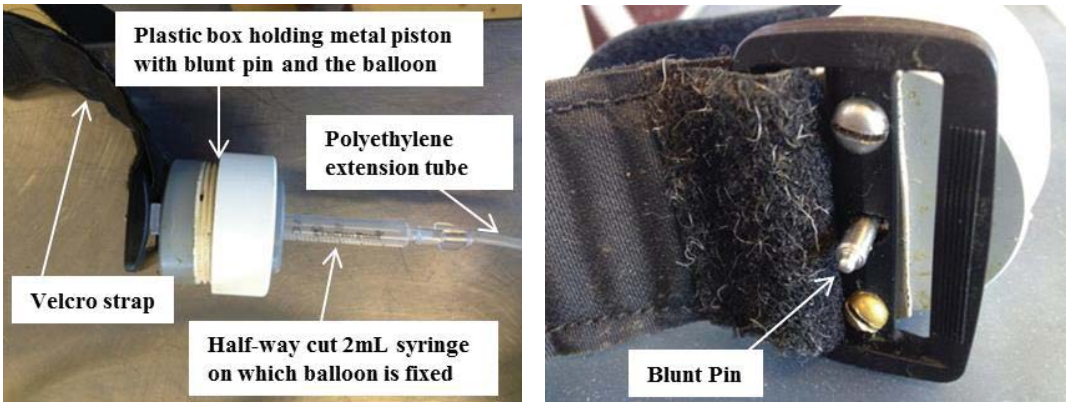

Fig 4.1b: *The leg unit of MNT device with its various components and blunt pin of the device which is pressed against the sheep's leg to produce a nociceptive stimulus*

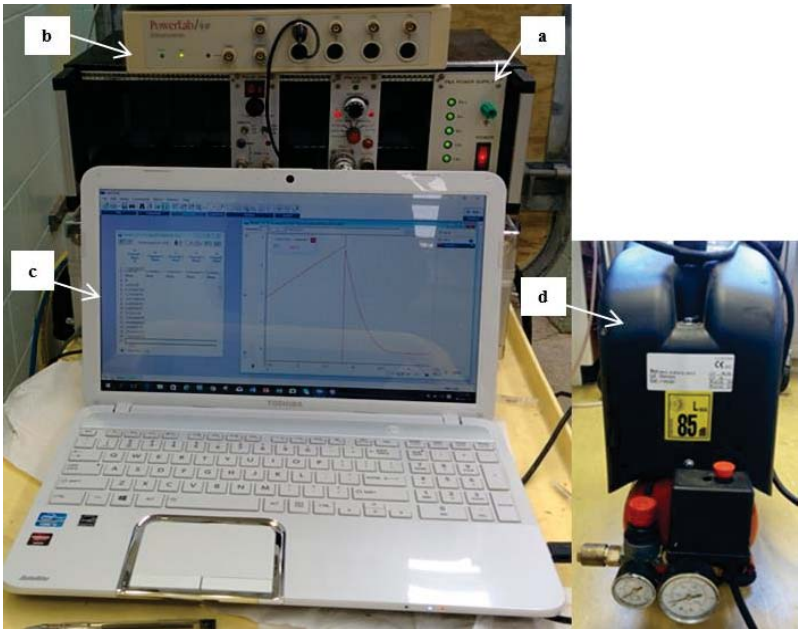

Fig 4.1c: *MNT device as a whole assembly including (a) J-rack (b) PowerLab (c) Computer and (d) Compressor*

4.2.3 Calibration of the device

The device was calibrated by measuring the pressure created by pneumatically driven pin. The pressure was recorded against weight (in grams) on the electronic weighing scale (METTLER PM 4600 DeltaRage®). The pressure applied several times with different intensities was recorded as a series of weights (grams force) and incorporated into the lab-chart software for conversion to Newtons. In the Lab-Chart, the Volts (V) corresponding to the pressure readings were converted to Newtons. Therefore, to check the validity of calibration, the calibration curve (Figure 4.2) was prepared and linearity was assessed by linear regression. The r^2 value was 0.9888.

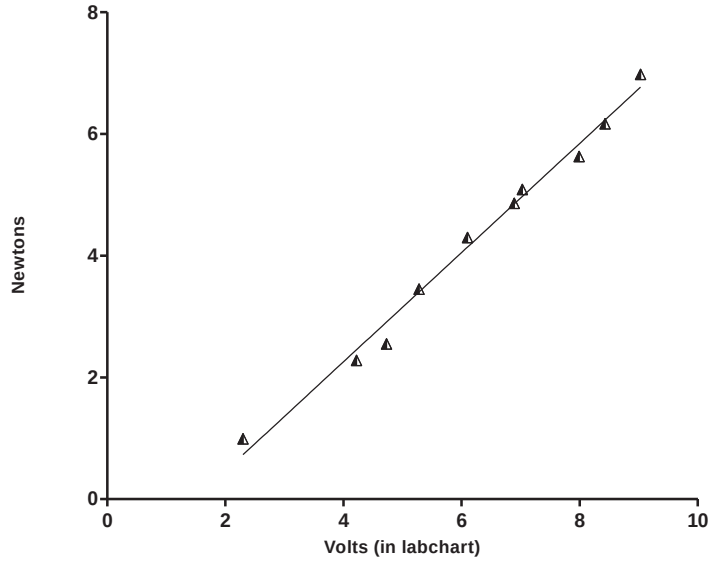

Fig 4.2: *Calibration curve for MNT device displaying the values in Newtons against the Volts measured in the Lab-Chart. X-axis represents volts (V) recorded in the Lab-Cart software and Y-axis represents the corresponding Force in Newtons (N)*

4.2.4 Drug administration

Each sheep received four different doses of NaS (10mg/kg, 50mg/kg, 100mg/kg and 200mg/kg) each dose diluted down to 20 mL, and 0.9% saline (20 mL/sheep) intravenously (I/V) in a cross-over design. The sequence of the dose of NaS administered was randomised for each animal; a one week wash out period was allowed between the treatments for each animal.

4.2.5 Mechanical nociceptive threshold testing

The MNT device described above was used to apply a nociceptive stimulus on the dorsal metacarpal region of the sheep's foreleg. The stimulus was applied at the same site in all sheep. Behavioural response indicating the threshold was leg lifting, and the force at which the sheep lifted its leg was recorded as the end point. A single, blinded operator measured the threshold responses throughout the study to avoid inter-observer variability. The sheep were not able to see the operator to avoid any distress.

MNT values were recorded for about 20 minutes to obtain constant baseline values before drug administration. These values were recorded at 8, 6, 4 and 2 minute time intervals. Post-

treatment thresholds were measured at 5, 10, 15, 30, 45, 60, 75, 90, 105, 120 minutes after the drug administration.

4.2.6 Statistical analyses

Data obtained from the MNT study were tested for normality by using a D'Agostino & Pearson omnibus normality test. Non-normally distributed data were log transformed. Two-way mixed model ANOVA with post hoc Bonferroni test was used to assess the significant differences ($P<0.05$) between pre-and post-treatment MNT values, within and between the treatment groups. The mixed model of two-way ANOVA includes random effects of animals along with the treatment effect, and repeated measures. Since, this was a cross-over study, each animal acted as its own control. Thus, there was no confounding co-variate.

The data for area under the curve (AUC), calculated from the mechanical thresholds recorded at the specific time points during the experiment (until 120 minutes post-treatment) were tested for Kolmogorov-Smirnov normality test. Significant differences between the treatment groups with non-normally distributed data were analysed by using Friedman's test (non-parametric equivalent of one-way ANOVA) with post hoc Dunn's multiple comparison test.

All statistical analyses were carried out using the GraphPad Prism 5 (Prism 5 for windows, version 5.01, August, 2007, GraphPad Software, Inc. CA, USA). Significance was considered at $P<0.05$.

4.2.7 Results

None of the sheep showed any significant side effects. The differences in the age and body weights of sheep were not significant.

The overall mean (±SD) nociceptive thresholds of sheep, (n= 6/treatment group) recorded before and after injection of the test drugs are shown in table 4.1 and figure 4.3a. No significant differences were found between pre-and post-treatment MNT values, within and between treatment groups. AUC of the nociceptive thresholds calculated from all the time points in the experimental period (Figure 4.3b) were not significantly different between the treatment groups ($P>0.05$). Although the AUC of sheep treated with 100mg/kg NaS was

high, no statistical significance was found when compared with the AUC of other treatment groups.

Table 4.1: Overall mean (±SD) pre- and post-treatment MNT values (in N) of six sheep during pilot study

Thresholds (N, mean±*SD*)	**Treatment groups**				
	200mg/kg	**100mg/kg**	**50mg/kg**	**10mg/kg**	**Saline (20mL)**
Pre-treatment	7.18±0.22	10.56±0.92	8.46±0.26	7.19±0.50	9.34±0.20
Post-treatment	8.16±0.87	9.52±3.35	8.39±3.17	6.84±0.76	8.47±0.79

(Differences are considered significant when P<0.05)

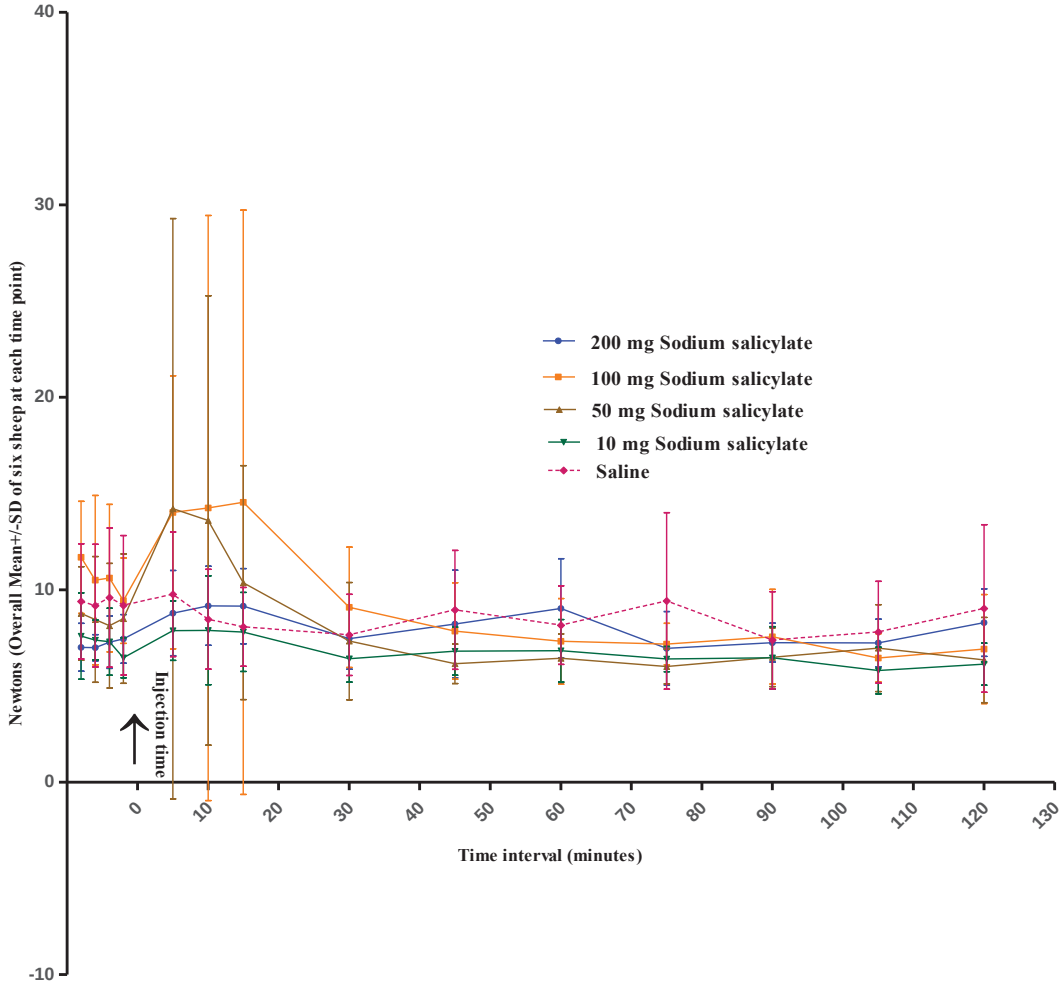

Fig 4.3a: *Pre- and post- treatment threshold responses of sheep recorded at different time points during pilot study. The time for drug injection is represented as time zero on X-axis*

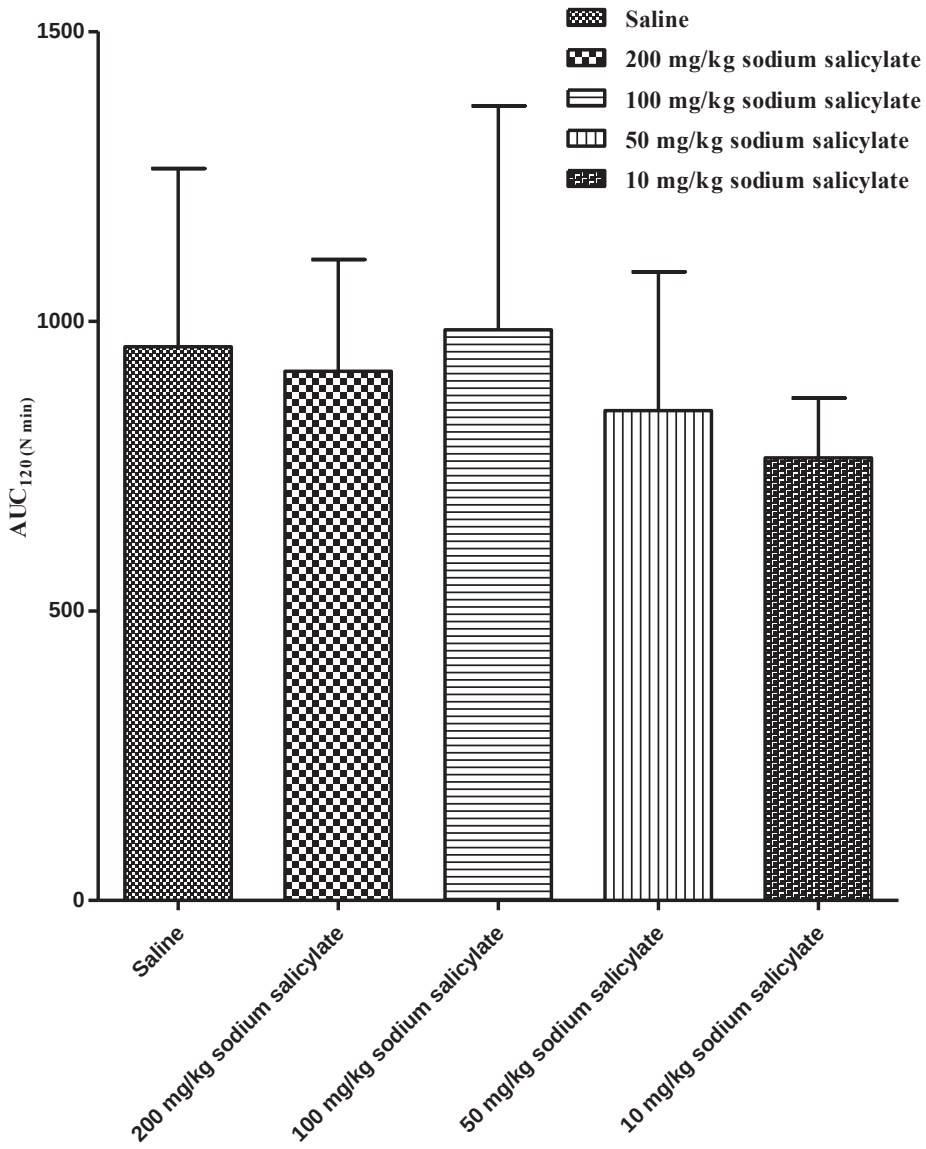

Fig 4.3b: *AUC for 120 minutes after each treatment. X-axis represents experimental treatments. Y-axis represents AUC in Newton-minute for each experimental treatment*

4.2.8 Discussion

This experiment was conducted using mechanical nociceptive threshold testing, which is considered a reliable method of pain assessment in animals (Viñuela-Fernández et al., 2007). During the experiment, a single observer blinded to the treatments given conducted the threshold testing to avoid the inter-observer variability. The observer was trained in

threshold testing method, and identifying sheep's responses to threshold test prior to the start of experiment.

No significant difference was found between pre- and post-treatment thresholds of sheep that received four different doses of NaS ($P>0.05$). Though the nociceptive thresholds of the sheep that received 100 mg/kg NaS were increased at 5, 10 and 15 minutes post-treatment, they did not approach significance when compared to pre-treatment thresholds, and that of other four treatment groups. The carry-over effect of the treatment was insignificant in our study as one week washing period was allowed in between the treatments.

Also, the AUC calculated for 100mg/kg NaS was higher but not significantly different to any other NaS treatment or negative control treatment (saline). These findings did not provide conclusive evidence of the efficacy of NaS in sheep in response to a painful stimulus. These inconclusive findings could be attributed to the noisy distractions at some time points during the experiment that stressed sheep, and stress induced hypoalgesia might have affected threshold responses of sheep. In the course of our study at LATU, teaching classes for veterinary students were conducted in the vicinity.

Another important factor which could be considered is the lack of training of the sheep prior to the start of experiment. It has been observed that animals accustomed to the experimental conditions usually respond consistently to the noxious stimuli (Lodola and Stadler, 2011).

In the present study sheep were not trained prior to the experiment; this could have resulted in inconsistency of the nociceptive thresholds. Also, inclusion of a positive control group treated with a traditional analgesic of well-known efficacy would have improved the accuracy of study outcome. Hence, further study was essential.

4.3 MAIN STUDY

This study was conducted to investigate the efficacy of NaS in a controlled environment using a positive and a negative control group. Prior to the start of this study, the animals were trained for MNT testing for three weeks to obtain consistent threshold responses.

4.3.1 Experimental design and preparation of animals

This study was approved by Animal Ethics Committee, Massey University, Palmerston North, New Zealand and was carried out at Large Animal Teaching Unit (LATU), Massey University, Palmerston North. Six healthy Romney cross ewes of age 4 years and weight (weight±SD) 67.83±10.10kg were assigned to receive test drugs in a randomised, crossover design. This study was conducted during the semester break to avoid any distractions due to any teaching classes near the study area. Experimental animals were housed and maintained under similar conditions described in the pilot study.

In this study along with the MNT testing, thermal nociceptive threshold (TNT) testing was also carried out simultaneously. Inclusion of TNT testing in the study could provide another pain modality to investigate the analgesic efficacy of test drug. Besides, it is highly recommended to consider the responses to different pain modalities for confirming the analgesic efficacy of drugs (Olesen *et al.*, 2012). In sheep, thermal and mechanical nociceptive threshold testing are established methods of pain assessment, and a combination of more than one pain assessment methods rather than a single technique is useful to confirm efficacy (Lizarraga and Chambers, 2012). To conduct MNT and TNT tests, two nociceptive threshold testing devices were used.

4.3.2 Description of devices

4.3.2.1 Mechanical nociceptive threshold device

MNT testing device used in the pilot study was used during this study.

4.3.2.2 Thermal nociceptive threshold device

A custom built TNT device (designed by Dr. Mike Gieseg and A/P. Paul Chambers, IVABS, Massey University, Palmerston North, New Zealand) consisting of a thermode, controller and power supply attached was used in our study. The thermode (Figure 4.4a) consisted of a 40mm square Peltier chip with a 50mm square aluminium plate containing a digital temperature sensor (Dallas DS18B20; RS Components, Auckland, New Zealand) on the skin side. On the other side was a large heat sink with a fan (DC Brushless EFB0512HA; Delta

Electronics Inc., Tainan City, Taiwan) to draw air through the heat sink to enable rapid cooling.

The controller unit consisted of a microcontroller board (Arduino Uno; RS Electronics) running a microprocessor (ATmega328P; Atmel Corporation, San Jose, CA, USA) and a N-channel MOSFET (FQP30N06L; RS Components) to control heating of the thermode using pulse width modulation, and a Bluetooth modem (BlueSMiRF Silver; SparkFun Electronics, Niwot, CO, USA). Connections and wiring to the microcontroller board were custom built on a prototyping shield (ProtoShield Basic; Freetronics, Croydon South, Victoria, Australia). The microcontroller board was programmed to increase or decrease the power to the Peltier chip in response to the temperature sensor output to achieve linear heating. The Bluetooth modem allowed the controller to communicate with a laptop computer to display the thermode temperature at 0.5 second intervals and also to receive commands from the observer via a laptop computer. The controller unit was housed in a polycarbonate box (160 x 80 x 55 mm) with a single on-off switch. Connections from the controller to the thermode were made with break apart plugs to allow the thermode to be removed from the controller.

The thermode and controller were powered by two 12 V 6.5 A sealed lead acid rechargeable batteries (DiaMec DM12-65; DiaMec Industrial Battery Ltd., Huiyang City, Guangdong Province, China) running in series. Temperature of the thermode increased at the rate of 0.8°C/second.

The batteries and the controller (Figure 4.4b) were fitted in fabric cases with Velcro fasteners and this whole assembly was hung on the side of the cage containing the sheep with nylon webbing and buckles for extra stability of the equipment and to reduce the stress of the sheep. The thermode was fixed on the clean shaved sheep leg on either lateral shank region (for thin legged sheep) or on the lateral metacarpal bone (for heavy sheep) to provide the maximum surface area for the contact with thermode. After application of thermode on the sheep's leg, the temperature of sheep skin was displayed as the thermode temperature and it was considered as the base temperature which remained constant. The temperature of thermode declined to the base temperature in about two minute's time after it has reached to the maximum/cut-off (60ºC) temperature. The cut-off temperature was safe and did not cause any tissue damage in sheep.

The software that ran and controlled the TNT device was written in an open-source software application (IDE v1.0.5; Arduino Software, Arduino) and uploaded to the microprocessor using a USB serial port. Communication with the controller was established by a Bluetooth connection on a laptop computer running the software ZTerm (v1.2; Freeware from http://www.dalverson.com/zterm).

No calibration was required for this device. The assembly was not complex. Indeed, very few components were required. However, the time taken by the device to cool down to normal temperature (2 minutes) was the only limitation. Nevertheless, it did not affect the experimental procedure.

Fig 4.4a: *Thermode of TNT testing device which is heated to produce thermal noxious stimulus and is constantly in contact with the sheep's skin to stimulate the thermal nociceptors*

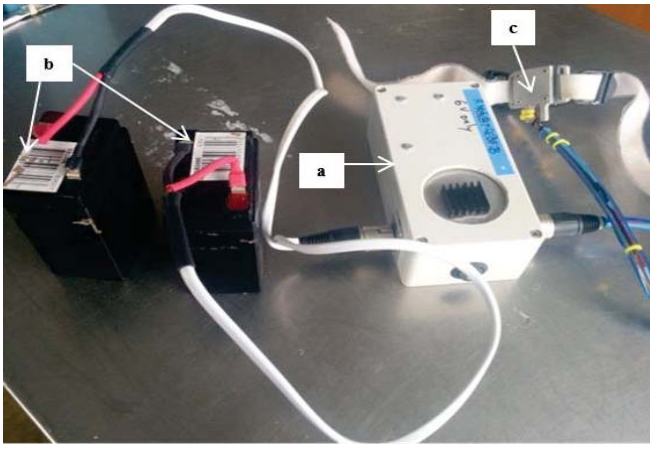

Fig 4.4b: *The assembly of thermal device: (a) A polycarbonate box comprising of circuitry and blue tooth (b) The set of 6V batteries connected from one side to the heating box supplies power to control the heating (c) Thermode (which has to be applied on the sheep's leg) is attached through extended set of wires from another side*

4.3.3 Experimental set-up

For the purpose of nociceptive threshold testing, sheep were moved to a metal crate located on the opposite side of the pen so that the sheep in the crate could see the other sheep. The MNT testing device was applied on the metacarpal region of the right forelimb of sheep and TNT testing device was applied on the lateral metacarpal region of the left forelimb about fifteen minutes before starting any experimental procedure to accustom the sheep to the device. Figure 4.5(a, b, c) shows the location of both devices on sheep's leg during experimental procedure.

The heating box and batteries of the TNT device were hung on the metal crate. The MNT device was connected to its extension tube. Thereafter, both devices were operated from a distance to avoid sheep becoming stressed due to human presence, which could affect the results.

4.3.4 Response of sheep to noxious stimuli

Thermal and mechanical nociceptive stimuli instigated the sheep to respond by lifting its leg. This response was considered as the end point and the force or temperature was the threshold.

As mentioned above in the experimental set-up, the thermal nociceptive device was placed on the left foreleg; while the leg unit of the mechanical nociceptive device was positioned on the right foreleg of the sheep. Sheep responded to the thermal and mechanical nociceptive stimuli by lifting left foreleg and right foreleg respectively when the temperature of thermal device and the force exerted by blunt pin of the mechanical device on the sheep's leg were elevated to the sheep's pain threshold.

The same trained observer as in the pilot study blinded to the treatments given recorded the responses to avoid inter-observer variability.

4.3.5 Drug administration

Test drugs were administered intravenously into the jugular vein. Each sheep received 20 mL normal saline (0.9%), 200mg/kg and 100mg/kg NaS, and 3mg/kg ketoprofen

(Ketoprofen™ 10%, Merial Ltd. Manukau city, New Zealand) randomly with one week interval (washout period) between each treatment.

Lower doses of NaS (50 and 10 mg/kg) tested in the pilot study, were not tested in this main study. From the pharmacokinetic study described in chapter 2, it was apparent that NaS given in lower doses did not achieve high enough plasma SA concentrations (16.8µg/mL; this concentration of SA is reported to be analgesic in humans) for longer duration (the effective plasma concentration was maintained for about 15 minutes when 200 and 100mg/kg of NaS were given). This decision was made after obtaining the results of the pharmacokinetics (PK) study of NaS (Chapter 2), which was conducted after the pilot study.

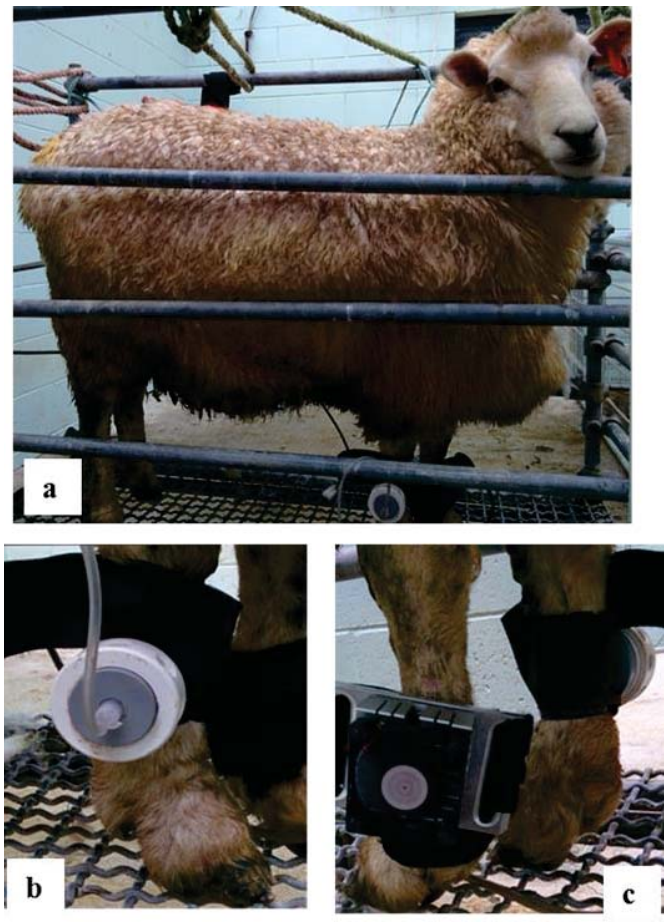

Fig 4.5: (a) *Sheep with both MNT and TNT testing devices on right and left foreleg respectively* ***(b)*** *Right leg of sheep zoomed in to display the leg unit (box with blunt pin) of the MNT testing device* ***(c)*** *Left leg of sheep zoomed in to display leg unit (thermode) of TNT testing device*

4.3.6 Data recording

4.3.6.1 Pre-treatment thresholds

Once sheep were accustomed to the testing devices, baseline thresholds to thermal (^{0}C) followed by mechanical nociceptive stimuli (N) were recorded. Baseline threshold measurements were taken until three consistent readings were obtained with each type of stimulus. Two-minute time intervals were allowed between each pair of recordings.

4.3.6.2 Post-treatment thresholds

The thermal and mechanical nociceptive thresholds of sheep were recorded at 4, 9, 14, 19, 24, 29, 44, 59, 74, 89, 104, 119, and 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105 and 120 minutes respectively, after the test drug administration.

4.3.7 Statistical analyses

Data obtained from both mechanical and thermal nociceptive threshold testing were initially analysed for normality with D'Agostino & Pearson omnibus normality test. Normally distributed data were analysed using Two-way ANOVA (mixed model) with post hoc Bonferroni test to find any significant differences ($P<0.05$) between pre-and post-treatment values, within and between the treatment groups. During this statistical analysis, data obtained from one sheep showing abnormal signs was excluded to avoid the erroneous outcomes. In our experiments, carry-over effect was insignificant as a one week wash out interval was allowed between the treatments. Two-way mixed model ANOVA considers effect of treatment and random effects.

Data for area under the curve (AUC), calculated from the mechanical as well as thermal thresholds recorded at the specific time points during the experiment (until 120 minutes post-treatment) were initially assessed for Kolmogorov-Smirnov normality test. Non-normally distributed data were analysed for significant differences between the treatments with Friedman's test (non-parametric equivalent for one way ANOVA) with post hoc Dunn's multiple comparison test. Normally distributed data were tested for significant differences between the treatment groups using a one-way ANOVA with post hoc Bonferroni test.

All statistical analyses were carried out using the GraphPad Prism 5 (Prism 5 for windows, version 5.01, August, 2007, GraphPad Software, Inc. CA, USA). Significance was considered at $P<0.05$.

4.3.8 Results

No side effects were observed during this study, although one sheep exhibited some signs such as grinding of teeth and restlessness after administration of 100mg/kg NaS which were considered as side effects and were nullified later when further analysis was carried out. No abnormal signs were observed after administration of NaS at the rate of 200mg/kg body weight to the same sheep.

There was no significant difference in the age and body weights of experimental sheep. Similarly, the random effects during study were insignificant and were excluded.

4.3.8.1 Thermal nociceptive thresholds

Mean pre- and post-treatment baseline threshold values with statistical significance are shown in the table 4.2. No significant differences were found between pre- and post-treatment thresholds within any treatment groups. Similarly, no significant difference was observed in the post-treatment TNTs between the treatment groups including negative and positive control treatments.

Figure 4.6a shows TNT values at each time point during experimental period. Also, AUCs (ºC minutes) calculated for post-treatment TNTs over the experimental period of 120 minutes (figure 4.6b) were not significantly different than each other when compared between the treatments.

Table 4.2: Pre-and post-treatment TNT values (mean±SD) in ºC

	Treatments			
Thresholds (mean±*SD)*	**Saline**	**100 mg/kg SS**	**200 mg/kg SS**	**Ketoprofen**
Pre-treatment	54.20±0.46[a]	48.94±1.17[b]	52.05±0.91[a]	48.89±2.76[b]
Post-treatment	52.00±1.71	49.52±3.14	51.00±2.67	51.00±1.19

4.3.8.2 Mechanical nociceptive thresholds

Mean±SD pre- and post-treatment threshold values are shown in table 4.3. Post-treatment thresholds of the sheep treated with NaS and ketoprofen were significantly higher compared to their respective pre-treatment values. This trend was not observed in sheep of the saline group.

Post-treatment thresholds of sheep of 3mg/kg of ketoprofen and 200mg/kg of NaS groups were significantly higher than that of the saline and 100 mg/kg NaS groups. There was no significant difference in the nociceptive thresholds between sheep of 100 mg/kg NaS and saline (20 mL) treatment groups. Figure 4.7a shows MNT values at each time point during experimental period.

Area under the curve calculated for post-treatment MNT values (figure 4.7b) over 120 minutes of experimental period for ketoprofen treated group of sheep was significantly higher than saline treated group of sheep. All other the treatment groups were non-significantly different when compared to each other. Though, the AUC of the ketoprofen group was not statistically significant yet, numerically higher (723.02± 235.20) than 100 and 200mg/kg NaS treated group of sheep. Similarly, the AUC of 100 mg/kg and 200 mg/kg NaS groups were not statistically yet, numerically higher (mean±SD) (520.08±62.67 and 597.70±76.42, respectively) than that of saline group (482.11±36).

Table 4.3: Pre- and post-treatment MNT values (mean±SD) in N

	Treatments			
Thresholds (Mean±SD)	**Saline**	**100 mg/kg SS**	**200 mg/kg SS**	**Ketoprofen**
Pre-treatment	4.25±0.16	3.76±0.11	4.30±0.26	4.12±0.16
Post-treatment	4.25±0.23[a]	4.73±0.55[a]	5.53±0.99[b]	6.22±1.00[b]

(Differences are considered significant when P<0.05). Different superscripts indicate significant difference.

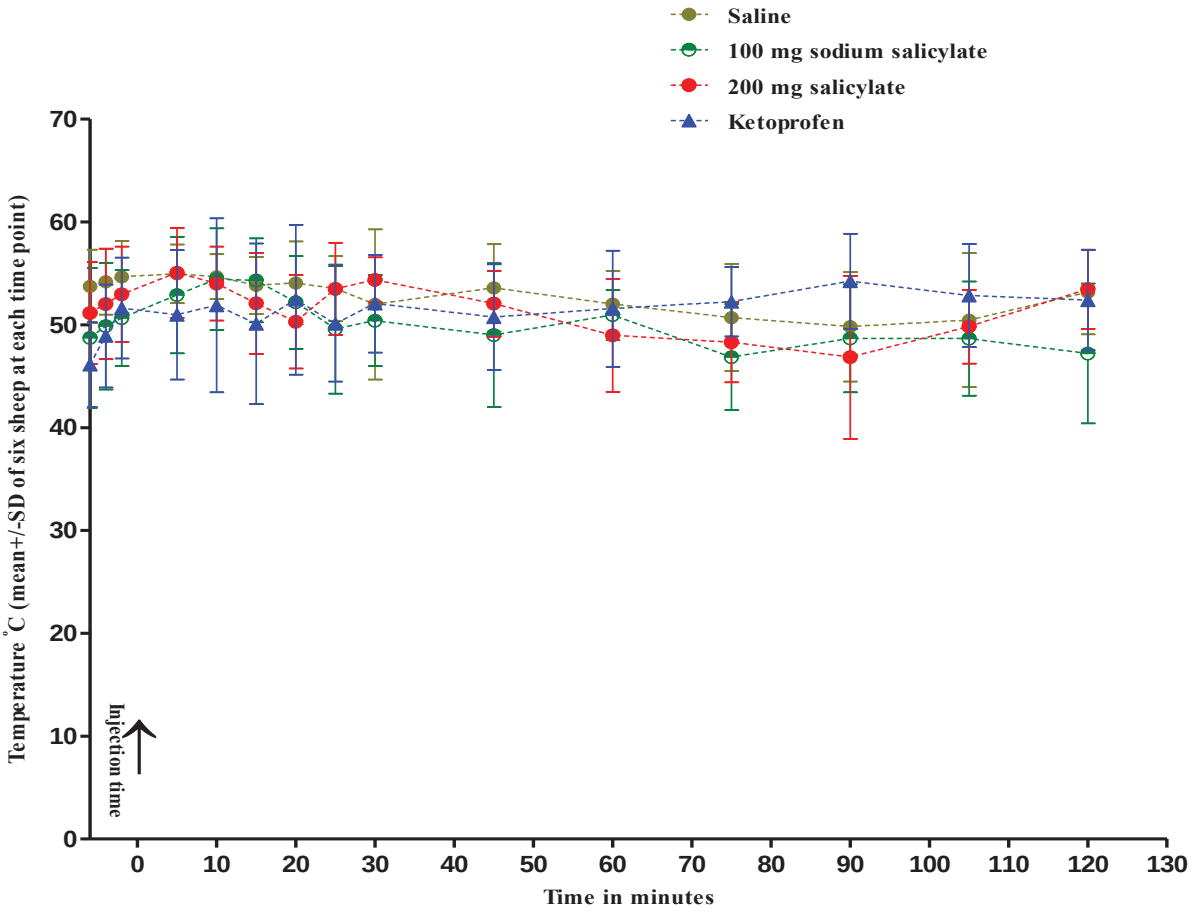

Fig 4.6a: *Pre- and post- treatment TNT responses of sheep recorded at different time points during main study. The black arrow indicates drug administration time*

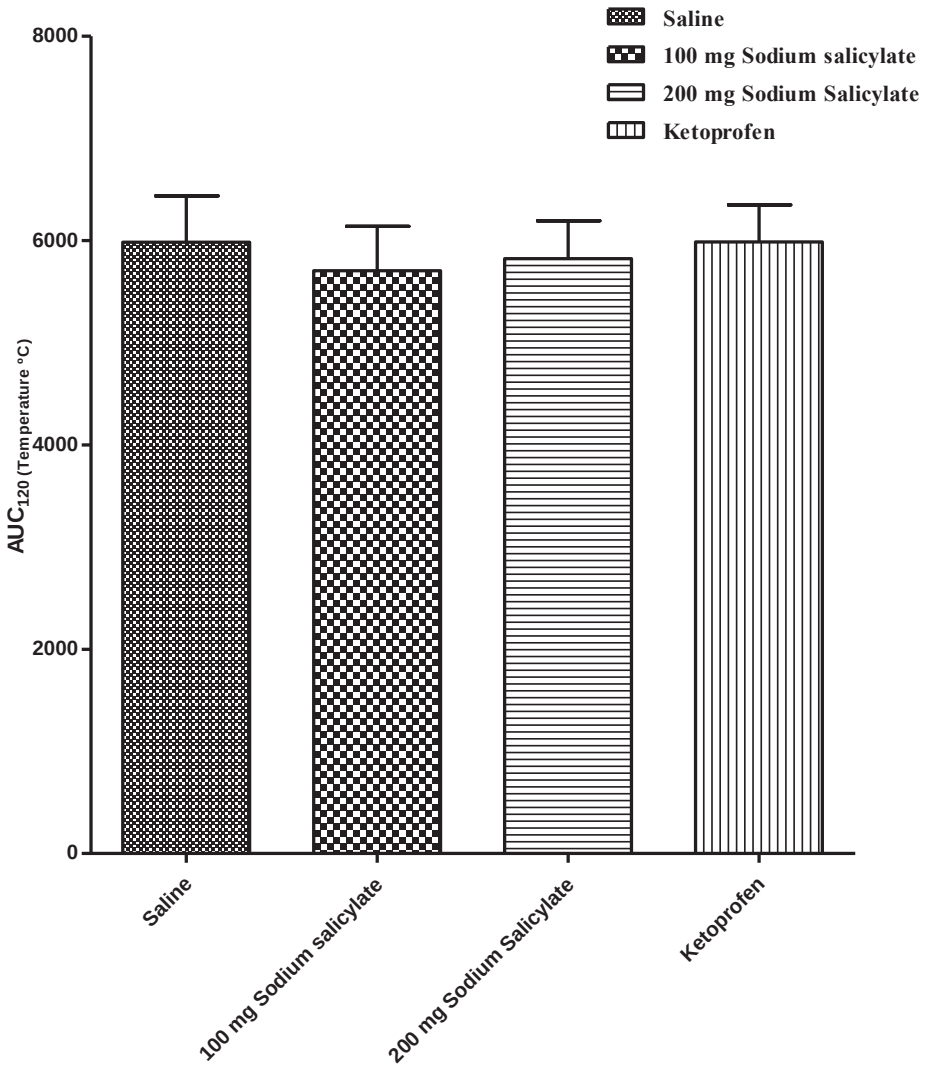

Fig 4.6b: *AUC calculated for TNTs of sheep, over the experimental period of 120 minutes post each treatment. X-axis represents each experimental treatment. Y-axis represents values of area under curve for each treatment*

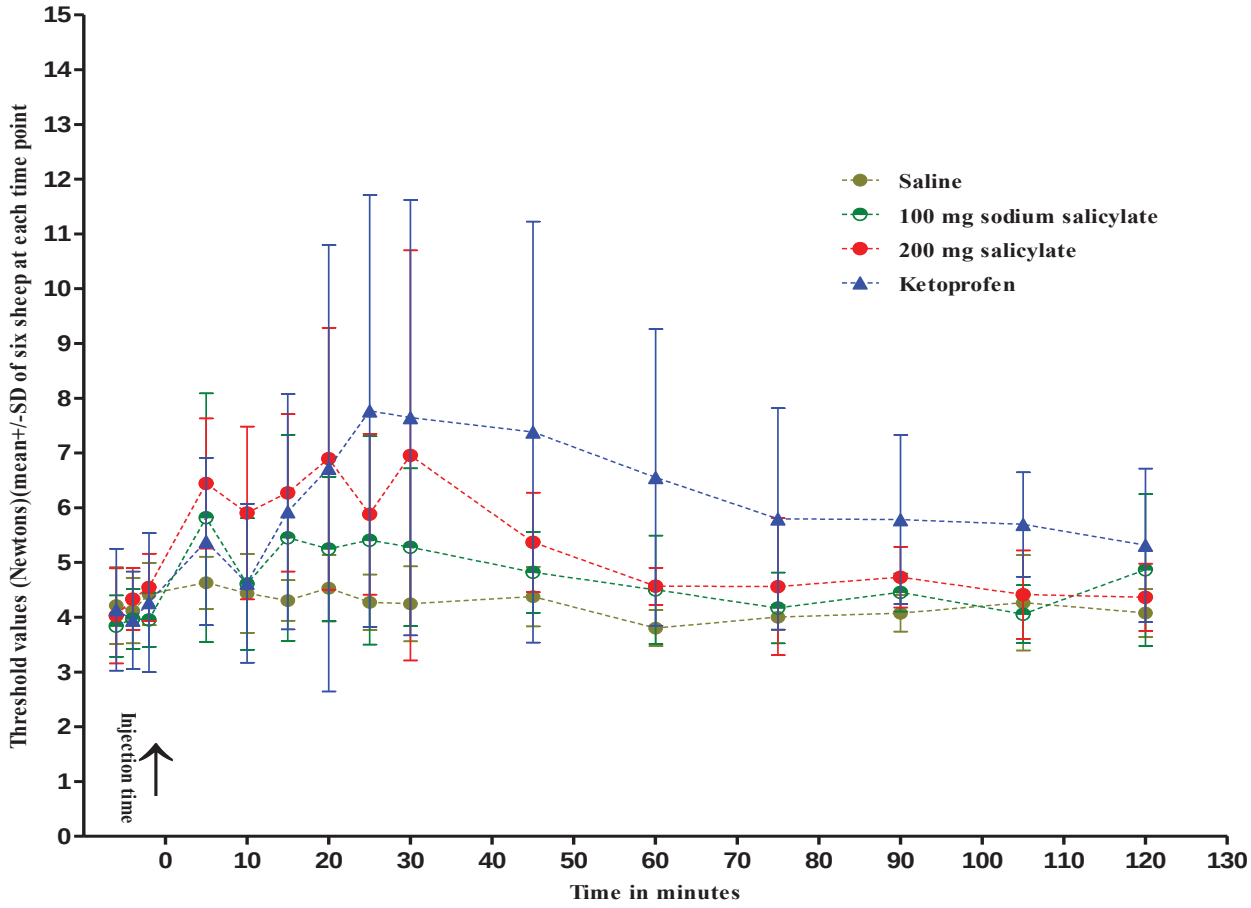

Fig 4.7a: *Pre- and post-treatment MNT responses of sheep recorded at different time points during main study. The black arrow indicates drug administration time*

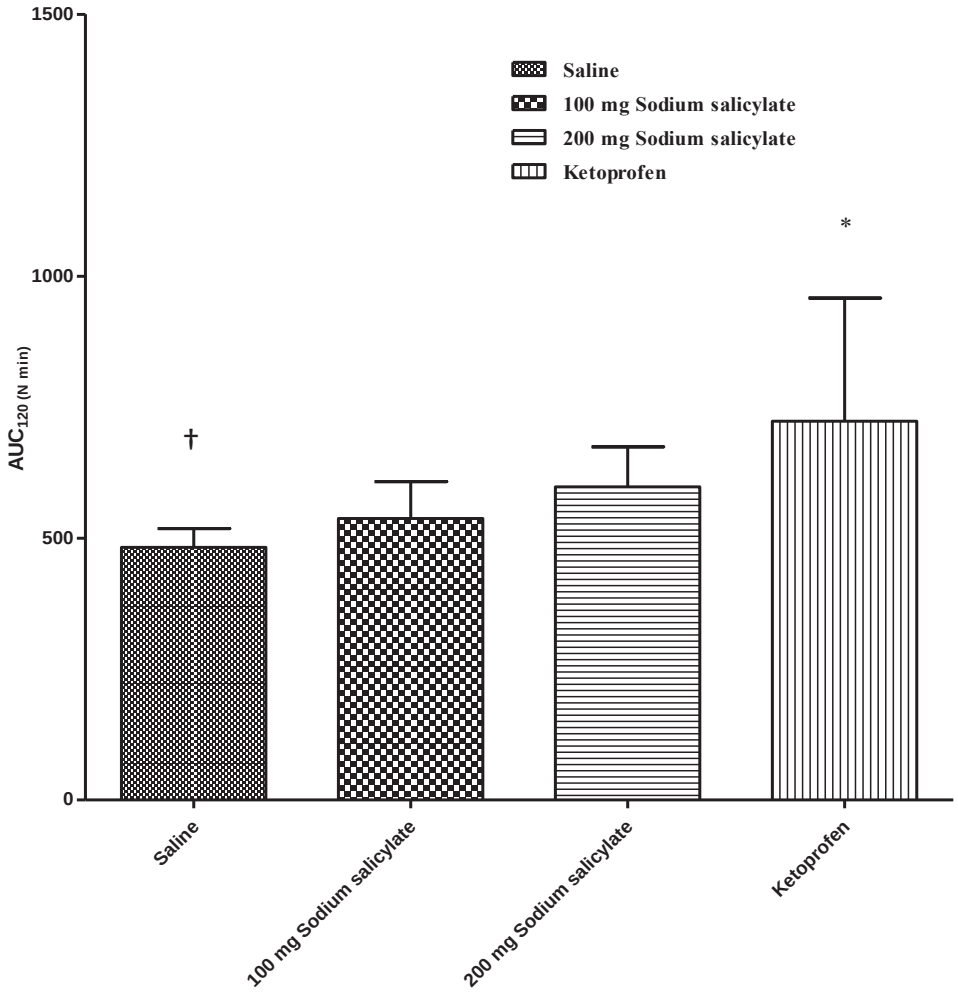

Fig 4.7b: *AUCs calculated for MNTs during main study over the experimental period of 120 minutes post each treatment. X-axis represents different treatments. Y-axis represents actual area under curve values for each treatment*

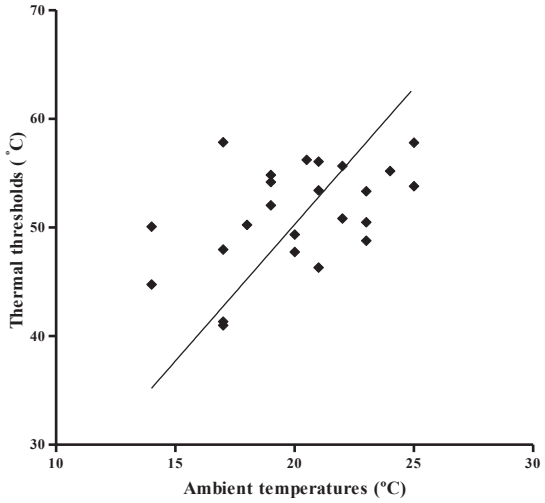

Fig 4.8: *Baseline thermal nociceptive thresholds and corresponding ambient temperatures. X-axis represents different ambient temperatures during experimental period. Y-axis represents corresponding baseline thermal threshold values of the experimental sheep*

4.3.9 Discussion

In this study, nociceptive thresholds of sheep in response to thermal and mechanical stimuli were tested to detect the analgesic efficacy of NaS. NaS administered in two different doses (100 mg/kg and 200 mg/kg) could increase the mechanical but not the thermal thresholds of sheep recorded at specific time-points in the experimental period.

All the necessary pre-requisites such as controlled environment, trained observer and device handler, allowance of optimum time for animals to adapt to the experimental environment were taken into consideration and implemented during the study.

4.3.9.1 Thermal nociceptive thresholds

Inconsistent responses were observed during the TNT testing study. Also, pre-treatment TNT temperatures for individual sheep varied between the experimental days. This was unanticipated. Under similar experimental conditions, the baseline MNTs of the individual sheep were constant between the experimental days. Prior to the start of each experiment, more time was allocated for sheep to adapt to the experimental setup in order to obtain consistent pre-treatment thresholds.

The inconclusive results of TNT testing in the present study are similar to findings of other studies in animals. In sheep with chronic foot-rot lesions, Ley et al (1989) could not find any change in thermal thresholds. On the other hand their mechanical nociceptive thresholds were decreased significantly.

Other researchers also observed the inefficiency of TNT testing to determine the analgesic activity of NSAIDs in animals (Koch, 2006). The ineffectiveness of these tests is believed to be either due to increased tolerance or over-sensitisation of animals to the TNT testing resulting in lowered thresholds (Tapper, 2011, Koch, 2006). Over-sensitisation results in lowered thermal thresholds. Hence, effect of analgesic administration on thresholds becomes insignificant and in spite of drug being effective, its analgesic effect may not be exhibited

Also, the location of the TNT device on animal's body can contribute to the variation in the responses due to high/lower sensitivity of that particular area of animal's body to thermal nociceptive stimulus (Caplen *et al.*, 2013, Love *et al.*, 2011, Ley *et al.*, 1989, Kamerling *et al.*, 1986). In this study, the device (thermode) was placed on the sheep's leg. In sheep, the ear pinna has been suggested to be a reliable site to elicit optimum response to TNT (Lizarraga and Chambers, 2012, Nolan *et al.*, 1987a). However, the device was too heavy to be placed on the ear pinna and it was located on the leg of the sheep during this study.

The pre-treatment TNT values were not consistent during the experimental days. This inter-day inconsistency in TNT values between the treatments could be due to lack of training to sheep prior to the experimental procedure. During training session, sheep were trained for MNT testing. Unfortunately, due to the unavailability of TNT device in this period, simultaneous training for TNT testing was not performed. The TNT values of sheep given 3 mg/kg ketoprofen (positive control) did not differ significantly from baseline values, and that of the other treatment groups. Thus, in the present study unacquaintance of animals to this technique might have resulted in these inconsistencies in responses even after administration of an analgesic (ketoprofen). Pre-experimental training is important to accustom the animals to the experimental device and technique, which is helpful to obtain consistent results (Lodola and Stadler, 2011). This was evident in the experiments conducted by various researchers in horses subjected to thermal nociceptive threshold testing (Chambers *et al.*, 1994, Kamerling *et al.*, 1986). In contrast, it was observed that sheep

usually respond variably to a thermal stimulus (Chambers *et al.*, 1994). This supports the findings of our study as the responses to thermal nociceptive stimulus were highly inconsistent.

Another possible confounding factor to consider is the ambient temperature. Our experiment was conducted at ambient/atmospheric temperatures and these temperatures were variable on daily basis (Figure 4.8). Ambient temperatures are reported to affect the thermal thresholds of sheep. Chambers et al. (1994) observed the changes in thermal thresholds of sheep when ambient temperature was below 8ºC. The ambient temperatures during the present study were ranging from 14ºC to 25ºC. Thus, it is unlikely that the variation in ambient temperatures affecting the thermal thresholds of sheep in this study. Skin temperature changes can also affect the thresholds to thermal nociceptive stimulus. (Bessou and Perl, 1969) reported the changes in low threshold receptors' response when skin temperatures changed in the range of 25 to 40ºC. Skin temperatures in our study were recorded and were found to be consistent throughout the experiment as the thermode of the device maintained the skin temperature of the sheep during test period (Device was constructed in such a way that after attaining highest/threshold temperature, instantaneously cooling process was started and temperature was plummeted to match the skin temperature of sheep and maintained further until next threshold testing procedure). Other possible factors affecting the thermal thresholds could be the thickness of the skin of individual sheep; variability in the size of leg (fat or lean) etc. These variations could be circumvented by increasing the number of experimental sheep (Chambers *et al.*, 1994). Due to ethical reasons, minimum required number of sheep (6) was used in this study to perform both mechanical and thermal nociceptive testing.

The selection of source of heat during this test is also considered to be an influential factor in the inconsistent results. Thermal stimulus produced by contact thermode has its own advantages and disadvantages. The thermode is used mostly as a source of thermal stimulus due to its convenient and inexpensive use. It also provides a linear heating slope (Le Bars *et al.*, 2001). Apart from these advantages, there are some limitations such as the area of skin/body covered by the thermode for optimum skin contact is always larger as compared to the area occupied by blunt pin used to create mechanical stimuli. The heat is transferred

from the thermode by means of conduction. During the process of conduction, along with the cutaneous nociceptors, heat specific thermoreceptors of low thresholds are also activated that can initiate inhibitory pain pathways (Kuhtz-Buschbeck *et al.*, 2010). Therefore, the response to the thermal noxious stimulus is delayed (Le Bars *et al.*, 2001) and might result in higher basal nociceptive thresholds as observed in our study. Also, when the heating process of thermode is slow i.e. less than 1ºC/second, the chances of Aδ fiber nociceptors getting activated could be reduced. These (Aδ-fiber) nociceptors are activated only in response to the intense and swift nociceptive stimuli such as mechanical nociceptive stimuli (Bessou and Perl, 1969). During this study, the thermode was heating up slowly at 0.8ºC/second. Thus, activation of Aδ fiber nociceptors is unlikely to occur. The measured responses to thermal stimulus could be due to the activation of two types of nociceptors, firstly, slow conducting C-fibre nociceptors and secondly, low threshold thermoreceptors. On the contrary, responses to mechanical noxious stimulus were more likely due to activation of the Aδ type nociceptors. Thus, the disparity in responses obtained during thermal and mechanical nociceptive threshold testing could be attributed to the activation of different types of nociceptors. The nociceptors activated by thermal stimulus are effectively blocked by opioids as compared to NSAIDs due to their mechanism of action (Steagall *et al.*, 2007). Also, absence of inflammatory pain in experimental sheep is major factor in this study. The NSAIDs are reported to exhibit their effects during inflammatory conditions rather than acute thermal nociception (Sukhtankar *et al.*, 2014). Many other studies have reported the effectiveness of thermal nociceptive threshold testing to detect the analgesic efficacy of different drugs during inflammatory pain in animals (Musk *et al.*, 2013, Slingsby and Taylor, 2008).

4.3.9.2 Mechanical nociceptive thresholds

Responses exhibited by sheep to the mechanical nociceptive stimulus were consistent and distinct as described in the results. Post-treatment MNT values were significantly higher than pre-treatment values within each treatment group except saline. This indicates the analgesic activity of NaS and ketoprofen. Ketoprofen is a NSAID in sheep and is used as analgesic during painful conditions. In the current study, after intravenous administration of ketoprofen, a delayed response to mechanical nociceptive stimuli was observed confirming

its analgesic activity. Similarly, post-treatment nociceptive thresholds of sheep administered with 100mg/kg and 200mg/kg NaS were elevated as compared to their pre-treatment thresholds, indicating the analgesic efficacy of NaS in this study. Statistical analyses resulted in non-significant difference between post-treatment MNTs of sheep treated with ketoprofen (3mg/kg body weight) and 200mg/kg body weight of NaS. Also, these two treatment groups (ketoprofen and 200mg/kg body weight NaS) had significantly higher post-treatment MNTs than that of the saline (negative control) group. These results confirm the analgesic efficacy of NaS at 200mg/kg body weight.

Further statistical comparisons provide non-significance between the post-treatment MNT values of sheep treated with saline (20mL) and 100 mg/kg body weight of NaS; nonetheless, post-treatment MNTs of the sheep treated with 100mg/kg body weight NaS were numerically higher than the post-treatment MNTs of the sheep treated with saline (20mL). Post-treatment MNT values of sheep treated with 100 and 200mg/kg body weight of NaS treatment were significantly different; where, 200mg/kg body weight of NaS exhibited higher MNTs in sheep as compared to 100mg/kg body weight of NaS. However, post-treatment MNT values of sheep treated with 100mg/kg NaS as compared to their pre-treatment MNT values were higher and exhibited statistical significance. Though, the analgesia in this case is evident, it is not equivalent to the analgesia produced by either 200mg/kg body weight of NaS or 3mg/kg body weight of ketoprofen in sheep. Thus, analgesic efficiency of NaS may be established (even at the lower dose 100mg/kg body weight of NaS) if larger number of sheep are subjected to the experimental treatment. However, in our study due to ethical considerations, minimum required number of sheep were used.

Studies showing the analgesic efficacy of NaS in sheep have not been published to date. Its anti-nociceptive activity has been reported in other animals such as rats and sows. (Tapper, 2011, Baluchnejadmojarad *et al.*, 2005). In these studies, experimental animals were suffering from inflammatory pain; conversely, in our study, nociceptive pain was induced in healthy sheep (absence of inflammation). Generally, analgesic effects of NSAIDs are presumed to be due to their anti-inflammatory effects that inhibit the formation of cyclooxygenase (COX) enzymes involved in production of prostaglandins (PGs). Inhibition

of PGs decreases inflammation and eventually minimises pain due to blocking of pain signals transmitted by PGs (Voilley, 2004). This is attributed to the peripheral mechanisms of NSAID action during inflammatory conditions. Researchers have also reported a central mechanism of NSAID analgesia such as their action dependent on G-protein function and blocking acid sensing channels (Voilley, 2004, Walker, 1995, McCormack, 1994b). These actions of NSAIDs dissociate their anti-inflammatory and analgesic activities (Voilley, 2004, Walker, 1995). The present study was conducted in healthy sheep and there was no apparent tissue damage by the MNT testing device probe. Therefore, the analgesic activity of NaS observed during this study could be due to its central mechanism of action.

It is also apparent, that NSAIDs such as ketoprofen and NaS inhibit the nociceptive pain initiated by the activation of thinly myelinated Aδ fiber nociceptors by their central mechanism of analgesia (Malfait and Schnitzer, 2013, Ferreira *et al.*, 1978). In contrast, the nociceptive pain by thermal stimulus through a weak source of heating (thermode) activates mixed nociceptors (low threshold thermoreceptors, and might activate C fibers). C fiber nociceptors are more sensitive to inflammatory conditions and tend to activate in the presence of inflammation (Kuhtz-Buschbeck *et al.*, 2010, Le Bars *et al.*, 2001). Thus, analgesic efficacy of ketoprofen or NaS after thermal nociceptive threshold testing was not evident in our study which may be due to absence of inflammatory pain.

A study in normal rats demonstrated the efficacy of aspirin in reducing the pain related behaviours in response to mechanical nociceptive stimuli (LaBuda and Fuchs, 2001). Researchers of the study opined that mechanisms other than inhibition of peripheral inflammation might contribute to analgesia produced by aspirin. Moreover, many researchers consider that the central mechanisms involved in the analgesic activity of NSAIDs are more effective than their peripheral anti-inflammatory activities (Burian and Geisslinger, 2005).

The MNT testing method in the current study demonstrates two important prerequisites; firstly, training and acclimatisation of animals to the nociceptive threshold testing prior to the start of experiment, secondly, a quiet environment during the testing. When the present study was conducted in a quiet environment after training the sheep for three weeks, more consistent and conclusive results compared to those of the pilot study were obtained. In the

pilot study, due to a noisy environment, stress induced analgesia or distraction might have masked the analgesic effects of NaS. Stress induced analgesia is also evident in the main study for shorter duration (about 5minutes). Drug administration during the main study was carried out by jugular venepuncture without catheterisation. On the other hand, sheep were catheterised for drug administration in the pilot study. Unfortunately, some sheep pulled out the catheters resulting in further stress during this study. Hence, in the main study catheterisation was avoided. Jugular venepuncture for drug-injection in this (main) study consequently demonstrated stress in sheep immediately after injection (due to manual restraining of sheep for few minutes for the purpose of injection). It is usual and likely for animals such as sheep to become psychologically stressed due to handling, transport and other husbandry procedures (Grandin, 1997). The stress in sheep was evident from behavioural changes such change in posture, increased movements during restraining etc. which are known and general behavioural changes in sheep (Cockram, 2004). In order to manage any kind of stress, an internal mechanism of analgesia (stress-induced analgesia) is activated by secretion of endorphins (Shutt *et al.*, 1987, Owens *et al.*, 1984) In figure 4.6a (main study results) MNTs of all the treatment groups at 5 minutes time interval (very first threshold testing point after injection) are high as compared to baseline thresholds. This short increase of thresholds may be a result of stress-induced analgesia. Further nociceptive testing at 10 minutes (post-injection) time resulted in decreased thresholds as sheep were stable and stress-free. Thereafter, no stress induced analgesia was apparent for the rest of the study period since there were no other additional stress causing factors such as a noisy environment.

NSAIDs including salicylates are reported to produce various side effects such as gastric irritation and haemorrhages, diarrhoea, respiratory distress, nausea, skin rashes, renal dysfunction etc. (Katzung *et al.*, 2004). During the present study sheep were constantly monitored for the side effects of NaS if any. One sheep showed abnormal signs such as teeth grinding and restlessness at 90 minutes after administration of 100mg/kg NaS. Thus, 40 mL of 5% dextrose saline was given IV to this sheep, and was monitored carefully for the next two days. However, sheep was normal and healthy within 15 minutes of saline administration. Nonetheless, these symptoms were considered as side effects of NaS.

Further administration of 200mg/kg NaS, a week later in the same sheep, did not cause any abnormal signs. The plausible explanation for the absence of any abnormal symptoms after higher dose of NaS could be the temperament of the sheep. It is usually assumed that grinding of teeth in sheep is evidence of irritability and pain. During the 100mg/kg treatment, the sheep was given the test drug after several hours of waiting in the pen and later moved to the crate for experimental procedure which could have resulted in anxiety. Also, when the drug is not properly injected into the vein, it could be painful sheep due to extravasation. In this case, tit is likely that extravasation of 100mg/kg NaS resulted in the described signs. Thus, the signs exhibited by sheep post-100mg/kg NaS treatment were not considered as side effects of NaS.

4.4 CONCLUSION

Analgesic efficacy of NaS in sheep was evident after mechanical nociceptive threshold testing. NaS treatment at 200mg/kg body weight exhibited equivalent analgesic potency to 3mg/kg body weight of ketoprofen (positive control) in sheep. Conflicting results obtained from the thermal nociceptive threshold testing in this study could be due to activation of different types of nociceptors as compared to mechanical threshold testing.

From these findings it appears that mechanical nociceptive threshold testing is more appropriate method than thermal nociceptive threshold testing in healthy (without inflammatory pain) sheep to investigate the analgesic efficacy of NSAIDs like NaS.

The apparent analgesic efficacy of NaS in the absence of inflammatory condition in sheep specifies central mechanism involved in analgesia

To conclude, NaS (metabolised to SA) is analgesic in sheep. This provides strong groundwork for testing the analgesic efficacy of willow leaves in sheep.

5 ASSESSMENT OF SALICIN CONTENT IN WILLOW (*SALIX SPP*) LEAVES AND SEASONAL VARIATION

ABSTRACT

Willow (Family: Salicaceae, Genus: *Salix*) is a medicinal plant and has been used for analgesia in humans since ancient times because of the salicin it contains. The utility of willow as a fodder for livestock during droughts due to its abundant presence in New Zealand has been studied (Pitta *et al.*, 2007, Kemp *et al.*, 2001, McCabe and Barry, 1988). Its efficacy as an analgesic in animals has never been reported. Therefore, with the possibility of using willow leaves as analgesic in sheep, this study was conducted to assess the variation in salicin content. Willow leaves from 12 trees were collected monthly over the year. Leaves were dried and processed to determine the salicin content by using high performance liquid chromatography. Salicin content of willow leaves on a monthly basis was compared. Corresponding average monthly temperature and rainfall during the leaves collection period were obtained and correlated with the salicin content. The effect of location, species and age was noted but not studied in detail. Salicin content and temperature has positive correlation. Salicin content in willow leaves in New Zealand (North Island) varies significantly with season and is high (1.62±0.17 grams/kilogram of fresh willow leaves) from November to March (from late spring throughout the summer to early autumn).

5.1 INTRODUCTION

Willow (*Salix spp*) belongs to the family *Salicaceae* and has hundreds of species (Newsholme, 1992) (which hybridise) distributed worldwide (Ball *et al.*, 2005). Although it is not native to New Zealand, it was introduced in 1840s by early European settlers (Phillips and Daly, 2008, Charlton *et al.*, 2007). The primary purpose of willow trees in New Zealand was to control soil erosion. Now willows are often used as a fodder for livestock such as cattle, deer and sheep during draught period (Olsen and Charlton, 2003, Wilkinson, 1999, McCabe and Barry, 1988). Willow is a medicinal plant and has been used as analgesic in humans since ancient times because of the active metabolite salicin. Willow bark and leaves

contain high concentrations of salicin, which has analgesic properties (Vlachojannis *et al.*, 2011). The analgesic properties of salicin occur after conversion to salicylic acid which is its major metabolite (Kammerer *et al.*, 2005). In spite of use of willow as fodder in animals (Pitta *et al.*, 2005, McWilliam, 2004, Kemp *et al.*, 2001), its use as a medicinal plant has not been reported. To exploit its medicinal properties, it is essential to determine the concentration of its key component salicin and to assess the variation in its concentration with respect to different seasons/climatic conditions. In New Zealand (NZ) only a single study on seasonal variation was conducted by (McIvor *et al.*, 2015) as far as we know. However, they did not exclusively focus on the salicin variation as their experiment included other objectives. The current study was carried out with the sole objective to determine salicin content in the willow leaves and its variation over the year. The results of this study provide further insight on the use of willow (*Salix spp*) as a natural analgesic agent for sheep.

MATERIALS AND METHODS

5.1.1 Leaf collection, drying and grinding

The willow leaves were collected on mid of every month (15±2) from 12 different trees during October 2014 to September 2015. However, since willow is deciduous, leaves were unavailable from June to September, 2015, due late arrival of spring. Trees selected for leaf collection were numbered from T-1 to T-12. Ten (T-1 to T-10) trees were located at the Large Animal Teaching Unit, Massey University, Palmerston North (LATU), 40°23'38.4"S 175°38’02.4"E growing on heavy clay. Two trees (T-11 and T-12) were located at 25km West of Palmerston North; 40˚23’ S, 175˚23’ E in drought-prone sand country. T-11 (figure 5.1, a) was curly willow similar to *S. matsudana×alba,* phenotypically. T-12 (figure 5.1, b) was a white willow type (*S.alba).* Its leaves were brighter, wider and longer than T1 - 10 (figure 5.1, c). However, species verification by genomic analysis was not confirmed as this is difficult (Karp *et al.*, 2011) and costly. Moreover, species differentiation was not required for this study.

The leaves were plucked up to the level usually grazed by sheep. They were weighed fresh and dried in an oven at 50ºC. Dry weights were recorded once a constant dry weight was achieved.

Dry matter (DM) content was calculated by using fresh and dry weights (DM % = dry weight/fresh weight X 100). The leaves were then ground (Cylcotec, 1093 Sampler mill) to prepare powder for salicin analysis.

a. *b.* *c.*

Fig 5.1: *Leaves from different willow trees showing differences in morphology (different species)*

5.1.2 HPLC analysis of salicin content in willow leaves

5.1.2.1 Derivation and validation of HPLC method for salicin detection

The High Performance Liquid Chromatography (HPLC) method for detection of salicin in willow (*Salix spp*) leaves was derived from the method developed by (Zhang *et al.*, 2011) to estimate salicin in poplar buds. They followed International Conference on Harmonisation (ICH) (ICH, 1997) guidelines. Zhang's method was initially used, however, due to a different HPLC system, analytical column and sample preparation method, several modifications were essential to acquire precise results. Therefore, several sample preparation methods, temperatures of analytical column, flow rates, extraction medium of leaves/samples etc. were incorporated to achieve satisfactory outcomes. The best method of salicin analysis was selected to conduct a validation study for assurance of the accuracy, precision, linearity and detection limits within the laboratory conditions.

5.1.2.1.1 Selectivity/Specificity

The selectivity of the method was checked by running the blank sample (sample other than willow leaves which did not contain salicin) with the same method used to prepare sample for the salicin detection. Absence of any peak in the blank sample at the retention time of salicin confirmed the selectivity/specificity of the method. Figure 5.2 shows a

chromatograph of the blank sample without any peak and other two chromatographs with salicin peaks; willow leaves and pure salicin standard. This demonstrates specificity of the method to detect the presence of salicylic acid at the given retention time.

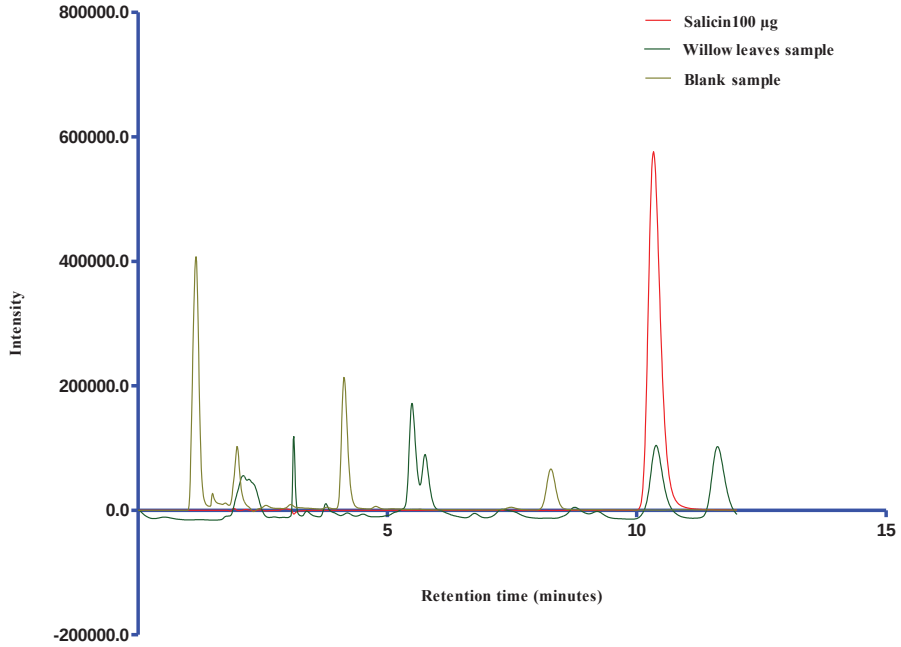

Fig 5.2: *Chromatographs showing presence and absence of salicin peak in salicin containing and blank samples respectively*

5.1.2.1.2 Accuracy/Recovery

Accuracy of the method was determined by spiking three different concentrations (200, 100 and 50 µg/mL) (100%) of pure (99.99%) salicin (from the stock solution) in water (Milli-Q). Samples of willow leaves with known salicin concentration were run individually and also were spiked in the pure salicin standards. Recovery of the salicin was obtained by deducting the peak area of the standard from the sample containing standard and willow leaves extract. This result was compared with independent peak area of the pure willow leaves extract sample. Linear regression model was used for estimation of the true recovery (µg/mL). Table 5.1 shows the result of recovery. Recovery for the given concentrations ranged between 99.86 to 103%.

Table 5.1: Recovery of salicin (µg/mL) after spiking in the leaves sample

salicin (µg/mL) spiked	Mean (n=9) salicin (µg/mL) recovered	Recovery (%)	SD (n=9)	RSD (%)
200	200.67	100.34	0.95	0.48
100	103.51	103.51	0.81	0.78
50	49.93	99.86	0.72	1.44

5.1.2.1.3 Precision

Precision of the HPLC method was assessed by intra- and inter-day variation. This was achieved by running three concentrations of salicin (400, 200 and 100 µg/mL) in water. Three samples of each concentration were prepared every day and analysed in three batches each day in triplicate to assess the intra-day variation. This was repeated for three different days to check inter-day variation. Table 5.2 and 5.3 show intra- and inter-day variation respectively.

Table 5.2: Intra-day variation (between batches) of salicin standards

Intra-day variation						
Day 1						
SA (µg/mL)	**Batch 1 AUC**	**Batch 2 AUC**	**Batch 3 AUC**	**Mean AUC**	**SD**	**%RSD**
400	36931441	36931175	36879694	36914103	29800	0.08
200	20367561	20397779	20369145	20378162	17008	0.08
100	10444000	10494152	10488610	10475587	27495	0.26
Day 2						
SA (µg/mL)	**Batch 1 AUC**	**Batch 2 AUC**	**Batch 3 AUC**	**Mean AUC**	**SD**	**%RSD**
400	34442080	33725137	33810842	33992686.33	391538	1.15
200	20198084	20136625	20166278	20166995.67	30736	0.15
100	10564713	10595233	10603804	10587916.67	20547	0.20
Day 3						
SA (µg/mL)	**Batch 1 AUC**	**Batch 2 AUC**	**Batch 3 AUC**	**Mean AUC**	**SD**	**%RSD**
400	32928916	33659364	32958098	33182126	413558	1.25
200	19889467	19909789	19896944	19898733.33	10278	0.05
100	10498953	10502362	10492298	10497871	5118	0.05

Table 5.3: Inter day variation (between days) of salicin standards

	Mean AUC's (n=3)			AUC		
SA (µg/mL)	**Day 1**	**Day 2**	**Day 3**	**Mean**	**SD**	**% RSD**
400	36914103.33	33992686.33	33182126	34696305.22	1962963	5.66
200	20378161.67	20166995.67	19898733.33	20147963.56	240280	1.19
100	10475587.33	10587916.67	10497871	10520458.33	59474	0.57

5.1.2.1.4 Linearity

Five samples with different concentrations of salicin from 25 to 400µg/ml were run and their linearity was assessed by plotting the graph of peak areas against the concentrations by applying a linear regression model in the statistical software GraphPad Prism 5. The r^2 (determination coefficient) was 0.9945. Table 5.4 shows the slope, Y intercept and r^2 values for three different sets of salicin concentrations.

Table 5. 4: Results of validation of linearity for salicin detection method

	Slope	**y- intercept**	**r^2**
Set 1	91140	1000000	0.9972
Set 2	88566	1000000	0.9961
Set 3	88209	1000000	0.9958
Mean	89305	1000000	0.9964
SD	1599	-	0.00074
% RSD	1.79	-	0.074

5.1.2.1.5 Limit of quantification

Range of lower concentrations of salicin (3.125, 6.25, 12.5µg/mL) were tested along with the blank sample to detect the quantifiable limits (figure 5.3). Concentration 6.25µg/mL and above were quantifiable at signal to noise ratio of 1:10.

5.1.3 HPLC Analysis of samples

The analysis of samples was carried out with the validated method. The procedure of sample preparation and analysis is described as below.

5.1.3.1 Sample preparation

Oven dried and powdered willow leaves (1g) were vortex mixed in 10 mL of Milli-Q water and kept overnight at room temperature (12±3 hours). Supernatant was extracted and filtered in a test-tube through a 5 µ PFTE syringe filter. 0.1 mL of this supernatant was diluted 20 times with Milli-Q water to make a volume of 2 mL. Diluted sample was centrifuged at 14000g for 10 minutes and 50µL of the final sample was injected to HPLC column.

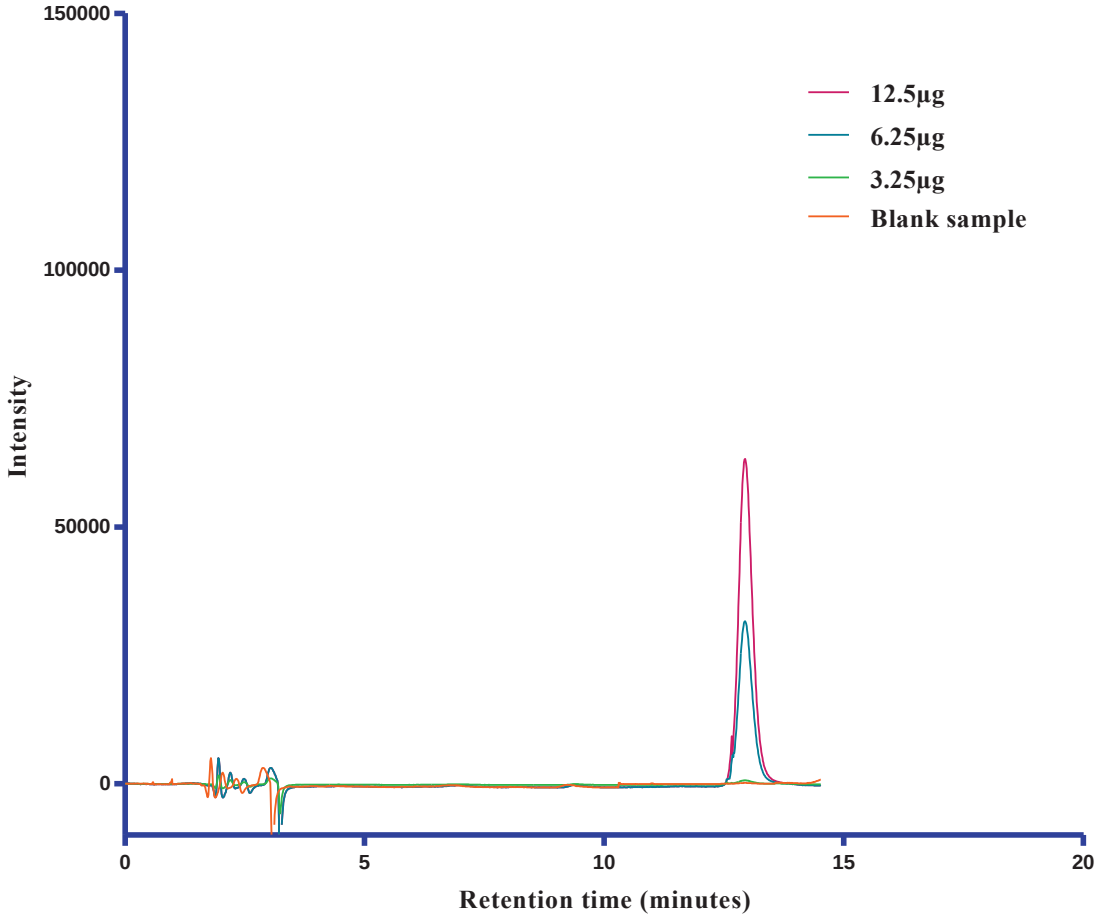

Fig 5.3: *Chromatographs of lower concentrations of salicin and blank sample for determination of lower limit of quantification*

5.1.3.2 Chromatographic conditions

The salicin content in the willow was analysed by reverse-phase HPLC. The HPLC system consisted of LC-20 AD pumps, SIL- 20 AC HT auto-injector, SPD- M20A diode array detector, CTO-20A column oven, DGU-20 A3 degasser (Shimadzu Japan).

Mobile phase for salicin analysis was prepared with Milli-Q water (Milli-Q PFplus system, Millipore Cooperation, USA), acetonitrile (HPLC-grade, Merck, KGaA; 64271 Darmastadt) and orthophosphoric acid (BDH Limited Poole England) respectively with the ratio 94.5:5:0.5. Reverse phase Phenomenex C18 Synergi Hydro® (4µ RP 80 Å, LC Column 150 x 4.6 mm) column was used for analysis. The temperature of column was maintained at 40ºC

under isocratic conditions with flow rate of 0.8 mL/minute. The detector was set at 213 nm wavelength. Retention time for salicin was 10±0.69 minutes.

5.1.3.3 Stock solution

Salicin stock solution was prepared by dissolving 0.1g salicin (Sigma Alderich, China) in 100 mL of Milli-Q water and this was used to prepare standards every day.

5.1.3.4 Standards

Five different concentrations of salicin standards 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, and 25µg/ml were prepared from stock solution in Milli-Q water to allow construction of a calibration curve.

The calibration curve was prepared using liner regression model ($r^2 = 0.9907$) in GraphPad Prism 5. A typical curve is shown in figure 5.4. The chromatographs for the salicin standard and willow leaves samples are shown in the figure 5.5

5.1.3.5 Calculations

AUCs obtained after HPLC analysis of willow leaves samples were multiplied by 20 (dilution factor) and then the salicin concentrations (µg/mL) were calculated from calibration curve in GraphPad Prism 5. As the leaves were soaked in 10 mL of water, the concentration in µg/ml was multiplied by 10/1 (1 g powder soaked in 10 mL water) to get salicin concentration in µg/g leaves which was consequently converted to g/kg of DM. Final salicin concentration per/g of fresh willow leaves was calculated from the dry matter of the leaves.

5.1.4 Weather data collection

Average rainfall and temperatures for the months of leaf collection were obtained from MetService (New Zealand's official weather reporting service) website for Palmerston North to observe the effect of temperature and rainfall on the salicin content of willow leaves.

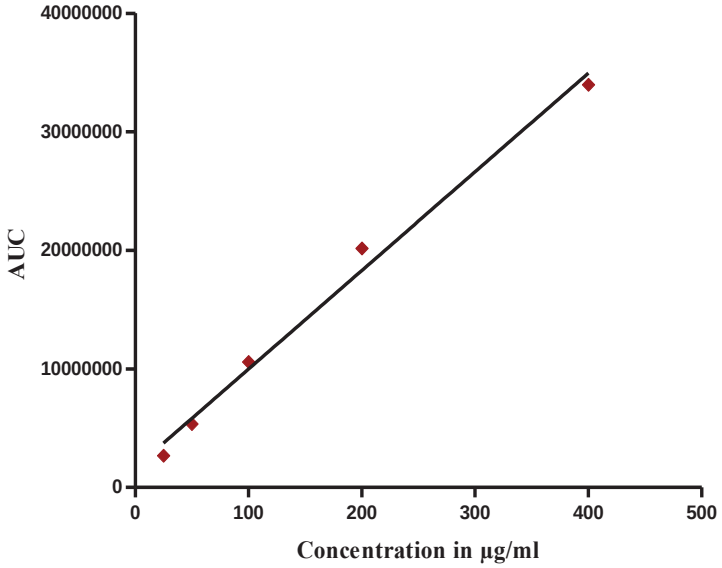

***Fig 5.4**: Calibration curve for salicin detection with HPLC*

5.1.5 Statistical analysis

The experimental data was analysed by one way ANOVA with post Tukey's multiple comparison test to compare the salicin content on the monthly basis. An unpaired t test with Welch's correction demonstrated the statistical difference between the two different locations. The Spearman correlation coefficient between temperature and salicin content was calculated to assess the effect of temperature on salicin variation. All statistical analyses were done in GraphPad Prism 5 (Prism 5 for windows, version 5.01, August, 2007) and were considered significant at $P<0.05$.

5.1.6 Results

5.1.6.1 Salicin content

Willow leaves had significantly ($P<0.05$) lower salicin content in the months of October-2014 and May-2015 as compared to other months. The monthly average content of salicin is shown in table 5.5.

It was observed that the salicin content in the two trees (T-11 and T-12) which were located distant from other 10 trees was consistently significantly higher ($P<0.05$) than other trees

throughout the year. Table 5.6 and figure 5.6 shows the variation in salicin content of individual trees throughout the study period as well higher salicin content in T-11 and T-12.

Table 5.5: Salicin (g/kg) content in fresh willow leaves (mean±SD)

Month-year	N (number of trees)	Salicin content (mean±SD) (g/kg)
October-2014	10	0.59±0.28
November-2014	12	*1.87±0.86*
December-2014	12	*1.51±1.01*
January-2015	12	*1.71±0.74*
February-2015	12	*1.43±0.97*
March-2015	12	*1.56±1.21*
April-2015	NS	NS
May-2015	11	*0.85±0.59*
June-2015	NS	NS
July-2015	NS	NS
August-2015	NS	NS
September-2015	NS	NS

NS - No leaves/sample available during this time

Table 5.6: Difference in Salicin (g/kg) content of fresh willow leaves from trees located on two different locations (mean±SD)

Month-year	Salicin content (g/kg)	
	T-(1to10)	T-(11+12)
October-2014	0.59±0.28	NS
November-2014	1.52±0.20	3.64±0.50
December-2014	1.15±0.27	3.64±0.65
January-2015	1.41±0.23	3.21±0.29
February-2015	1.01±0.12	3.49±0.12
March-2015	1.06±0.24	4.05±0.76
April-2015	NS	NS
May-2015	0.60±0.22	1.96±0.18

NS - No leaves/sample available during this time

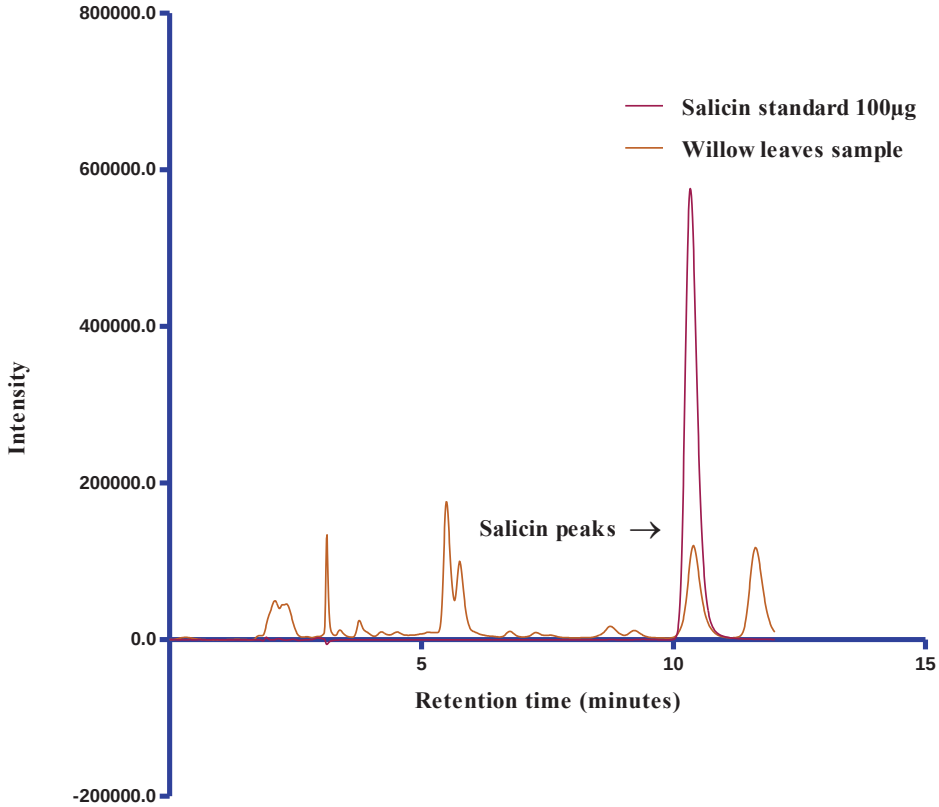

***Fig 5.5**: Chromatographs of salicin standard 100µg and willow leaves sample showing respective peaks at given retention time (10±0.69 minutes)*

The salicin content was lowered with increased rainfall. Increase in temeperature resulted in incresed salicin content in willow leaves (figure 5.7). Correlation coefficient (Spearman) between temperatures and salicin content in willow leaves was 0.5455.

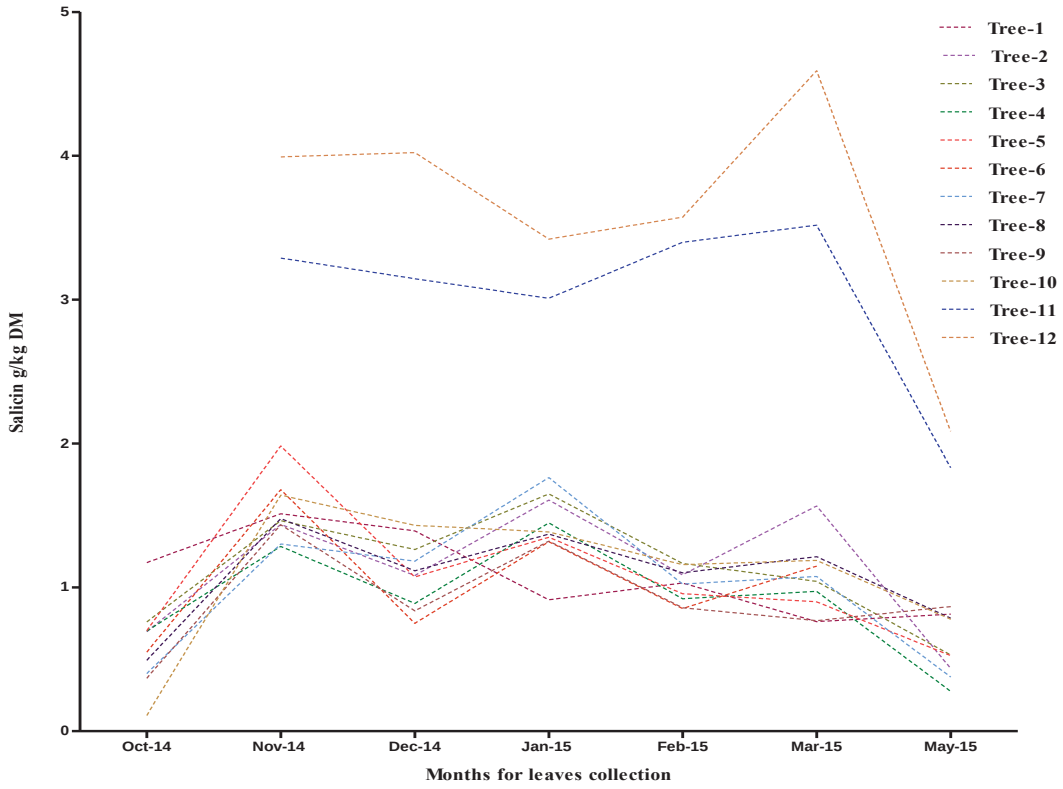

Fig 5.6: *Graphical representation of willow leaves salicin content (g/kg of fresh willow leaves) in individual tree*

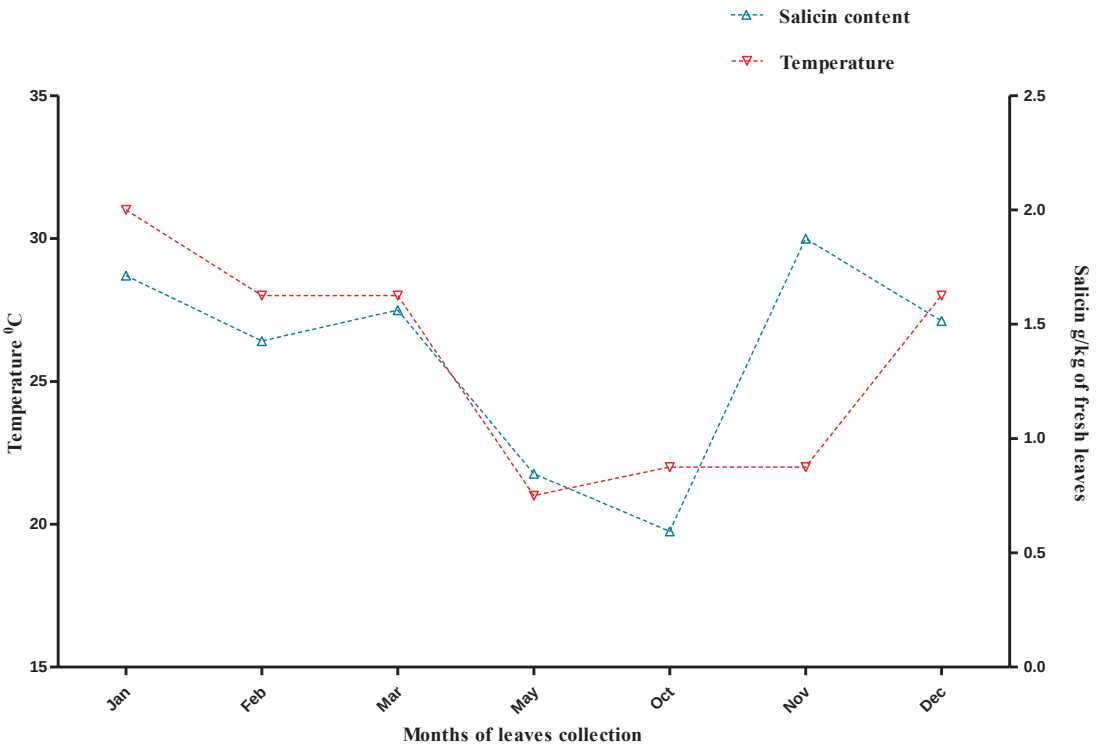

Fig 5.7: *Graphical representation of average monthly temperature vs. respective monthly willow leaves salicin content (g/kg of fresh willow leaves) during leaves collection period*

5.1.7 Discussion

Salicin content in the willow leaves over the year was variable with the season. During the winter months, salicin content was declined while; during summer months it was elevated. Similar observations were reported by (McIvor *et al.*, 2015) when they studied willows in New Zealand.

Location and species also influenced the salicin concentrations; as two of the distantly located trees in the current study had higher salicin concentration than other ten trees. However, McIvor *et al.* (2015) did not observe salicin variation due to change in geographical location.

Overall salicin concentration in willow leaves collected in this study was consistent through the month November to March. Therefore, as per New Zealand's climatic conditions, salicin in willow trees considered in this study was high during late spring, throughout the summer to early autumn. Rank (1991) observed a decline in salicin content in some species of willows over summer (May to July) in the USA, Nevada (central and eastern California). On the contrary, in the present study, salicin content in willows we studied had high salicin content over the summer (November to March) in New Zealand i.e. when plants were stressed. Therefore, climatic alterations in different regions/parts of the world influence salicin content of willow.

The increase in temperature results in the increased salicin content in the leaves. Willow leaves were present on the trees and accessible during 2014 from late May while in 2015 leaves on the trees selected for this study were inaccessible from mid-April as rainfall was high during that period, but in May some leaves were available. Though, unlike in 2014, leaves were not available from June till the end of September due to higher rainfall in 2015.

Literature suggests that salicin content in the willow varies a with age, season, location, species of the tree (Kenstaviciene *et al.*, 2008, Rank, 1991). Some studies have mentioned that the male plants have lower salicin content and phenolic glycosides than female plants (Elmqvist *et al.*, 1991, Palo, 1984); whereas, Nichols-Orians *et al.* (1993) observed no variation in salicin content as well as phenolic glycosides content due to sex difference but they found that genetic clonal differences in willow causes variation in the phenolic

glycosides. Therefore, to evaluate factors for salicin variation in willow is challenging due to the inconsistencies reported in different studies. Hence, in this study, variation with respect to regional weather and season was considered as the objective of the study.

This study is an important pre-requisite for the analysis of analgesic efficacy of willow leaves in sheep.

5.2 CONCLUSION

This study showed overall salicin variation in willow leaves in Palmerston North (Manawatu region), New Zealand, on a seasonal basis. Also, a simple method of processing willow leaves for salicin estimation with reversed-phase HPLC has been derived which will make future salicin estimation studies easier.

6 GENERAL DISCUSSION

In New Zealand, sheep farming is the second largest farming sector after cattle. According to Statistics New Zealand (government record) (Statistics, 2015) the country has six sheep per person (In year 2016, this has been declined to less than six sheep per person), a total sheep population of 29.5 million. Also, animal welfare is given high importance in this country and the New Zealand Animal Welfare Code (2005) recommends alleviation of pain in animals under all circumstances. Pain in sheep has deleterious consequences with respect to welfare and production (Montossi *et al.*, 2013, J. Fitzpatrick et al., 2006). Therefore, pain management in sheep is very important to improve welfare as well as production.

In general, animal pain management with analgesics is an effective approach and in many cases the only alternative to euthanasia. In our study, exploring a "new" analgesic for sheep was prioritised because of the existing scarcity of analgesics for sheep. The NSAID, salicylic acid which is derivative of salicin obtained in natural analgesic willow was selected. Salicin is the important metabolite present in the willow leaves which in turn produces SA which is analgesic in humans. However, to investigate the absorption, metabolism and excretion of the drug (SA) in sheep, a pharmacokinetic study is essential. Likewise, to assess the effect of the drug in sheep, a pharmacodynamics study is important. Hence, initially, pharmacokinetics and pharmacodynamics of SA were investigated in healthy sheep. These studies provided strong groundwork for testing the analgesic efficacy of willow in sheep in future.

6.1 PHARMACOKINETIC STUDIES

As described in the chapter 3, the pharmacokinetic study of SA after intravenous and oral administration in the form of its sodium salt in sheep provided the basis for further work and also showed significant differences when compared to the single similar published pharmacokinetic study conducted in sheep in India. During our experiment, a simple, robust and sensitive HPLC method was developed and validated in our laboratory for the analysis of SA in sheep plasma. Currently, no other HPLC method has been published to analyse SA specifically in sheep plasma. Thus, this method will be useful for further research to be

conducted in sheep with SA. The sensitivity of our HPLC method was higher than other published methods described for the analysis of SA in plasma samples of different animals.

The pharmacokinetic study of SA was conducted after intravenous and oral single doses of sodium salicylate. During this study, SA was administered in the form of its sodium salt rather than its free form. The administration of salt of a drug does not affect the release of its original moiety after administration; instead, it improves the absorption and availability of the drug in the body (Hedaya, 2012). However, while administering the salt of any drug, it is important to consider the free form of the drug available for absorption. Sodium salt of SA yields about 86% of SA (Saltzman, 1948). In our study, we selected four dose rates for intravenous administration as 200, 100, 50 and 10mg/kg body weight of sodium salicylate which produces 172, 86, 43 and 8.6mg/kg bodyweight of SA respectively. During pharmacokinetic analysis, plasma SA concentrations were analysed to investigate its pharmacokinetics. However, calculation of pharmacokinetic parameters was carried out for the respective doses of both sodium salicylate and SA. No significant difference was observed in the pharmacokinetic parameters of both compounds. Thus, pharmacokinetics of SA, with the mentioned dose rates of sodium salicylate was preferred and presented in this thesis.

In our pharmacokinetic study, maximum plasma concentration of SA was 37.91µg/mL which was maintained for shorter duration (less than 15 minutes) after administration of 200mg/kg body weight of sodium salicylate. Yet, no other study with this dose rate (200mg/kg) in sheep has been reported as far as our knowledge. Sulaiman and Kumar (1995) administered a single bolus/dose of sodium salicylate at the rate of 100mg/kg body weight both intravenously and orally. Doses in our study for intravenous administration (200, 100, 50 and 10mg/kg body weight of sodium salicylate) and two doses for oral administration (200 and 100mg/kg body weight of sodium salicylate), 100mg/kg dose of sodium salicylate included the single dose used by Sulaiman and Kumar in their study in sheep. Comparison of pharmacokinetic parameters after administration of 100mg/kg body weight in both studies intravenously and orally, reveals divergent results. Sulaiman and Kumar analysed total plasma salicylate concentration for calculating pharmacokinetic parameters. On the contrary, in our study plasma SA (free form) concentrations were analysed to calculate

pharmacokinetic parameters. This may account for the diversity in results. However, some of their findings were in agreement with the present study. For instance, they evaluated and recommended a one compartment model as a best fit for sodium salicylate plasma concentrations over time. Similarly, calculation of AIC and BIC values in our study explored the suitability of a linear one compartment model as the best fit for SA in sheep. Comparison of other pharmacokinetic parameters demonstrated high diversity due to analysis of total salicylate as opposed to plasma SA. Sulaiman and Kumar calculated the extrapolated concentration of total salicylates at time zero (Cp0) as 300.37µg/mL after intravenous administration of 100 mg/kg body weight of sodium salicylate in sheep. Correspondingly, in our study, after evaluation of one compartment model, Cp0 was found to be 25.64±2.41µg/mL for the same dose rate (100mg/kg body weight of sodium salicylate). This concentration was almost 12 folds lower than the total plasma salicylates concentration reported by Sulaiman and Kumar.

In our pharmacokinetic study, volume of distribution of SA was enormously high after intravenous as well as oral administration. In general, the volume of distribution is very low for NSAIDs (Riviere and Papich, 2013). Also, in other studies, of different animals including sheep, cattle, goat, camel, rat, cat, rabbit and chicken the volume of distribution for salicylates/SA after intravenous administration was in the range of 0.17 to 0.39L/kg; while in our study it was observed between 2.25 and 8.73L/kg after intravenous administration. The range of volume of distribution after oral administration of sodium salicylate in our study was even higher than intravenous study and was in the range of 7.97 to 28.73L/kg. While discovering the causes for these enormously high volumes of distribution in our study, the most probable factor appears to be lipophilic nature of the SA. Rainsford (2013), mentioned the lipophilic nature of salicylates at favourable pH conditions, usually above pH 5 (Bronaugh and Maibach, 1989). Sheep plasma pH is usually 7.42 which seem favourable for SA to be lipophilic. At the same time, availability of high tissue fats will entice SA for distribution in the tissues. SA is comparatively more soluble in fats than water. One gram of SA is soluble in 460mL of water; while, the same quantity can dissolve in only 80mL of fats (Beers *et al.*, 2003). In our study, the sheep had comparatively high body weights and consequently had higher body fats. This may explain the high volume of distribution in our study.

Further differences in pharmacokinetic parameters when compared to other studies could be attributed to the various factors in each study such as difference in analysis techniques, calculation methods and other influences such as climatic/temperature conditions, age, breed and species of the animals used in the study. For instance, Sulaiman and Kumar used local Bannur breed of sheep of average age 17 months; while in the present study Romney cross sheep of about 6 months age were used. These inter-breed differences must be considered (Riviere and Papich, 2013). Furthermore, Nawaz and Nawaz (1983) compared the pharmacokinetics of sulphadimidine in sheep after a single intravenous injection during two different seasons, summer and winter. They observed that zero time extrapolated plasma concentration of the drug was higher during summer as compared to winter. Therefore, climatic/temperature conditions are also decisive and should be taken in to consideration.

In New Zealand, or other temperate regions, no other study in sheep with sodium salicylate has been reported. Thus, comparison of pharmacokinetic parameters in our study under the similar climatic conditions was not possible.

6.2 PHARMACODYNAMICS STUDIES

As described in chapter 4, in the pharmacodynamic study, analgesic effects of sodium salicylate were apparent with 200mg/kg sodium salicylate and were similar to the positive control ketoprofen (3mg/kg) in sheep. Pharmacodynamics of sodium salicylate/SA in sheep after administration of high dose rates (200 and 100mg/kg) have not been reported in literature as far as we know. Lizarraga and Chambers (2006) administered a smaller dose of 10mg/kg SA in sheep intravenously and observed no effect on mechanical thresholds. This dose was not used in the main study; we considered that a higher dose was more likely to achieve an analgesic effect. These findings certainly looked promising for the study which may be conducted in lame sheep by feeding willow leaves.

In chapter 4, pharmacodynamics was evaluated with thermal and mechanical nociceptive threshold testing. These studies showed the ineffectiveness of thermal nociceptive threshold testing at detecting the analgesic effects of sodium salicylate in sheep. Simultaneously, mechanical nociceptive threshold testing was able to show analgesic effects of this drug.

These contradictory results may appear dubious. However, previous studies conducted in animals confirmed the validity of the results obtained from mechanical nociceptive threshold testing. For instance, Ley *et al.* (1989), Chambers *et al.* (1994), Hothersall *et al.* (2014) report similar findings to those of our study. Similarly, unreliability of thermal nociceptive threshold testing in detection of analgesia of NSAIDs in healthy animals (without an inflammatory condition) has also been reported. Chambers *et al.* (1994), in their experiment observed inconsistent responses of sheep to the thermal threshold testing. For those reasons, other methods of detecting pharmacodynamics/analgesic efficacy of drugs may be considered in future studies in healthy animals. These methods include neurophysiological techniques such as EEG (Murrell and Johnson, 2006) and COX inhibition in serum samples (Schäfer *et al.*, 2006), although neither of these measure analgesia directly. EEG analysis to detect analgesic effects of drugs can only easily be carried out in anaesthetised sheep (Johnson *et al.*, 2005): in awake, non-anaesthetised sheep movement artefacts render this technique useless. Similarly, COX inhibition is appropriate during inflammatory conditions to evaluate the peripheral mechanism/anti-inflammatory properties of the drug (Burian and Geisslinger, 2005) but these may not account for all the analgesic effects. The sheep in our study were healthy with no inflammation.

The fact that SA can provide analgesia in healthy sheep in the absence of inflammation implies the involvement of central mechanisms to produce analgesia. Central mechanisms of analgesia by NSAIDs have been evident (Burian and Geisslinger, 2005); therefore, these studies in healthy sheep may be considered as a foundation for the use of SA in pre-emptive analgesia in sheep prior to the surgical interventions or other painful practices.

SA is metabolised from both sodium salicylate and salicin. When willow leaves are considered as a source of SA, salicin is the element which is converted to SA through initial hydrolysis of salicin to saligenin (salicyl alcohol) and then oxidation of this compound to SA (Mahdi, 2014). When these chemical reactions are taken into consideration, theoretically salicin can produce about 49% of SA (calculated based on the molecular weight of both compounds). Hence, it is promising to feed willow leaves to sheep in pain to test the anaglsic efficacy of willow leaves.

6.3 ASSOCIATION AND SIGNIFICANCE OF ALL THE STUDIES CONDUCTED DURING THIS RESEARCH PROJECT

All the experimental studies conducted during this research work complement each other and add to our knowledge of analgesia in sheep.

NSAIDs are reported to produce various side effects in monogastric animals (Katzung *et al.*, 2004). Therefore, pilot studies were conducted prior to both pharmacokinetic and pharmacodynamics studies to ensure that there were no obvious risks to safety of the animals. Pilot studies can play an important role in avoiding bias in the definitive studies and also reduce overall cost (Hillman *et al.*, 1991). The pilot study on pharmacodynamics of sodium salicylate revealed some inadequacies which were amended during the main study. The main pharmacodynamic study confirmed that SA was analgesic in healthy sheep. The pharmacokinetic study provided plasma SA concentrations in healthy sheep along with other important pharmacokinetic parameters. Together these should allow prediction of the effect in the general sheep population. The aim of this thesis was achieved as we wanted to test if salicin derivative SA is analgesic in sheep.

6.4 FUTURE WORK

In this current research work, pharmacology of salicylic acid in sheep has been studied using nociceptive testing. Clinical pain may well respond to lower doses; this needs to be assessed. However, the use of natural source of salicylic acid i.e. willow leaves as analgesic in sheep should be further studied in future by feeding willow leaves to lame sheep. Some more work would be beneficial prior to willow feeding in sheep.

The concentration of salicin and ultimately of SA differs in individual willow plant depending upon location and season, and probably also age, climatic conditions, cultivar etc. Hence, collection of willow leaves from different willow cultivars from different parts of the country and perhaps different parts of the world to further investigate the seasonal variation in salicin content can add to the specific knowledge about the use of willow leaves with respect to their salicin content in sheep. This future work may validate the use of willow leaves in sheep for pain relief. In this particular research project we aimed to test only if salicylic acid is analgesic in sheep; thus, these factors were not investigated.

6.5 CONCLUSION

Overall, in this research, we used well established method of pain assessment such as nociceptive threshold testing by a blinded, trained observer (Molony and Kent, 1997, Ley *et al.*, 1989, Carlsson, 1983) to assure the accuracy and reliability of the results. During all the studies, behavioural changes were also monitored carefully and all variable factors including altered responses of an individual animal were considered. Hence, we consider the results reliable. Similarly, the HPLC method to determine SA and salicin in sheep plasma and willow leaves was thoroughly validated to ensure accurate analysis. Standard pharmacokinetic models were used to determine pharmacokinetic parameters to aid the development of accurate dosing regimens and intervals. No side effects apart from possible pain on inadvertent subcutaneous injection were seen.

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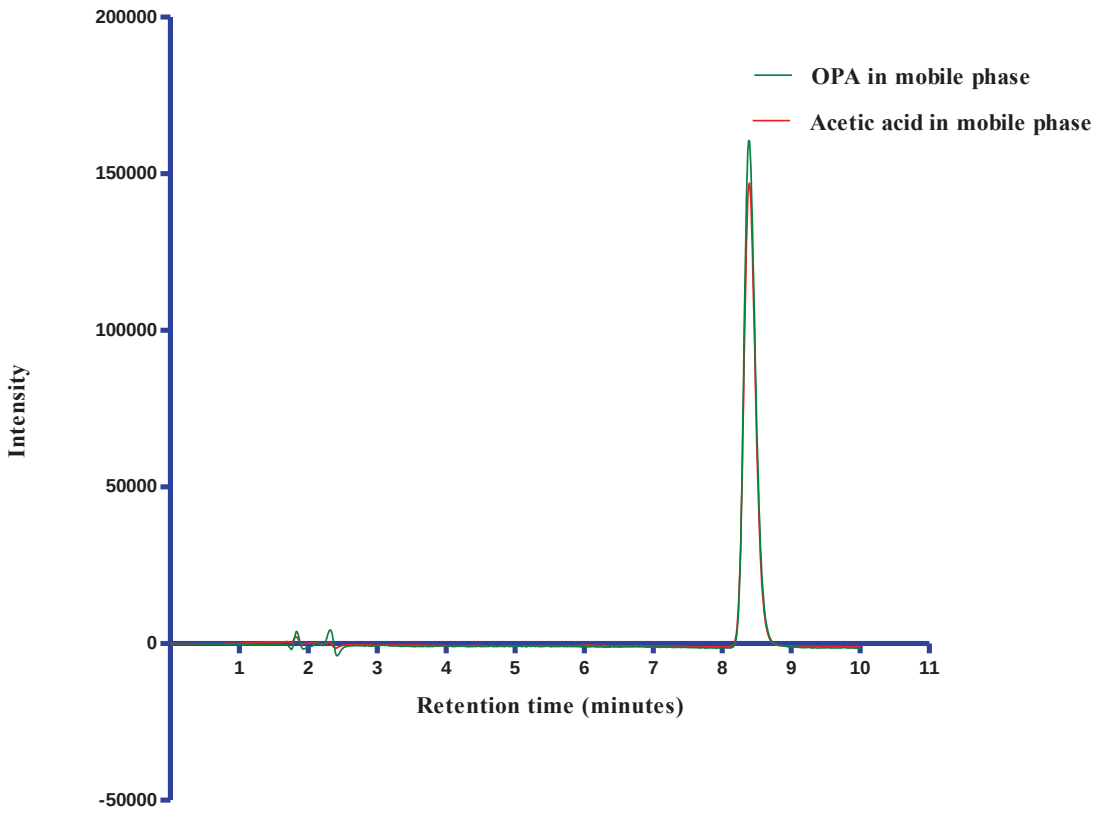
Appendix: 1

Chromatographs showing better peak of salicylic acid after replacement of acetic acid in the mobile phase with OPA for HPLC analysis of salicylic acid



Chromatographs of 50µg SA standard in the mobile phase. SA extraction with Synergi Hydro C18 column and altered mobile phase mobile phase composition as (a) water (71): acetonitrile (28): OPA (1) and (b) water (71): acetonitrile (28): acetic acid (1). The chromatograph with mobile phase composition 'a' has better peak resolution than mobile phase composition 'b'

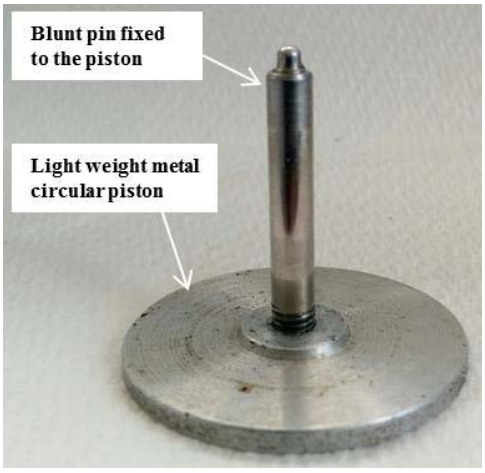
Appendix: 2a

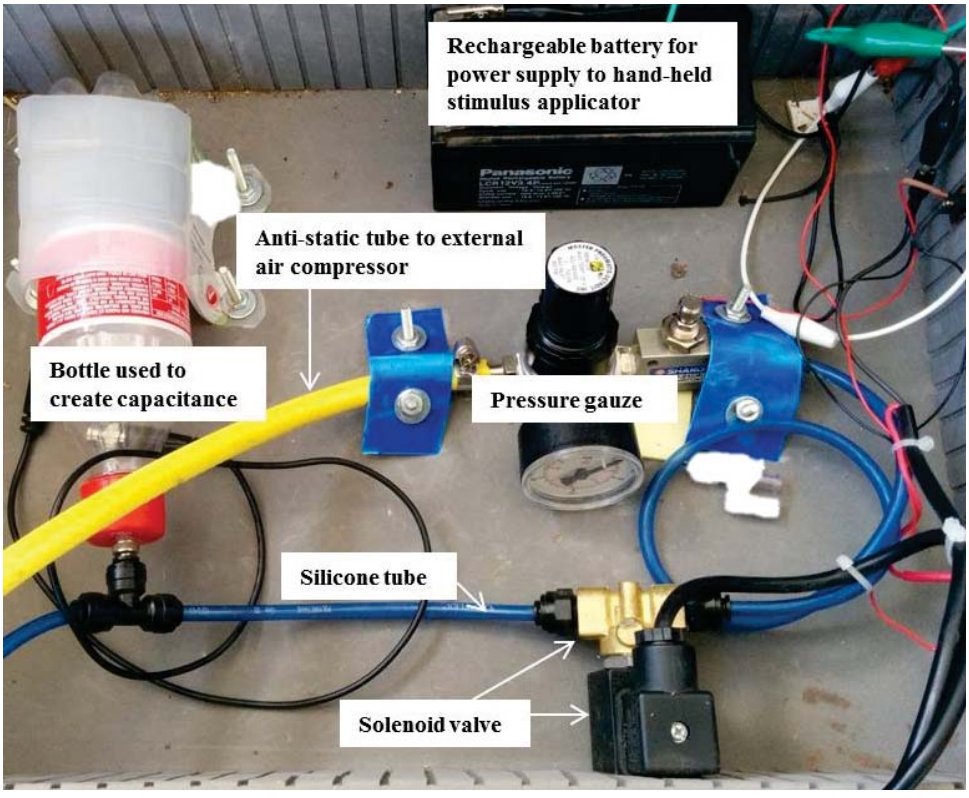
Details of the components of mechanical nociceptive threshold testing device

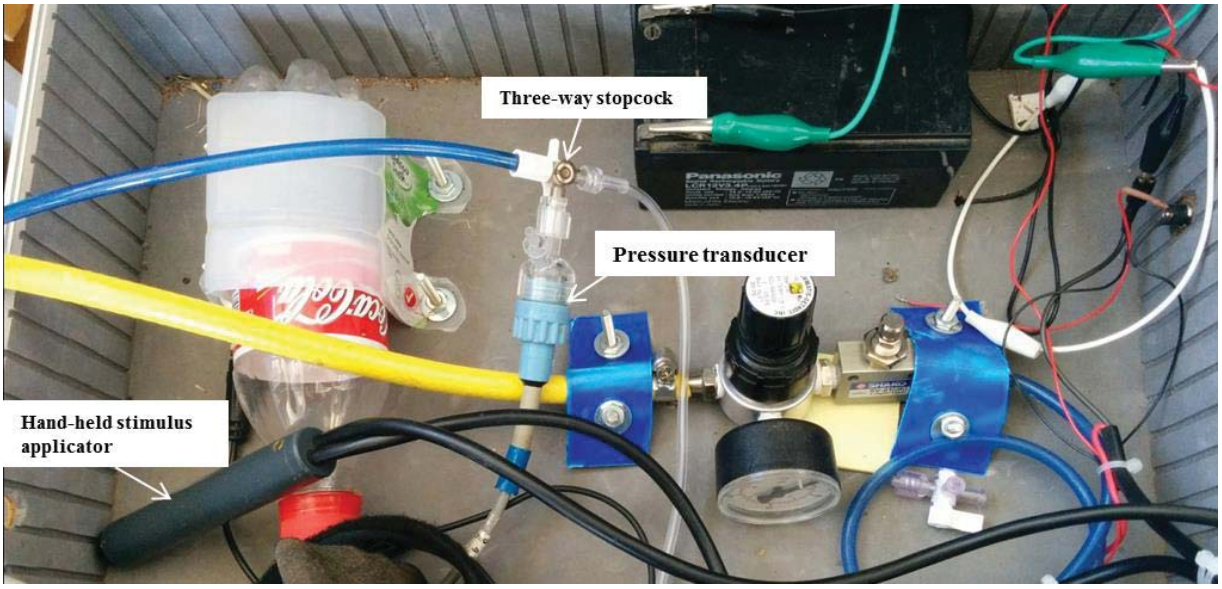
Sr. No.	**Component**	**Details**	**Function/utility in the device**
1	Metal piston (circular) with blunt pin	Aluminium piston of diameter 3.8cm on which a blunt pin of 2mm is fixed	The blunt pin on the piston is forced against sheep's leg to generate nociceptive stimulus
2.	2mL half-way cut syringe	Normal 2mL injection syringe with loss of resistance	A medium sized balloon is fixed on the front end. 180cm long extension tube is attached to the rear end
3.	Plastic box/leg unit	Circular light weight and firm with screwed lid	Holds the metal piston with blunt pin and balloon inside. The Velcro strap is screwed on the outer front surface
4.	Velcro strap	Black, Velcro strap of 20cm	To hold the leg unit of the device on sheep's leg
5.	Extension tube	Polyethylene, 180cm long, 2mm internal diameter.	Fixed on the rear end of the syringe and further attached to the three-way stopcock
6.	Silicone tube	50cm long, 5mm internal diameter	One end attached to pressure transducer through the three-way stopcock and other end is connected to the pressure gauze via plastic bottle
7.	Pressure gauze	Operating range 0-100 PSI; Master Pneumatic-Detroit, Inc. USA	To control and measure the pressure. It is connected to the silicone tube and Solenoid valve
8.	Solenoid valve	3/2 way Universal Direct acting, 12 volt DC, Italy	Controls the inflow and outgo of the compressed air to inflate the balloon to create the force
9.	Pressure transducer	NT-128, Spectramed, Statham, Singapore	Converts the pressure into electrical signals
10.	Three-way stopcock	Medical use, Disposable	To inter-connect pressure transducer, leg unit and Solenoid valve
11.	Hand-held stimulus applicator	Ebonite cylindrical thin rod with the knob on the	One lead is connected to the rechargeable battery to get the power. Another lead is connected to the

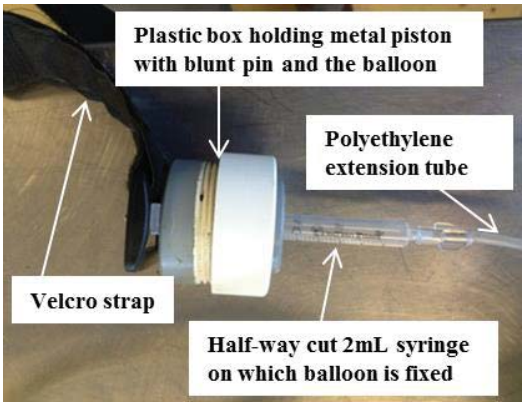
		top to press. It has two rubber leads	solenoid valve. Therefore, when the knob on the applicator is pressed, the air inflow from the valve to the leg unit is operational
12.	Rechargeable battery	Panasonic sealed rechargeable battery, LCR12V3.4P, Matsushita Electric, Japan	Supplies power to the hand-held stimulus applicator
13.	Plastic bottle	500mL, normal soft-drink bottle	Used to create the capacitance. It is attached to the pressure gauze and pressure transducer.
14.	External air compressor	Portable Oil Free 1HP/6 Litre Air Compressor, ReddiAir, Redditch, UK	Uses electric energy to generate the pressure through compressed air.
15.	Anti-static garden pipe	½ inch internal diameter and 2metre long	Connects the external air compressor with pressure gauze
16.	Wheatstone bridge circuit	Constructed at IVABS, from the design developed J RACK pressure amplifier (Biosignals, Melbourne, Australia)	Connected to the pressure transducer for amplification of the pressure. Also, connected to the PowerLab
17.	PowerLab	4/SP ADInstruments, Dunedin, New Zealand	Converts complex analogue to digital data which is decipherable in computer
18.	Computer	Windows 8, TOSHIBA satellite Notebook, Mfd. China	Receives the data in the software LabChart
19.	Lab Chart	Windows 8 (32-bit and 64-bit editions) (7.3.7)	Detection of data signals from PowerLab and exhibition on the computer

Figures of the different components of mechanical nociceptive threshold device






Three-way stopcock
Pressure transducer
Hand-held stimulus applicator


Plastic box holding metal piston with blunt pin and the balloon
Polyethylene extension tube
Velcro strap
Half-way cut 2mL syringe on which balloon is fixed


Velcro strap screwed on the plastic box
Blunt Pin

Appendix: 2b

Significant internal components of thermal nociceptive threshold device

Sr. No.	Component	Details	Figure	Function/utility in the device
1	Digital temperature sensor	Dallas DS18B20 RS Components, Auckland, New Zealand		Thermostatic control, can be used as thermometer and used in thermally sensitive systems
2	Resistor	10 Ohm 1/4w 0.25W 5% Tolerance		To control the current flow throughout the circuit
3	Microprocessor	ATmega328P; Atmel Corporation, San Jose, CA, USA		A microcontroller single chip used to combine 32 KB flash memory with read while write capabilities
4	Transistor	N-channel MOSFET FQP30N06L; RS Components		It is a transistor used to manage (amplify/control) power in the device
5	Bluetooth modem	BlueSMiRF Silver; SparkFun Electronics, Niwot, CO, USA		Used to pass streams/commands from computer to the target
6	Microcontroller board. Consists of components 1, 2 and 3	Arduino Uno; RS Electronics		Controls the heating of device
7	Prototyping shield	ProtoShield Basic; Freetronics, Croydon South, Victoria, Australia		Used as a base for wiring of microcontroller and as a connector between Bluetooth modem and microcontroller