Pharmacology of Analgesic Drugs in Birds

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Abstract

Analgesics drugs are widely used to alleviate pain in mammals and birds. However, in the case of birds, there is a scarcity of information on their usage and dosing regimen. A lack of pharmacokinetic knowledge can result in under or over-dosing of drugs with subsequent loss of efficacy or side-effects. Complete understanding of a drug requires knowledge of its pharmacokinetics as well as pharmacodynamics. Considering the various voids in pharmacological research in birds and in an effort to know more about pain and welfare in birds, this study was designed to study the pharmacokinetics of morphine, butorphanol, aspirin and salicylic acid in broiler chickens. Broiler chickens were used as a model for wild and rare birds. Morphine and butorphanol were injected intravenously at 2 mg/kg, while aspirin and salicylic acid were injected intravenously at 50 mg/kg.

All the analgesic drugs were well distributed in chickens. The plasma clearance for these drugs was much higher than in mammals, resulting in shorter half-lives. All the drugs remained within the theoretical therapeutic range for 2 hours.

For analgesic efficacy testing, all the drugs except aspirin were injected in lame broiler chickens at similar dose rates as in the pharmacokinetics experiment. The results from the efficacy tests suggest that butorphanol and salicylic acid provided adequate analgesia which lasted for less than 2 hours. Morphine at 2 mg/kg intravenously induced sedation and drowsiness in chickens, which might be due to the high dose. It may have analgesic effects at lower dose rates, however this needs to be further evaluated. The approximate therapeutic range in broiler chickens for butorphanol is 50 to 80 ng/mL and for salicylic acid is 50 to 110 ng/mL. The therapeutic range for butorphanol is much higher in birds as compared to mammals while for salicylic acid it is in the mammalian range. The duration of analgesia in birds could be increased by using sustained released formulation or drug delivery systems, which warrants further research.

Plasma concentrations after butorphanol given at 4 mg/kg in an injured Northern Royal Albatross under surgical conditions were also evaluated. This is the only pharmacokinetic

study of an analgesic drug in a sea bird. The pharmacokinetics of butorphanol in this albatross differed significantly from chickens, with slower clearance and lower tissue distribution, although these were much higher than in mammals. The difference in pharmacokinetic parameters could either be due to species variation or due to the continuous fluid therapy along with butorphanol administration. This albatross was suffering from a major femur fracture, which potentially altered its normal physiology and metabolism. Chickens may be used as a model of drug research for wild and rare avian species, especially for preclinical trials. The dosing regimens can be extrapolated from chicken pharmacokinetics data, but this should be done with extreme caution as pharmacokinetics are highly variable between the species.

KEYWORDS: Morphine, butorphanol, aspirin, Salicylic acid, Broiler chickens, Albatross analgesia.

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List of Publications

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NMDA-N-Methyl-D-Aspartate
NRPG- nucleus recticularis paragigantocellularis
NRM-nucleus raphae magnus
PAG-Periaqueductal Gray
I/T- intrathecal
S/C-subcutaneous
I/M-intramuscular
I/V- intravenous
MAP- Mitogen Activated Kinases
DAG- Diacylglycerol
PKC- Protein Kinase C
MAC-Minimum Alveolar Concentration
MEC-Minimum effective concentration
NSAIDS- Non Steroidal Antiinflammatory Drugs
COX-cyclooxygenase
TXA2 and TXB2-thromboxane
M-3-G- morphine-3-glucoronide
M-6-G- morphine-6-glucoronide
LLQ-lower limit of quantification
LOD-Limit of Detection
LC/MS- Liquid Chromatography/Mass spectrophotmeter
AUC-area under the curve
AUMC- area under the moment curve
MRT- mean resident time
V_d- volume of distribution
V_c-volume of distribution, central compartment
V_p-volume of distribution, peripheral compartment
V_t- total volume of distribution
V_{dss}- volume of distribution, steady state
$t_{1/2\alpha}$- Distribution half life
$t_{1/2\beta}$- Elimination half life
$t_{1/2\lambda 2}$- Terminal half life
LOD-lower of detection
DAD-diode array detector
HPLC-high performance liquid chromatography
RSD- relative standard deviation
μ-mu Opioid receptor
κ-kappa Opioid receptor
σ-sigma Opioid receptor
δ-delta Opioid receptor
β-elimination Rate constant
α-distribution Rate constant
ED-Electrochemical detector
SPE-solid phase extraction
PEG-Polyethylene glycol
MSU-Microcrystalline sodium urate

OC- Obstacle course
LTL-Latency to lie
TD-Tibial dyschondroplasia

to my parents
Late Dr Inderjeet Singh
Late Balwinder Kaur

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General Introduction

BACKGROUND

PAIN

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 such terms of damage" (Merskey and Bogduk, 1994). It is a complex and multidimensional experience with sensory, motor, emotional and physiological characteristics (Stucky et al., 2001). Numerous subjective pain scales based on verbal communication, physiological and behavioural changes, are used in humans, such as the Visu al. Analogue Scale (Collins et al., 1997), with moderate success (Marquié et al., 2003).

Birds lack verbal communication and cannot tell whether they are in pain. Most of the avian species, in order to avoid predators, do not vocalise while experiencing pain (Paul-Murphy 2003). In such cases, the birds show tonic immobility. Tonic immobility is not only related to pain but also to fear (Hohtola, 1981). Some behavioural changes shown by the domesticated avian species are weight loss, inappetence, reduced walking, lack of grooming etc (Paul-Murphy 2003). These changes may vary from species to species e.g. dust bathing is a normal behaviour of healthy chickens and hens but it is not shown by those in pain. Thus, lack of dust bathing is a good indicator of pain in chickens (Vestergaard and Sanotra, 1999). However, other avian species like parrots and eagles do not dust bathe. The species differences in exhibition of a particular behaviour related to pain also makes it difficult to ascertain pain in birds. Therefore, in our study, we used lame broiler chickens in evaluation of analgesia. Lame birds can be easily recognised from the flock and there are various validated methods which can evaluate the degree of lameness in broiler chickens (described in Section 1.3.4). Using lame broiler chickens reduced ethical complications as we did not have to induce pain.

Before evaluating the effects of any drug it is desirable to study the pharmacokinetics of that drug in the species of interest. Pharmacokinetics knowledge allows us to predict the plasma concentration of a drug in the body at any particular time, which can be correlated with the various useful or side-effects exhibited (Gilbaldi and Perrier, 2007). Therefore, prior to the analgesic studies in lame broiler chickens we studied the pharmacokinetics of the chosen drugs; mor-

phine, butorphanol, aspirin and salicylic acid in healthy broiler chickens. The combination of pharmacokinetics and pharmacodynamics experiments allowed us to predict the approximate therapeutic range of these drugs in broiler chickens.

1.1 ANALGESIC DRUGS

Analgesic drugs can be defined as medicines given to reduce pain without resulting in loss of consciousness. The main classes of general analgesic drugs are opioids, nonsteroidal antiinflammatory drugs, α-2 adrenergic agonists and N-Methyl-D-Aspartate (NMDA) receptor anatagonists. Local analgesics may also exert some general analgesic effect after absorption.

1.1.1 Opioids

Opioids are drugs that bind to the opioid receptors and can be displaced by naloxone. Opiates are extracted from opium poppy *Papaver sominiferum*. The main effect of opioid alkaloids is pain relief. The most commonly used opiates are morphine and codeine, which are natural alkaloids extracted from opium poppy, while hydromorphone, oxymorphone etc are semisynthetic opiates derived from natural opiates. Fully synthetic opioids such as pethidine and the fentanyl series, methadone and tramadol are also widely used (Rang et al., 1995).

Historical evidence of the use of opium goes back to 3400 BC. During that period opium was cultivated in Mesopotamia by Sumerians. They also knew the art of isolating opium from their seed capsules and called opium "gil" which meant joy (Brownstein, 1993). In those times it was used as an euphoriant in religious rituals only. Sumerians passed on the plant and art of isolation of opium to Assyrians and from the Assyrians it was passed to the Babylonians and Egyptians. Hippocrates in 460 BC acknowledged its use as narcotic and styptic in treatment of various internal diseases. Opium and laudanum (opium in alcohol) were used to treat almost all known diseases. Nobody knew the active ingredient behind the myriad effects of opium till 1806, when the 21-year-old German, Friedrich Wilhelm Adam Serturner, isolated an organic alkaloid from *Papaver somniferum* and named it morphine after the god of dreams, Morpheus (Brownstein, 1993).

Mechanism of Action of Opioid drugs

Opioid drugs exert their effects by mimicking the naturally occurring endogenous peptides endorphins (Morley, 1983). These distinct opioid peptides are β-endorphins, enkephalins and dynorphins. The precursor for these peptides are pre-proopiomelanocortin (β-endorphins), pre-proenkephalin (enkephalins) and pre-prodynorphins (dynorphins) which are encoded by separate genes (Morley, 1983; McNally and Akil, 2002). The gene for pre-proopiomelanocortin also encodes other non-opioid peptides such as adrenocorticotropic hormone, α-melanocyte-stimulating hormone and β-lipotrophic pituitary hormone (McNally and Akil, 2002). Recently two short peptides showing high affinity for μ were identified as endomorphin 1 and 2 but their precursors are not yet known (Zadina et al., 1994). The large number of opioid effects lead to the discovery of multiple opioid receptors. Martin and co-workers (Martin et al., 1976; Gilbert and Martin, 1977) reported the existence of μ, κ and σ receptors in a study conducted on chronically spinalised dogs. The receptors were named after the agonist drugs used in that study; μ- for morphine, κ- for ketocyclazocine and σ- for SKF, 10, 047. Later on, the δ receptor was identified by Lord in 1977 (Lord et al., 1977), during the demonstration of its effects in guinea pig ileum and mouse vas deferens. The fourth opioid receptor named ORL1 was cloned by (Mollereau et al., 1994), but this receptor is not opioid in function as it does not interact directly with other opioids (Morley, 1983). Naloxone, an opioid antagonist, binds to μ, δ and κ receptors but not ORl-1 receptors (Zaveri, 2003). This receptor was isolated by two groups independently (Meunier et al., 1995 and Reinscheid et al., 1995) and named as nociceptin and orphanin FQ, respectively. In recent years, with the rise of alternative splicing techniques such as oligodeoxynucleotide (ODN) mapping, various subtypes of opioid receptors have been identified. Two subtypes of μ and δ and four subtypes of κ receptors were identified (Pasternak, 1993), but there is controversy about their physiological relevance of κ receptor subtypes.

Opioid receptors belong to the superfamily of G-protein coupled receptors and the subfamily of rhodospin receptors (Law et al., 2000). Activation of opioid receptors leads to inhibition of adenyl cyclases (Sharma et al., 1977), blocks the voltage regulated calcium channels (Surprenant et al., 1990; Dolphin, 2003) and activates the inwardly rectifying potassium channels (Yamada et al., 1998). All these events lead to hyper-polarisation and can block pain transmission. Re-

cently, it has been demonstrated that opioid receptors can also activate the Mitogen Activated Protein Kinases (MAP) and phospholipase C mediated cascade leading to formation of inositol tri-phosphate and diacylglycerol (DAg) (Law et al., 2000). Inositol phosphate mobilises the intracellular calcium stores while DAg activates the other isoforms of Protein Kinase-C (PKC) (Standifer and Pasternak, 1997). Desensitisation of μ receptors can also occur via activation of PKC isomers (Ingram and Traynor, 2009).

Site of Opioid Action

Opioid drugs act at supraspinal, spinal and at peripheral levels. The sites for supraspinal action are: the nucleus recticularis paragigantocellularis (NRPG), the nucleus raphe magnus (NRM) and the periaqueductal gray (PAG) (Zubieta et al., 2001). The sites for spinal analgesia are lamina I and II of the dorsal horn of spinal cord. Satoh et al., (1983) examined the supraspinal multiple opioid receptor analgesia in rats by microinjecting the μ, δ and κ receptor agonists in NRPG, NRM and PAG. They found that all the agonists produced analgesic effects but the analgesic effect of κ receptor agonist was weakest at all sites. Parolaro et al., (1986) supported the above findings by microinjecting the μ, δ and κ receptor agonists in PAG. They demonstrated the inhibition of gastrointestinal transit and antinociception by μ agonist and antinociception only, by δ agonist. There was neither antinociception nor the gastrointestinal effects after injection of κ receptors. There was no cross-tolerance between μ and δ agonists (Porreca et al., 1987a) supporting the contribution by both receptors in providing analgesia as assessed by the mouse tail flick test. Porreca et al., (1987b) also demonstrated involvement of μ and δ receptors in the supraspinal regions and spine while κ receptors were only involved in spinal analgesia. They also found that μ and δ receptors are effective against both chemical and thermal nociception, but κ receptors provided analgesia only after chemical nociception. Przewlocki et al., (1983) demonstrated the spinal analgesic effects of κ receptors by injecting a κ receptor agonist (intrathecal) I/T and the analgesia was reversed by κ receptor antagonists. Millan et al., (1989) found that κ receptors also mediate analgesic effects both through supraspinal and spinal sites of action. Autoradiographic studies on the distribution of opioid receptors in different regions of the brain revealed that all three receptor sites are differentially distributed in the brain, μ being widely distributed after δ and then κ, in the fore and mid brain regions of rats (Mansour et al., 1987). In

rat spinal cords, the opioid binding sites are concentrated more in the superficial layers (laminae I and II) with approximately 70%, 20% and 10% binding for μ, δ and κ receptors, respectively (Besse et al., 1991). Recently, it was documented that the opioid receptors can mediate analgesia by activating the receptors present outside the central nervous system, at the peripheral sensory nerve terminals (Stein et al., 2003). The population of these opioid receptors increased after a noxious stimulus on the peripheral tissue (Stein and Lang, 2009).

Use of opioid drugs in birds

Early literature regarding the use of opioid in birds is quite confusing. It was believed that morphine was not an efficient analgesic agent in broiler chickens. Schiender (1961) concluded from his study that morphine produced analgesic effects in chicken only above 200 mg/kg body weight. He found that below 200 mg/kg, there was no difference in response towards multiple toe pinching when compared with the control group. However, some studies have reported the analgesic effects of morphine in chickens at a dose of 30 mg/kg (Bardo and Hughes, 1978) and some have reported strain variations in analgesic responses to morphine (Fan et al., 1981). They found that in the Rhode Island Red cross Light Sussex strain morphine produced analgesia in only 57% of the total sample at dose rate of 10 mg/kg while in all the birds of two strains of White Leg horn chickens morphine produced analgesia at 30 and 15 mg/kg respectively. In their experiment Rager and Gallup demonstrated no effect of morphine at 5, 20 and 30 mg/kg to the shock elicited vocalization stimuli but observed decreases in wing extension and flapping in response to painless stimulation (Rager and Gallup, 1986). They concluded that analgesic effects of morphine were due to motor impairment. In another study it was demonstrated that morphine produced hyperalgesic effects at the dose rates of 2.5, 15 and 25 mg/kg while codeine produced analgesic effect at similar dose rates (Hughes, 1990b). In this study it was also noted that naloxone at 5 mg/kg reversed the hyperalgesic effect of morphine at 30 mg/kg and potentiated the effect of codeine. He suggested that due to selective breeding of the White Leghorn strain for low body fat, there could be changes in binding and population of μ and κ receptors resulting in the hyperalgesic effect of morphine. In another study conducted by Hughes (1990a) on White Leghorn, Cal-White and Rhode Island Red strains of domestic fowl, he observed that morphine produced analgesia in Rhode Island Red and hyperalgesia in White Leghorn and

Cal-White strains and both the effects were reversible by naloxone. He concluded that hyperalgesia caused by morphine is strain dependant and may reflect the effects of selective breeding on opioid receptors. Hyperalgesic effects of morphine in domestic fowl were again confirmed by Sufka and Hughes (1990) in a study and observed that the temporal characteristics of hyperalgesia were depicted as U-shaped function similar to the dose and temporal characteristics of morphine-induced analgesia in other species (Sufka and Hughes, 1990). But more recently some studies have documented analgesic effects of morphine. Concannon et al. (1995) used the isoflurane sparing technique to determine the analgesic effect of morphine in birds. In this study, birds were anaesthetised with isoflurane and Minimum Anaesthetic Concentration (MAC) was determined before and after injection of morphine at the dose rate of 0.1, 1 and 3 mg/kg. Each injection of morphine resulted in significant reduction of MAC indicating analgesia induced by morphine. Hoppes et al., (2003) evaluated the analgesic efficacy of fentanyl, which is a μ receptor agonist and 80 to 100 times more potent than morphine on psittacine birds. They compared the response of white cockatoos to electrical and thermal stimuli before and after administration of fentanyl at the dose rate of 0.02 mg/kg I/M and 0.2 mg/kg S/C. There was no difference between control and fentanyl injected groups after 0.02 mg/kg I/M. The dose of 0.2 mg/kg given S/C produced significant analgesia but some birds had shown hyperactivity (Hoppes et al., 2003). This study supported that the psittacine birds have more κ receptors than μ receptors (Mansour et al., 1988). Butorphanol a μ antagonist and κ receptor agonist (Heel et al., 1978; Horan and Ho, 1989) produced analgesic effects at 1 mg/kg in African Grey parrots while buprenorphine a partial agonist for μ receptors failed to produce any analgesia (Paul-Murphy et al., 1999). Butorphanol also produced analgesic effects at 0.5 mg/kg in turkeys suffering from degenerative joint disease (Buchwalder and Huber-Eicher, 2005).

Opioid Receptors in birds: A brief overview

There is not much literature on the isolation and identification of opioid receptor subtypes in the avian brain. Mansour et al., (1988) reviewed the existence of the opioid receptor subtypes namely μ, δ and κ. The pigeon brain had significantly higher proportion of κ receptors (76%) as compared to μ (14%) and δ (10%) (Mansour et al., 1988). In a chicken the proportion of μ, δ and κ receptor types is 23, 30 and 26%, respectively (Danbury, 1999). Reiner et al., (1989) and

Csillag et al., (1990) demonstrated the isolation and distribution of μ, δ and κ receptor binding sites in the forebrain and midbrain of pigeon and turkey, respectively (table 1). The differences in the binding receptor binding between pigeon and chicken might be due to species difference or experimental technique.

There was greater binding of δ and κ receptors than μ in the forebrain and midbrain of the pigeon. Similarly, there is higher density of δ receptor binding sites in the forebrain of passerine songbirds (Deviche et al., 1993). But in the chicken forebrain the proportion of μ and κ receptor binding sites are higher than δ while in midbrain μ and δ receptor binding sites are higher than κ (Csillag et al., 1990). There was also a mismatch in the distribution of μ, δ and κ receptors in pigeon brain with the distribution of opioid peptides, similar to one seen in mammalian brains (Reiner et al., 1989)

In the present study, we chose morphine because it is a typical μ receptor agonist and a reliable analgesic in mammals, and butorphanol as an antagonist to μ receptors and agonist to κ receptors and commonly used for analgesia in birds. As described in previous studies on use of opioid drugs in birds, the role of morphine in avian analgesia is vague and still needs to be supported by evidence. Butorphanol is considered good analgesic in birds as they have more κ receptors than μ and δ. Being a κ agonist and μ antagonist, its mechanism of action is totally opposite to morphine. The comparison of pharmacokinetics of both these opioid drugs will provide us some interesting conclusions for their further use in broiler chickens and other avian species.

To our knowledge, there are no published reports on the pharmacokinetics of morphine and butorphanol in broiler chickens. This experiment will help in evaluating the dose rate, plasma levels of the drug and the duration of analgesia in chickens which could further help in rational use of these drugs.

Brain Region*	Pigeon			Chicken		
	Mu	Kappa	Delta	Mu	Kappa	Delta
Hyperstriatum	++++	++++	++++	++++	++++	+
Medial Neostriatum	++++	++++	++++	++++	++	++
Paleostriatium Augmentum	++++	++++	++++	++	+	++
Lobus Parolfactorius	++++	++++	++++	#	#	#
Nucleus Basalis	#	#	#	++++	+++	+
Dorsolateral Cortical Area	++++	+++	+	+++	+	+
Archistriatum Dorsale	-	++++	-	++++	++	++
Nucleus Rotundus	+	+	+	+	+	++++

Table 1 : Differences between the opioid receptor binding in pigeon and chicken (Reiner et al. 1989 and Csillag et al. 1990)

 # Not mentioned
+ Low binding
++ Mild binding
+++ Moderate binding
++++ High binding
- No binding
** Regions having significant differences in the binding are mentioned*

1.1.2 Nonsteroidal Antiinflammatory Drugs (NSAIDs)

There is a class of drugs with antiinflammatory, analgesic and antipyretic actions which are not steroids, but have similar eicosanoid inhibiting, antiinflammatory action, and are therefore termed "Nonsteroidal Antiinflammatory Drugs".

These drugs have been in use almost as long as opioids. In ancient Greek times, the bark and leaves of the willow tree were known to exhibit antipyretic properties and Hippocrates in 400 BC recommended the chewing of these leaves for pain relief and fever. The willow tree bark and leaves are rich in salicin, a precursor of salicylate. The modern history of NSAIDs started with the commercial production of salicylic acid by Kolbes and colleagues in 1874 (Vane and Botting, 2003). Unfortunately, salicylic acid was not free from side effects and caused gastric irritation, nausea and indigestion. The quest to produce a safer and stable alternative of salicylic acid advanced in 1897, when Felix Hoffman, a German chemist working for Bayer, synthesized the stable form of salicylic acid by acetylation. Later, it became the active ingredient in aspirin, "a" from acetyl, "spir" from spirea plant (which yields salicin) and "in" a common suffix for medications, hence aspirin (Andermann, 1996).

Mechanism of action and side-effects of NSAIDs

Sir John Vane, and independently, Smith and Willis, first proposed the mechanism of action of aspirin in 1971: inhibition of cyclooxygenase (COX) enzymes thereby inhibiting the formation of prostaglandins and thromboxane, which are potent mediators of inflammation (Vane, 1971; Smith and Willis, 1971).

Cell membrane damage releases phospholipids which are converted to arachidonic acid by phospholipidase-A_2 (figure 1). Arachidonic acid is converted to prostaglandin hydroperoxide (PGG_2) and PGH_2, by the action of COX enzymes. PGH_2 is precursor for various prostaglandins; PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and also thromboxane (TXA_2 and TXB_2) (Vane and Botting, 1998). Prostaglandins cause vasodilatation and hyperalgesia by sensitising the pain receptors to other mediators of inflammation e.g. histamine, bradykinin, thromboxane, platelet activating factor, leukotrienes and interleukin-1. Inhibition of prostaglandin production causes

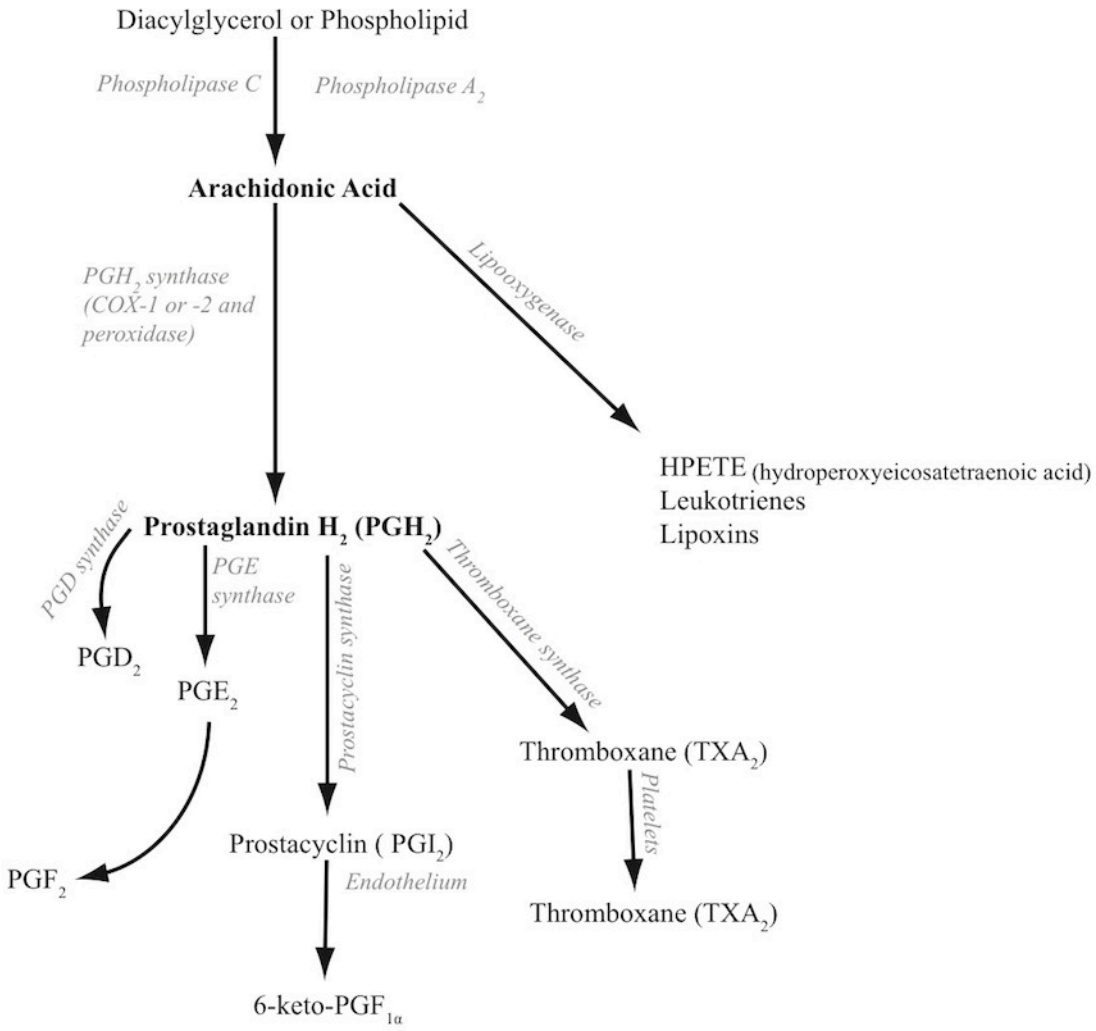

Figure 1: Biosynthesis of Eicosonoids from Arachidonic Acid (Basic and Clinical Pharmacology, Katzung 2007)

analgesia and also reduces fever as it inhibits the action of interleukin-1, which is an endogenous pyrogen (McNamara andMayeux, 1996).

In 1991, Xie and co-workers (Xie et al., 1991) proposed the existence of another isoform of COX, and the two isoforms of the enzyme were named COX-1 and COX-2. COX-1 is the constitutive form and its activity is enhanced during cell development and differentiation (Otto and Smith, 1995). It is responsible for normal physiological functions such as cytoprotection in the gastric mucosa, maintaining renal blood flow and mediating the production of TXA-2 in platelets which promotes vasoconstriction, platelet activation and aggregation (Vane and Botting, 1987). COX-2 is inducible, although some studies have reported its constitutive nature (Zimmermann et al., 1998), and its expression is triggered by injury or inflammation, resulting in production of prostaglandins including PGI_2, which is a potent vasodilator and inhibits platelet aggregation. This discovery lead to the belief that the side effects of NSAIDs are due to inhibition of COX-1 and the analgesic and antipyretic action is because of COX-2 inhibition. Theoretically, COX-2 inhibitors should provide good analgesia, reduce inflammation with fewer side effects especially those on the gastric mucosa. NSAIDs with better COX2/COX1 ratios and higher affinity for COX-2 are considered safer. Thus aspirin and indomethacin which are two strongest inhibitors of COX-1 and they also cause most damage to gastric lining (Vane, 1996), although there are probably species differences. There is lower incidence of gastric ulcers after use of meloxicam as it has better COX-2/COX-1 ratio and is thus considered safer (Vane, 1996). The lack of side effects associated with COX-2 selective NSAIDs has lead to production of highly selective COX-2 inhibitors, and the first to come in the market was rofecoxib (Vioxx), then celecoxib (Celebrex) and later on valdecoxib (Bextra) (Warner and Mitchell, 2004). These drugs were thought to have lesser side effects than traditional non-selective NSAIDs. Silverstein et al. (2000) conducted a study named CLASS (Celecoxib Long-term Arthritis Safety Study) to demonstrate the safety profile of celecoxib, one of the specific COX-2 inhibitors. It was funded by Pharmacia, the manufacturer of the drug. Gastric ulcers, bleeding, perforation and obstruction was monitored for 6 months after the oral administration of celecoxib, ibuprofen or diclofenac. The results suggested that celecoxib was well tolerated by all patients and the incidence of gastrointestinal complications was significantly less than the other NSAIDs. This

study did not include any other potential complications that can arise after long term administration of celecoxib e.g. renal or cardiovascular side effects. This study design and the results of CLASS was questioned by many researchers. Hrachovec et al., (2001) and Wright et al., (2001) raised some concerns about the CLASS study in the same journal: JAMA. They reported that the protocol and results submitted to FDA were different from what were published in the journal and the authors deliberately omitted some data showing serious gastrointestinal complications. The original protocol submitted to FDA was to follow up for 12 to 15 months, but after 6 months incidence of gastric ulcers with celecoxib increased and there was no difference in the various treatment groups. The authors in reply (Silverstein et al., 2001) to the questions raised by Hrachovec and Wright said that the 6 months data was the most scientifically and clinically valid, because the most susceptible patients dropped out from the study. This reduced the percentage of incidence of peptic ulcers. The authors thought that the 6 months data were less biased and showed the true picture of celecoxib effects. The increase in number of GI lesions could be due to concurrent use of low dose aspirin. Jones (2002) also reported serious flaws in the CLASS as it did not say anything about long term effects, number of deaths and cardiovascular or renal complications. Selective inhibition of COX-2 enzyme reduces prostacyclin production, disrupting the balance between thromboxane and prostacyclin, which is required for normal cardiovascular function. Prostacyclin is a vasodilator and inhibitor of platelet aggregation. After selective COX-2 inhibition, there will be increase in platelet aggregation due to higher thromboxane levels, along with vasoconstriction (Martínez-González and Badimon, 2007). This cascade can cause increased vascular occlusion resulting in stroke or myocardial infarction. The selective inhibition could also result in an increase in blood pressure due to retention of water, salt and lack of vasodilators like prostacyclin (Jones, 2005).

The major question raised after CLASS was whether the side effects and complications associated with the use of celecoxib were due to flaws in the study design and analysis or insufficiencies in celecoxib. In an attempt to answer this query, a smaller study was conducted by Chan et al. (2002) to compare the incidence of gastric ulcers with treatment of celecoxib and diclofenac along with omeprazole. Chan found 4.9% incidence of gastrointestinal ulcers with celecoxib as against 6.4% with combined therapy of omeprazole and diclofenac for 6 months time period.

The renal adverse effects were of similar magnitude for both the cases. They concluded that both regimens were unable to completely eliminate the risks associated with NSAIDs treatment.

A study similar to CLASS was conducted on rofecoxib (Vioxx, Merck) too. This study was popularly called VIGOR (Vioxx Gastrointestinal Outcomes Research) and this was also controversial. The objective of this research was to investigate the association of rofecoxib with the lower incidence of GI tract side effects as compared to naproxen (a non-selective NSAID) (Bombardier et al., 2000). As the COX-2 inhibitors do not inhibit platelet aggregation, the patients undergoing its therapy would have more thrombotic cardiovascular effects as compared to naproxen. Therefore, in the VIGOR study, cardiovascular changes were also monitored. They found that there was significant decrease in gastrointestinal abnormalities and significant increase in myocardial infarctions in the rofecoxib group as compared to naproxen. They explained the increase in cardiovascular abnormalities in the rofecoxib treatments groups as a coronary protective effect of naproxen, which is absent in rofecoxib owing to its selective inhibition of COX 2 enzyme. In 2005, the editors of the same journal published an expression of concern regarding discrepancies in data submitted to FDA and published in the journal (Curfman et al., 2005). They reported that there were more deaths caused by myocardial infarctions which were not reported at the time of publication. Addition of these deaths in the statistical analysis would significantly raise the percentage of deaths due to cardiovascular abnormalities associated with rofecoxib therapy. In response to the expression of concern (Bombardier et al., 2006), the authors said that the deaths which were not reported occurred after the cut off time point of data collection and should not be included in the analysis. Later on, the editors of the journals reaffirmed their expression of concern as the cut off date was changed by the sponsors to one month earlier for reporting the gastrointestinal side effects (Curfman et al., 2006). In 2004, Merck had already withdrew Vioxx from the market after the reports of higher incidence of adverse cardiovascular effects.

Some smaller studies have shown that COX-2 inhibitors like valdecoxib (Goldstein et al., 2003), etoricoxib (Hunt et al., 2003) and lumiracoxib (Rordorf et al., 2003) are well tolerated and had fewer gastrointestinal lesions after short term therapy. A larger study called MEDAL (Multinational Etoricoxib and Diclofenac Arthritis Long-term Programme) was conducted by

Cannon and co-workers (Cannon et al., 2006; Laine et al., 2007). This programme had three randomised, double-blind clinical trials; MEDAL, EDGE I (Etoricoxib v/s Diclofenac Gastrointestinal Tolerability and Effectiveness) and EDGE II. There were fewer gastrointestinal complications with etoricoxib as compared with diclofenac (Laine et al., 2007). The incidence of cardiovascular manifestations was similar for both the drugs (Cannon et al., 2006). In another clinical trial on lumiracoxib; TARGET (Therapeutic Arthritis Research and Gastrointestinal Event Trial) (Schnitzer et al., 2004), the rate of GI risks associated with lumiracoxib was significantly lower than ibuprofen and naproxen. Overall incidence of cardiovascular complications in the total population was 0.55% and 0.65% for ibuprofen or naproxen and lumiracoxib, respectively.

All these studies have confounded the clinician about prescribing traditional NSAIDs and COX-2 inhibitors. The FDA and American Heart Foundation have recommended safe regimens and consecutive therapy for the use of these drugs (Rocca and Davì, 2007; Antman et al., 2007). Both the traditional NSAIDs and COX-2 inhibitors should be used on short-term basis at lowest possible dose. If a patient has history of GI complications, then traditional NSAIDs with proton pump inhibitors (omiprazole) should be considered. An alternative to this regimen could be the use of COX-2 inhibitors, which should be avoided in patients with any history of myocardial infarctions, ischaemic cerebrovascular events, angina, chronic heart failure or stroke.

Use of NSAIDs in birds

NSAIDs are the preferred group of drugs to provide antiinflammatory and analgesic therapy in both human and animal subjects. Of many NSAIDs available for human and animal use, only a few have been tested in birds. Further research is still required to determine the pharmacokinetics of NSAIDs and subsequently their dosage in birds. There is much contradiction in the dose rates used for various analgesic agents in birds. Birds have higher a metabolic rate and the drugs used in mammals may be metabolised too fast to produce the desired effect. Carprofen has been used in birds at the dose of 1 mg/kg administered S/C and was claimed to produce good analgesia without any side-effects (McGeown et al., 1999) but Hocking (2005) calculated the minimum effective dose of carprofen as 30 mg/kg (Hocking et al., 2005). If carprofen is

mixed in the broiler feed at the rate of 34 mg/kg feed, it produces good analgesia and also was preferentially consumed by broiler chickens in pain (Danbury et al., 2000).

Aspirin is the most popular of all the NSAIDs. It has been used as dietary supplement in poultry flocks to maintain higher growth rate, alleviate heat stress and broiler ascites (Shlosberg et al., 1996; Proudfoot and Hulan, 1983). Other indications for aspirin are in cases of *E. coli* infection given with vitamin E (Likoff et al., 1981) and maintaining egg production and egg shell quality (Mcdaniel et al., 1993; Balog and Hester, 1991). Baert and Backer (2002) studied the disposition of sodium salicylate (aspirin is metabolised to salicylate) after injecting 50 mg/kg I/V. A plasma concentration of 50 μg/mL (the effective plasma concentration of sodium salicylate) was maintained for 5 hours (Baert and De Backer, 2002). Unlike mammalian species, there was only one metabolite of sodium salicylate i.e. salicylic acid rather than salicyluric acid and gentisic acid. The half-life for salicylic acid was longer in birds than in mammalian species.

Flunixin is a popular NSAID for use in animals (Horii et al., 2004). The pharmacokinetics of flunixin can be described by a two compartment model with a very low volume of distribution in chickens as compared to horses and camels (Baert and De Backer, 2002). The duration of analgesia provided by flunixin at dose rate of 5 mg/kg was 12 hours (Machin et al., 2001). It is relatively safe at a low dose of 3 mg/kg in domestic fowl (Hocking et al., 2005) but at higher doses of 10 mg/kg in Budgerigars (*Mesopsittacus undulatus*), it causes gastric ulceration and bleeding (Bauck, 1990). In Bob White quails (*Colinus virginianus*) and Siberian Cranes (*Grus leucogeranus*), low doses of flunixin at 0.1 mg/kg I/M for 7 days and 5 mg/kg once caused renal ischaemia and necrosis (Klein and Charmatz, 1994; Clyde and Paul-Murphy, 1999).

Toxicity caused by diclofenac, another commonly used NSAID in animals in some parts of the world, has been reported in White Backed Vultures (*Gyps bengalensis*) in the Indian subcontinent (Green et al., 2004; Meteyer et al., 2005; Oaks et al., 2002; Shultz et al., 2004; Swan et al., 2006b) with lesions in kidneys, liver and spleen with extensive uric acid crystal deposition. Reddy and coworkers (Prakash Reddy et al., 2006) proposed that nimesulide is completely safe to use in birds as compared to diclofenac. But Richard and coworkers (Cuthbert et al., 2007) condemned the experimental design of Reddy et al. and proposed that their study lacked the

proper dosage and exposure of wild vultures to nimeluside residues similar to diclofenac residues in dead livestock. Meloxicam is considered a safer alternate to diclofenac as it showed no toxic effects and there was no elevation of serum uric acid concentration in Gyps vultures (Swan et al., 2006a). It is also considered safe to use in broiler chickens and in psittacines. The volume of distribution for meloxicam in chickens is lower and clearance is slower as compared to horses and humans. The bioavailability is 100% after I/V administration (Baert and De Backer, 2002; Wilson et al.).

Ketoprofen can be used safely and effectively as an antiinflammatory and analgesic agent at 5 mg/kg I/M in Mallard Ducks (Machin et al., 2001). At this dose rate there were no side-effects seen and the duration of analgesia was longer than 70 minutes but it took 30 minutes to produce analgesia (Machin and Livingston, 2002). The minimum effective dose of ketoprofen in the case of domestic fowl is 12 mg/kg (Hocking et al., 2005).

Roder and his co-workers investigated the intravenous, intramuscular and oral pharmacokinetics of ibuprofen in broiler chickens (Roder et al., 1996). In their pilot study they administered ibuprofen at 50 mg/kg I/V, and observed toxic effects, mainly central nervous system excitation. Birds exhibited hyperexcitability, respiratory distress and death. Therefore, for the pharmacokinetic trial they administered the drug at 25 mg/kg I/V and 50 mg/kg by I/M and oral routes, respectively. Volume of distribution at steady state, total systemic clearance, elimination half-life and mean residence time after I/V injection was 0.303 L/kg, 482.3 mL/h.kg, 2.71 h and 1.02 h, respectively. The MRT after I/M and oral routes was 1.65 and 2.28 h, respectively. The maximum plasma concentration after I/M and oral routes was 42.17 and 23.91 μg/mL after 0.37 and 0.31 hours, respectively. There was low bioavailability after I/M (46.7%) and oral (24.2%) administration. They found the pharmacokinetics of ibuprofen in broiler chickens is very different from other species.

Cristofol and co-workers (Cristofol et al., 2000) studied the pharmacokinetics of indomethacin at 2 mg/kg I/V and orally in broiler chickens. Elimination half life and mean residence time after oral intake was 3 and 6 times higher than the I/V administration. They concluded that the oral administration of indomethacin in drinking water would be effective treatment for various

problems associated with pain and inflammation in broiler chickens. Also there was no residual indomethacin left in tissue after 3 days of oral administration at 2 mg/kg dose rate (Cristofol et al., 1998).

In the present study we investigated the pharmacokinetics and analgesic effect of aspirin and salicylate in broiler chickens. Salicylate is one of the metabolites of aspirin and the analgesic effects of aspirin are probably due to salicylate. Both aspirin and salicylate were assayed.

1.1.3 NMDA Receptor Antagonists

N-Methyl-D-Aspartate (NMDA) receptors play an important role in controlling synaptic plasticity and memory function (Chizh et al., 2001). These are ionotropic receptors and composed of different sub-units; NR_1, NR_2 (A, B, C and D), NR_3 (A and B) (Petrenko et al., 2003). Activation of the NMDA receptors require simultaneous binding of glutamate, an excitatory neurotransmitter, and glycine (Petrenko et al., 2003). Physiological depolarisation and agonist binding removes the Mg^{2+} block and opens the calcium channel. This facilitates influx of Ca^{2+} causing central sensitisation and nociception (Coderre and Melzack, 1992). Drugs that inhibit these receptors block the Ca^{2+} influx, thus blocking the depolarisation of the post-synaptic neurone, hence reducing pain. NMDA blockers act on four different sites to produce their effect; 1. the competitive (primary transmitter site) e.g. R-AP5, 2. the strychnine sensitive glycine site, e.g. GV 196771A, 3. the polyamine site (NR_2B selective), e.g. ifenprodil and 4. the phencyclidine site (uncompetitive channel blockers), e.g. ketamine, memantine, amantadine (Parsons, 2001).

NMDA blockers such as ketamine are routinely used as anaesthetic agents in veterinary medicine. At low doses ketamine produces analgesia but it is associated with some serious side effects like memory impairment, psychotomimetic effects, ataxia and motor incoordination (Eide et al., 1995).

1.1.4 α_2 Adrenoceptor Agonists

α_2 adrenergic agonists selectively activate the α_2 adrenergic receptors. They produce dose dependent analgesia at spinal, supraspinal (Buerkle and Yaksh, 1998) and peripheral sites (Khasar et al., 1995). Their analgesic effect is mediated by an action on the α_2 adrenergic receptors present

on the projection neurones. They act through Gi protein coupled K^+ channels to increase their conductance, which causes hyperpolarisation. They also inhibit the Ca^{2+} channels and adenyl cyclase activity (Buerkle and Yaksh, 1998). The various known subtypes of α_2 adrenergic receptors are α_{2A}, α_{2B} and α_{2C}. (Buerkle and Yaksh, 1998), out of these α_{2A} are responsible for analgesia and hypotension (Millan, 1992) while α_{2B} at higher doses causes hypertension (Kamibayashi and Maze, 2000). Clonidine is used for analgesia after intrathecal administration in people (Buerkle and Yaksh, 1998; Kamibayashi and Maze, 2000). It provides a good plane of analgesia, but is recommended only in clinical settings owing to its hypotensive effects. The other side effects include respiratory depression, partial atrioventricular block and dose dependent hypothermia. These side effects can be reversed by using α_2 adrenergic antagonists, e.g. yohimidine. Owing to the serious side effects associated with the use of α_2 adrenergic agonist, these are rarely used as analgesic agents in people (Machin, 2005).

1.2 PHARMACOKINETICS

Pharmacokinetics is the prediction of the time dependent concentrations of a substance in a living system. It can be defined as time course of drug absorption, distribution, metabolism and excretion (Gilbaldi and Perrier, 2007). A drug must attain its therapeutic levels and reach its site of action to produce its desired effects. Knowledge of the pharmacokinetics of a drug enables us to administer it at a proper dose rate and frequency, so that it remains above the minimum effective concentration (MEC) for desired time period. The knowledge of pharmacokinetics is required for the rational use of drugs in an animal. (figure 2).

1.2.1 Pharmacokinetic Principles

Absorption

Absorption is the passage of drug molecules from the site of administration to the vascular compartment. Drugs cross the cell membranes by the process of diffusion, which could be passive (along the concentration gradient) or active (requires energy and movement across electrochemical gradient) (Godin, 1996).

Passive diffusion follows the Fick's Law of diffusion:

Rate= $D\,A\,\Delta C\,K/\Delta x$

D = Diffusion coefficient of the molecule

A = Surface area

ΔC = Concentration gradient across the membrane

K = Drug partition coefficient

Δx = Thickness of the barrier

The rate of passive diffusion is directly proportional to the concentration, lipid solubility and degree of ionisation of the drug. Highly lipid soluble drugs and nonionised drugs rapidly cross the cell membrane barrier and are absorbed faster.

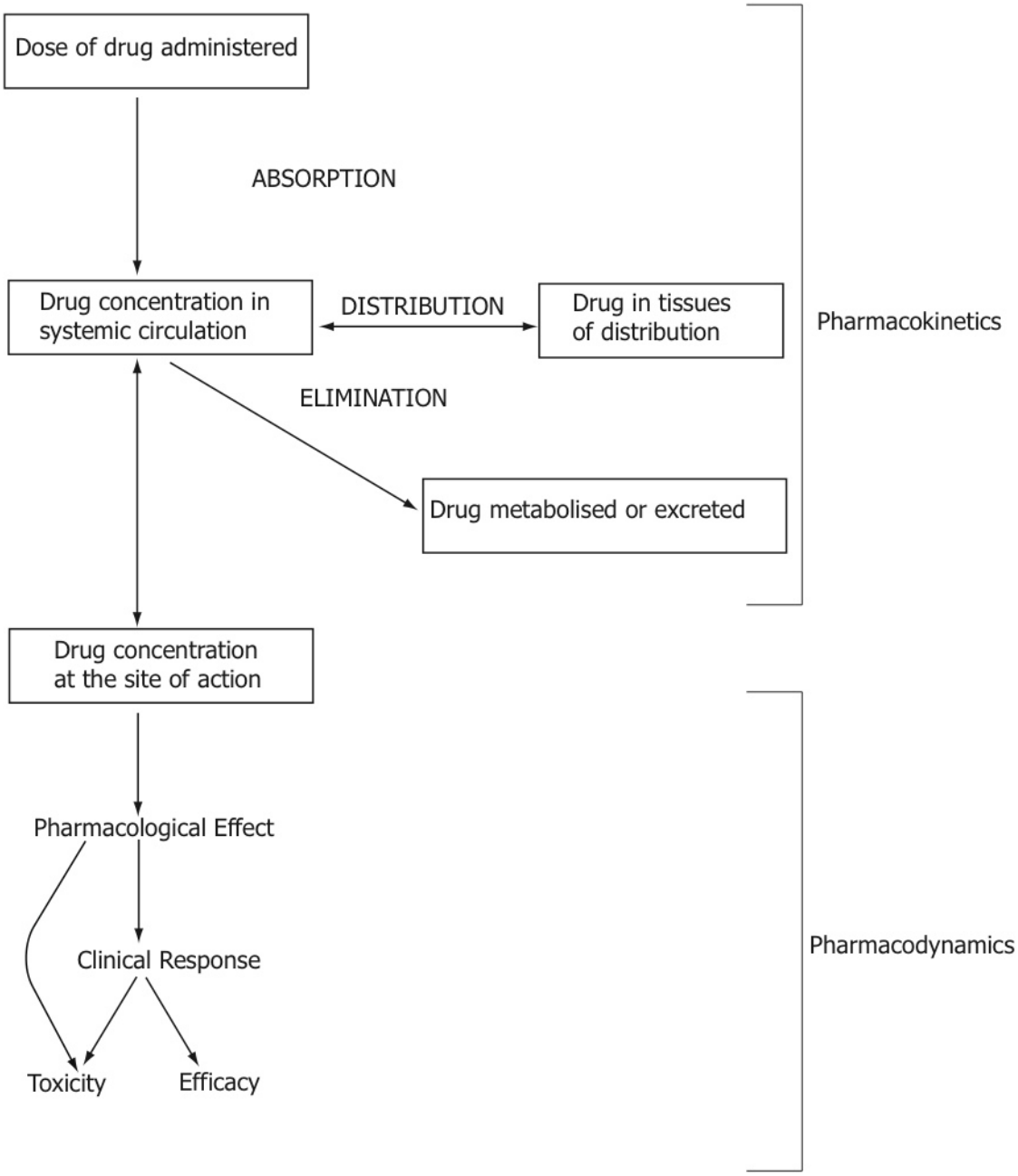

Figure 2: Relationship between dose and effects. (Basic and Clinical Pharmacology, Katzung, 2007)

Active passage of the drug is facilitated by energy dependent membrane carrier mechanisms such that the transport can occur against the concentration gradient. Active transport requires a transmembrane protein, which is usually called a transport protein and source of energy which is ATP. It exhibits structural selectivity, saturability and competition between similar substrates. All the ions, vitamins, sugars and amino acids are absorbed by active transport. Some small hydrophilic organic molecules with low lipid solubility (e.g. glucose into erythrocytes) are transported by a process termed as facilitated diffusion. The carrier and the transporter molecule forms a reversible complex outside the cell membrane and diffuses into the cell, releasing the substrate inside the cell. The interaction between the carrier and the transporter resembles the interaction between the enzyme and the substrate (Godin, 1996).

The rate of absorption also depends on the route of administration. Intravenously injected drugs have zero absorption, as they are directly injected in the blood. These drugs have 100% bioavailability. Bioavailability is the fraction of dose which reaches the systemic circulation. Drugs given orally have to be absorbed through GI tract and may be metabolised in the liver before entering the systemic circulation. This phenomenon is called first pass metabolism and is partially responsible for the lower bioavailability of orally administered drugs as compared to I/V. Low bioavailability means that the dose of the oral drugs has to be much higher than parenteral drugs to acheive the same plasma concentration. The absorption of drug after I/M and S/C injection depends on the blood flow to that region. Gentle massage after I/M injection facilitates absorption from the site by increasing the surface area of drug exposure. Aseptic conditions should be maintained while injecting drugs to avoid the formation of an abscess at the site of injection. Irritant drugs should be avoided by this route as they may cause sloughing and severe pain. Inhalant drugs given by the pulmonary route are rapidly absorbed owing to the larger surface area of lungs. The bronchodilators used for treatment of asthma are given by pulmonary route because of the rapid onset of action and avoidance of first pass metabolism in the liver. Hepatic first pass metabolism can also be avoided by rectal administration of drugs. This route is sometimes used when the patient is unconscious or non-cooperating, or when the drugs cause irritation to the gastric mucosa, but the absorption is irregular and unpredictable (Buxton, 2006).

Distribution

Distribution of the drugs to various organs and tissues depends on the blood flow to that particular organ and the plasma protein binding of that drug. The drug is distributed faster to the highly perfused organs; brain, kidneys, liver and then to most viscera, muscles, fat and skin. Tissue distribution also depends on the lipid solubility. Highly lipid soluble drugs distribute faster to the tissues as they cross cell membranes more easily. The classic example of this is thiopentone, which is an ultra short acting anaesthetic agent and has a rapid onset of action after bolus I/V injection. The rapid action is due to the high lipid solubility of thiopentone and higher blood flow to brain. Another anaesthetic drug; pentobarbitone is 70 times less lipid soluble than thiopentone and takes a much longer time for induction.

Plasma protein binding also plays a very important role in the distribution of drugs. Albumin is a major circulating protein and all the weakly acidic drugs (anionic) bind to this plasma protein. The other minor proteins; lipoproteins, α_1 acid glycoproteins bind with the cationic or weakly basic and neutral drugs. The extent of plasma protein binding depends on the concentration of the drug, affinity for the binding sites and number of binding sites (albumin concentration). Drugs with higher plasma protein binding remain in circulation in their inactive form as bound to the plasma proteins and consequently have lower volumes of distribution and longer half lives as compared to the drugs with lower plasma protein binding. The concept of volume of distribution will be dealt in later sections (Buxton, 2006)

Metabolism/Biotransformation

Metabolism or biotransformation of drugs play an important role in terminating the biological activity of drugs or in some cases transforming then into the active forms of the parent compound, which are more potent than the latter. e.g morphine-6-glucuronide is more potent than its parent drug morphine. Some prodrugs, e.g. paracoxib, are converted into its active form valdecoxib by metabolism. Biotransformation usually makes the drug more water soluble so that it can be excreted by kidneys. The smaller molecules or more polar drugs, such as benzylpenicillin, are simply excreted by kidneys but most drugs are either highly lipid soluble or bound to plasma proteins. Highly lipid soluble drugs diffuse faster into hepatocytes and accumulate in cytosol

and endoplasmic reticulum. Here they undergo hepatic metabolism to more polar metabolites suitable for excretion (Buxton, 2006). Hepatic metabolism takes place in two phases. Phase I reactions are the simple oxidation, reduction and hydrolysis reactions. The enzymatic components of Phase I reactions are collectively termed as mixed function oxidases. There are two major components of mixed function oxidases; NADPH along with molecular oxygen and two enzymes, a flavoprotein (cytochrome P450 reductase) and a haemprotein (cytochrome P450). Phase I reactions make metabolites which are generally more reactive than the parent drug and most pro-drugs undergo Phase I reactions to make their active forms. Most of the Phase I metabolites undergo Phase II (conjugation) reactions to make more highly polar and water soluble metabolites which are readily excreted from the body. Phase II reactions require energy and specific transfer enzymes. These reactions include; glucoronidation, sulphate conjugation, glycine conjugation, acetylation, glutathione conjugation, although there are species differences. For instance, Phase II metabolism of morphine involves glucoronidation to M-3-G and M-6-G: M-6-G is believed to be the active metabolite and more potent analgesic agent than morphine, whereas M-3-G may have an anti analgesic effect. Aspirin is metabolised to salicylic acid in blood. In mammals, almost 80% SA is metabolised in liver by conjugation with glycine resulting in salicyluric acid. It also forms conjugates with glucoronic acid resulting in salicyl acyl and phenolic glucoronides (Katzung, 2007).

Excretion

Metabolism makes the drug metabolites highly polar and water soluble in order to enhance the renal excretion. The kidneys are the major organs for excretion of drugs and their metabolites through urine. Renal excretion is governed by three mechanisms; glomerular filtration, tubular secretion and tubular reabsorption. The rate of excretion depends on the glomerular filtration rate and plasma protein binding. Only an unbound drug is filtered through kidneys.

High molecular weight, polar and lipophilic drugs are usually excreted in bile. These metabolites are thus secreted into the intestines and some can be reabsorbed from the intestinal lumen and enter circulation. This phenomenon is called enterohepatic recycling and it can prolong the presence and effects of drug in the body. It is particularly important for opioids. Other routes of

excretion are meat, saliva, milk and lungs (Katzung, 2007)

1.2.2 Pharmacokinetic Parameters

Area Under the Curve (AUC)

Area under the concentration time curve represents the total amount of drug present in the body over a given period, often from administration to infinity. It can be easily calculated by the trapezoidal rule or integration. The accuracy of the *AUC* increases with higher number of concentration time points. It is expressed as mass (usually ng) x time (hour) / volume (L). *AUC* from zero time point to infinity is used to calculate bioavailability of a drug (Foster, 2007).

Bioavailability (F)

Absolute bioavailability can be defined as the fraction of drug administered which arrives in the central compartment. It can be calculated by comparing the *AUC* of the dose after administration by an extravascular route with the *AUC* obtained after I/V administration. Bioavailability of a drug after I/V injection is 100% as it is injected directly into the central compartment; blood. It gives us the extent of drug loss by routes other than I/V (Baggot, 2001).

$$F\ \% = AUC\ route \times Dose\ IV / AUC\ IV \times Dose\ route$$

Relative bioavailability refers to the availability of drug product as compared to another dosage form or product of the same drug given in the same dose. It determines the effect of formulation differences on drug absorption

$$Relative\ Bioavailability = AUC\ A \times Dose\ B / AUC\ B \times Dose\ A$$

Volume of Distribution or Apparent Volume of Distribution (Vd)

This is not a real volume but a theoretical volume of fluid required to contain the amount of drug in the body at the same concentration as that present in plasma. Drugs with higher lipid solubility and low plasma protein binding are widely distributed and have a higher volume of distribution as compared to poorly lipid soluble drugs and highly plasma bound drugs, which remain in circulation for long time. The knowledge of volume of distribution and required concentration of drug in blood allows accurate estimation of the dose to administer (Birkett, 1988).

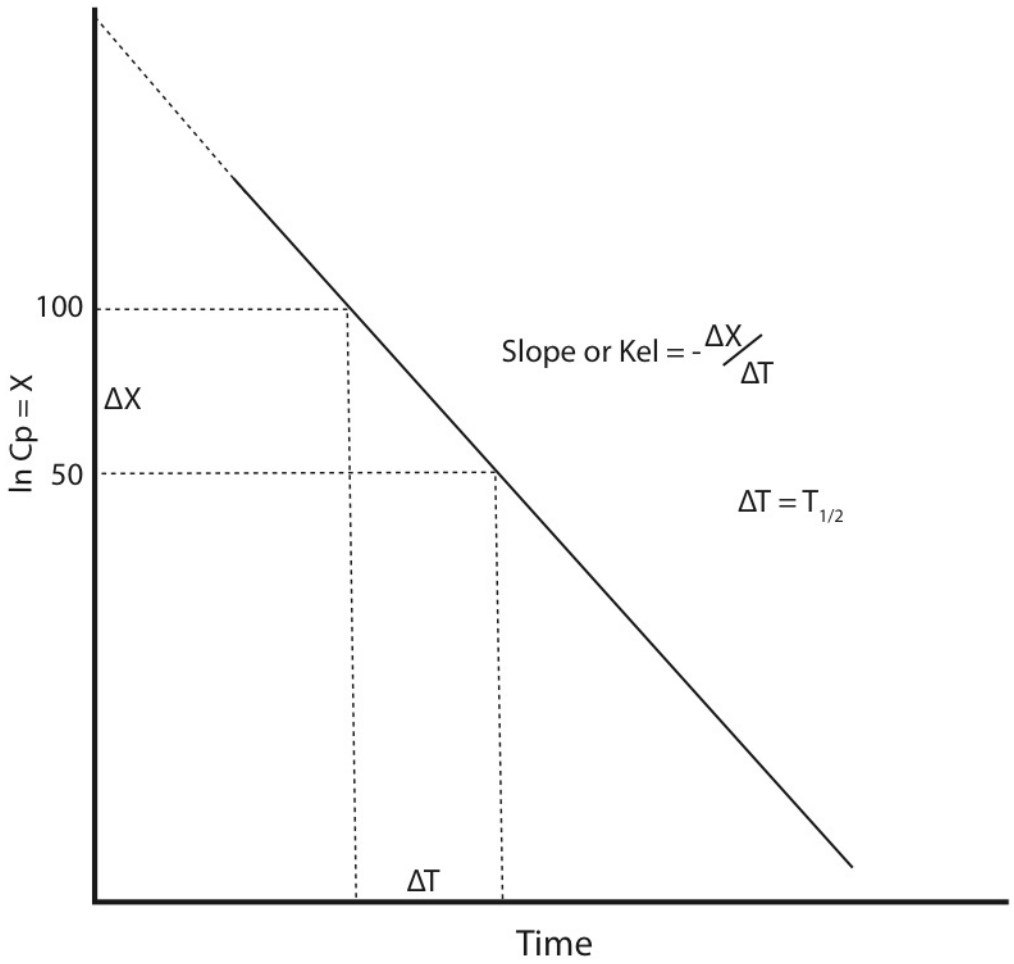

Figure 3: Graph showing the method for calculating half-life from the semi-log plot of concentration time curve. X-axis represents log of concentration (x) and Y-axis is Time (t)

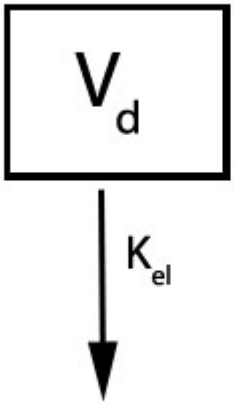

Figure 4: One compartment open pharmaockinetic model (Riviere 1999)

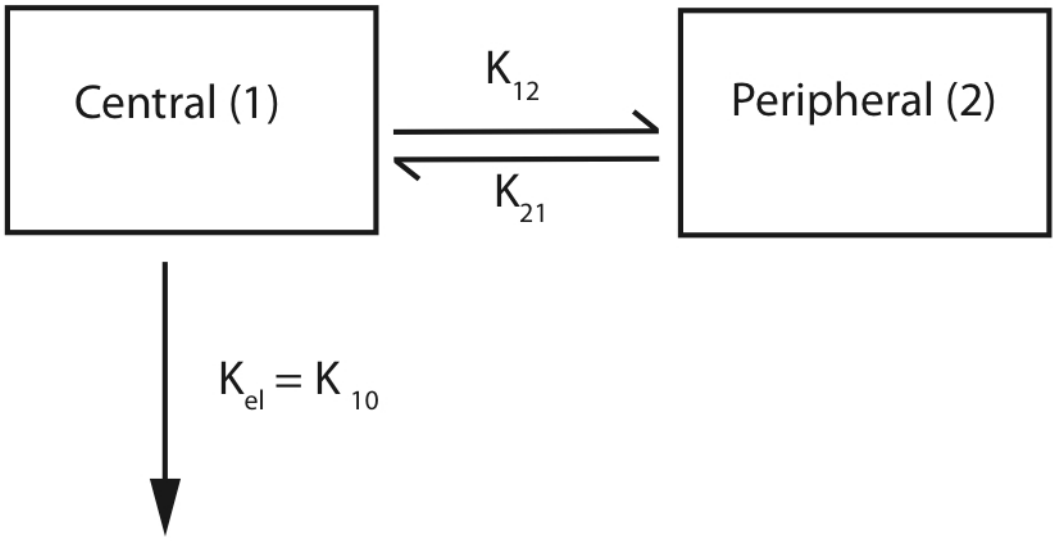

Figure 5: Two compartment open pharmacokinetic model after intravenous administration with elimination (K_{el}) from the central compartment. K_{12} and K_{21} represent interCompartmental micro-rate constants.(Riviere 1999)

$Vd = Dose/Cmax$ and is expressed as mL/Kg.

Clearance

Clearance is the volume of plasma from which the drug is completely removed per unit time. Systemic clearance is the sum of the clearances by the various organs of elimination.

$Cl = F \times Dose/AUC$

For drugs given I/V bioavailability *(F)* is 100% or 1, so for I/V route

$Cl = Dose/AUC$.

It can also be expressed as a function of volume of distribution and elimination constant (Kel)

$Cl = Vd \times K_{el}$

The elimination rate constant is the fraction of drug eliminated per unit time and it is the slope of the log concentration / time curve.

or

$K_{el} = ln\ C_1 - ln\ C_2 / t_2 - t_1$ (figure 3)

Clearance is a constant fraction for the drugs obeying first order or linear kinetics. Drugs following non-linear kinetics or dose dependent clearance, it will vary with the concentration of drug present in plasma per unit time. Examples of such drugs with zero order kinetics are phenylbutazone in dogs and horses and salicylate in cats (Riviere, 1999b)

Half-Life ($t_{1/2}$)

Half-life is the time required for the amount of drug to fall to half its concentration during elimination is called the half-life. Four half-lives equals 94% elimination of drug from the body. It measures the overall elimination of the drug and is helpful in deciding the dosing intervals of a drug (Baggot, 2001). Frequency of administration depends on the clinically relevant half-life which is associated with the therapeutic range of the drug in an animal. For drugs with first order kinetics the half-life is independent of the dose administered, but in cases of zero order

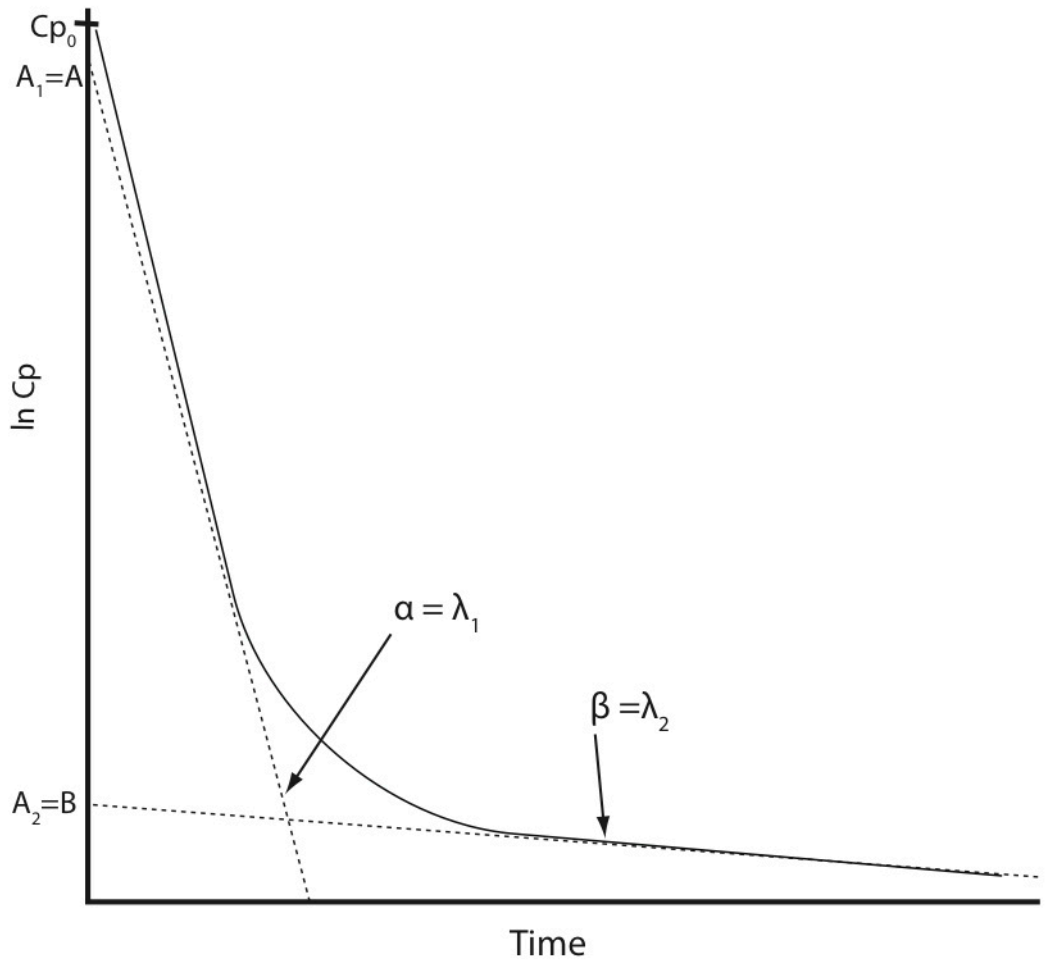

Figure 6: Semi-logarithmic plasma concentration versus time profile of a drug described by a two compartment open model. (Riviere 1999)

A and B are the intercepts, α; Distribution rate constant, β; Elimination rate constant, Cp_0 ; concentration at zero time point

kinetics, e.g. salicylate in cats, half life progressively increases with increase in dose rate. It can be calculated from the concentration time curve plotted on the semi-log scale, as shown in the figure 3.

$t_{1/2} = 0.693/ K_{el}$

Mean Residence Time (MRT)

It can be defined as the mean time for the intact drug molecules to transit through the body and involves all kinetic processes including release from the dosage form, absorption and disposition (Riegelman and Collier, 1980).

1.2.3 Compartmental Pharmacokinetics

In compartmental analysis, the body is assumed to be composed of a number of theoretical compartments with no anatomic or physiological reality (Gilbaldi and Perrier, 2007). These compartments are assumed to represent non-specific body regions and the rate of transfer between the compartments is constant. The elimination takes place only from the central compartment and also the rates of elimination and distribution follows first order or linear kinetics (Riviere, 1999a). A one compartment model (figure 4) is the simplest model in which body is represented by a single compartment and it is assumed that the rate of drug elimination from plasma is similar to other body fluids and tissues (Gilbaldi and Perrier, 2007). The equation for one compartment model is

$Cp = Cp0\ e^{-Kelt}$

Volume of Distribution can be calculated by extrapolating the line t=0, which will gives concentration at time zero.

$Vd = Dose/Cp0$

Clearance of the drug is calculated by using the following equaiton

$Cl = Vd \times K_{el}$

And half-life $t_{1/2}$

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

Though the one compartment model is very simple and is easy to derive pharmacokinetic parameters from it, the plasma concentration-time cuves of most drugs are a better fit for two or multiple compartment models. In the two compartment model (figure 5), the drug is injected into the central compartment and distributed slowly to the peripheral compartment (Riviere, 1999a). The central compartment approximates to brain, liver, kidneys (the highly perfused organs) and the peripheral compartments to the other visceral organs, muscles and skin.

The equation for two compartment model is

$Cp = A_1.e^{-\lambda 1t} + A_2.e^{-\lambda 2t}$

here, A_1 and A_2 are the mathematical coefficients (see figure 6)

λ_1 or α is the distribution rate constant

λ_2 or β is the elimination rate constant

In case of two compartment models there will be three volumes of distributions, of a central compartment (Vd_c), peripheral compartment (Vd_p) and the total volume of distribution (Vd_t).

$Vd_c = Dose/Cp0\ or\ D/A_1+A_2$

$Vd_{ss} = Vd_c\ ((k_{12} + k_{21})/k_{21})$

$Vd_p = Vd_{ss} - Vd_c$

$Vd_t = Vd_c + Vd_p$

The half-lives for distribution and elimination in two compartment models can be calculated from the respective rate constants

$T_{1/2\alpha} = 0.693/\lambda_1$

$T_{1/2\beta} = 0.693/\lambda_2$

1.2.4 Noncompartmental Analysis

In recent years, non-compartment derivation of pharmacokinetic parameters is gaining acceptance (Riviere and Williams, 1999). It follows the statistical moment theory, which is more of a stochastic approach. This was first used employed in pharmacokinetic analysis by Yamaoka in 1978 (Yamaoka et al., 1978). The moments "Area Under the Curve (*AUC*)" and "Mean Residence Time (*MRT*)" are calculated from the concentration time curve. *AUC* and *AUMC* (Area under the moment curve) are calculated by the trapezoidal rule and *MRT* is calculated from these two values.

$$MRT = AUMC/AUC$$

Bioavailability is usually calculated from *AUC* even in compartmental analysis and it is a compartment independent pharmacokinetic parameter. Other parameters e.g. volume of distribution, clearance and half-life can be calculated from *AUC* and *MRT*

$$Cl = Dose/AUC$$

$$Vd = Cl\ (MRT)$$

$$T_{1/2} = 0.693\ (MRT)$$

Both the methods of pharmacokinetic analysis have their own advantages and disadvantages. Theoretically, the parameters calculated both ways should give the same results but this is not always true (Foster, 2007). In noncompartmental analysis, non-linear events are not addressed and it does not provide any information about the time course of drug concentration (Gilbaldi and Perrier, 2007). While noncompartmental analysis has limited application from the mathematical perspective, the fewer assumptions and lack of curve fitting makes it simpler and a popular choice among clinicians (Riviere and Williams, 1999). It calculates *AUC*, which usually offsets the error, while compartmental analysis uses differential equations, in which error has more impact on final outcome. Parameters calculated by noncompartmental analysis, such as *AUC* and C_{max}, provide information directly related to safety and efficacy required by a clinician. Multi-compartmental analysis sometimes becomes more complicated. The results also vary from one computer software to another. Pascual and Montoro (1997) found statistically significant difference in the half-life and volume of distribution calculated by four different pro-

grammes following one or two compartment models while no difference was seen in the calculations made by noncompartmental analysis. The compartmentalisation of body into kinetically homogenous models and its physiological correlation is difficult to interpret (Gillespie, 1991).

All the pharmacokinetic models rely on accurate plasma concentration data to be meaningful. Both the noncompartmental analysis and compartmental analysis have advantages and disadvantages which makes the choice difficult. Therefore, in this current research, we followed both the noncompartmental analysis and compartmental modelling to calculate the same parameters.

1.3 BROILER LAMENESS

Lameness can be defined as the inability to accomplish normal locomotion and deviation from normal gait.

Broiler lameness is one of the most serious problems affecting the broiler industry worldwide. It poses a large welfare hazard to broiler chickens as they live in continual pain. Because of impaired locomotion they find difficulty in reaching the drinker and feeder, and are therefore unable to fulfil their basic needs of eating and drinking and are also incapable of exhibiting normal locomotive behaviour (Kestin et al., 1992; Sanotra et al., 2001). The lame birds are also dominated by their sound compatriots and end up dying because of dehydration and starvation (Butterworth et al., 2002). It is not only a welfare issue but also an economic issue. Every year the broiler industry loses billions of dollars worldwide due to the culling and death of lame birds. Losses incurred by lameness are huge in terms of decreased production, resource wastage and expenses on treatment and prevention (Bennet et al., 1999; Mench, 2004). The annual loss to the UK broiler industry was £24 million (Bennet et al., 1999) and the US broiler industry is losing $120 million per year through broiler lameness (Cook, 2000). In this chapter, we will discuss the following points regarding lameness in broiler chickens:

1. Causes of lameness
2. Economics
3. Behaviour alterations in chickens and response to stressful environment
4. Diagnosis and evaluation of the severity of lameness
5. Management of lameness.

1.3.1 Causes of broiler lameness

Webster (1995) made a hypothesis that lameness, which develops in modern genotypes of broilers, is a result of their selection for high live-weights and rapid growth rates, resulting in abnormally high loads being placed on relatively immature bones and joints (Kestin et al., 2001) which is the main reason for altered gait and intense chronic pain (Danbury et al., 2000). Rapid

growth rates due to genetic selection and higher nutrient intake can cause severe lameness, skeletal defects and other deformities (Julian, 1998). There is a direct relationship between faster growth rate and skeletal deformities resulting in lameness (Mercer and Hill, 1984). The incidence of skeletal deformities and lameness is much lower in slow growing chickens and roosters (Havenstein et al., 1994; Kestin et al., 1999). The bone quality of the fast growing genotype is also poor in terms of porosity and mineral content which could be due to poor utilization of minerals because of genetic factors and higher growth rate (Williams et al., 2000). The common skeletal deformities found in fast growing broiler chickens are angular bone deformity (varus and valgus), tibial dyschondroplasia, spondylolisthesis (kinky back), epiphyseal separation, ruptured gastrocnemius tendon and rickets (Julian, 1998; Julian, 2004). Rapid growth rate does not give sufficient time for proper alignment and remodelling of bones and for strong tissue formation (Julian, 1998).

Infectious agents (bacteria and viruses) are also predominant causal factors in broiler lameness. Bacterial infections linked to lameness include chondronecrosis, osteomyelitis, synovitis, arthritis and gangrenous dermatitis (Butterworth, 1999). Bacterial chondronecrosis accounts for 0.5 to 0.7% of the losses from the total UK broiler production (Yogaratnam, 1995) and the major causative agent is *Staph aureus*, but *Salmonella* species and *E. coli* can also cause this disease (McNamee et al., 1998). The most common viral infections that can cause broiler lameness are reovirus and adenovirus (Butterworth, 1999). Reovirus causes bilateral inflammation of the digital flexor and tarsometatarsal extensor tendons causing discomfort and lameness. It is mostly seen in broiler chickens of 4-8 weeks of age (Islam et al., 1988). Adenovirus along with reovirus was isolated from the tenosynovitis outbreak in an Australian flock (Mackenzie and Bains, 1977). Lameness can also be associated secondarily with other virus infections like herpes virus (Marek disease) and aramyxoviridae (Newcastle disease). In these cases chickens may show signs of lameness at the terminal stage (Butterworth, 1999).

The farm management practices such as 24 hour light schedules, higher stocking densities and faults such as poor litter quality also increase the susceptibility of broiler chickens to lameness. (Sørensen et al., 2000; Manser, 1996; Sanotra et al., 2002). The high stocking densities deteriorate the litter quality due to higher faecal deposits, water spillage and poor ventilation thus

increasing the incidence of contact dermatitis (Kristensen and Wathes, 2000).

1.3.2 Economic impact of broiler lameness

According to Bennet et al. (1999) and McInerney et al. (1992), the direct cost associated with any disease (C) can be defined as the sum of the losses in expected output. For resource wastage due to disease (L), the treatment cost incurred to mitigate the effects of disease on production (T) and the cost associated with specific disease prevention (P), C can be given as:

$$C = L+T+P$$

Economic losses due to leg weakness or lameness in chickens are associated with higher rates of mortality and culled birds due to failure in proper growth. There is also higher rate of carcass condemnation due to lameness (Morris, 1993; Sullivan, 1994). According to Morris (1993) the overall impact of broiler lameness on the US broiler industry was an increase in mortality (1.1%) and carcass condemnations (2.1%), and an annual loss of $80 to $120 million (Cook, 2000). Sanotra et al., (2001) concluded from their survey on incidence and reasons for leg abnormalities in Denmark, that incidence of leg disorders in Denmark was very high and most of the broilers suffered from tibial dyschondroplasia and other skeletal deformities. According to Algers and Berg (2001), in 1996, 11% of the total broiler population in Sweden suffered from leg abnormalities, which dropped to 6% in 1999. In UK, Kestin et al., (1992) found that 26% of the total UK broiler population was suffering from leg disorders and 19.5% carcass rejections were due to lameness (Yogaratnam, 1995). The estimated annual losses to the UK broiler industry caused by lameness was £24 million (Bennet et al., 1999).

1.3.3 Broiler Behaviour and Response to Lameness

Behaviour is defined as the aspect of an animal's phenotype involving the presence or absence of definable motor activities, vocalizations and odour production by means of which it conducts its daily affairs of maintenance and social interactions (Banks, 1982). In simpler terms, "what the animal does" is its behaviour. The branch of zoology which studies the animal behaviour is called ethology. The basic survival needs of broiler chickens are adequate feed, water, favourable environmental conditions, lack of injuries and freedom from pain or disease (Broom, 2001). To

satisfy all these needs they exhibit certain behavioural patterns such as foraging, exercise, preening, dust bathing, social interactions, and response to potential danger signs (Broom, 2001; Vestergaard and Hogan, 1992). Under normal conditions of rearing and management, broiler chickens are relatively more lethargic than the laying stock. They like to spend most of their time (64% to 76%) resting (Murphy and Preston, 1988; Weeks et al., 2000; Bizeray et al., 2000). Eleven percent of their time was spent on feeding and the remainder in standing idle, preening, leg and wing stretching, and dust bathing. The time budgeting of broiler chickens (Bizeray et al., 2000) suggests that broiler chickens spend 67% of the time lying down, 28% active immobile (eating, drinking and standing) and only 5% walking. An individual chicken moves in an area of 20 sq m in one hour and the occurrence of dust bathing was for very short periods only (Murphy and Preston 1988). Contrary to this, Vestergaard and Sanotra (1999) found that dust bathing was quite a common behaviour in broiler chickens as it was seen everyday and almost in every chicken.

Deviation from the normal behaviour can be seen in broiler chickens suffering from lameness (Weeks et al., 2000). According to a study conducted by Weeks et al., (2000), lame chickens spent significantly more time lying (86%) than their sound compatriots. Sound birds chose a standing posture for eating whereas the lame ones were seen eating while lying down. Sound birds fed over 50 times in 24 hours, while the number of visits to the feeder for the broiler with gait score 3 was reduced to 30 times. Lame birds also spent significantly less time in standing preening, standing idle and dust bathing. Vestergaard and Sanotra (1999) found there was a significant decrease in time spent on dust bathing by lame chickens. Dust bathing involves forceful movements with legs and wings, which might be difficult for broilers in pain or having leg abnormalities. Lack of dust bathing is also associated with an increase in blood corticosterone levels in affected birds (Vestergaard et al., 1997)

1.3.4 Evaluation of Severity/Degree of Lameness in Broiler Chickens

After segregating the birds suffering from lameness due to pain from the flock, it is important to evaluate the degree of their lameness. It would help in determining the efficacy of analgesics in broiler chickens i.e. any improvements in the degree of lameness after treatment. It also helps

in interpretation of the behaviour of birds in relation to lameness; effect of various degrees or severity of lameness on the behaviour of birds. By evaluating the lameness in scores or degrees, it is possible to statistically analyse the prevalence of the condition.

There are various methods devised and validated by researchers to evaluate lameness in broiler chickens. Kestin et al., (1992) devised a scaling system to describe the degree of lameness in broiler chickens (gait scoring). In this method, birds were visually examined and rated on the scale of 0 to 5, 5 being the lamest and 0 perfectly sound bird. The gait score bird had a slight defect which was difficult to define. The gait score 2 bird had a definite and identifiable defect. The abnormal strides in gait score 1 and 2 birds did not affect the agility of these birds. The gait score 3 bird had obvious gait defect which affected its movements. The gait score 4 bird had severe gait abnormality but it was still capable of walking. Its manoeuvreability and speed both were severely affected. The gait score 5 bird was incapable of sustained walking (Kestin et al., 1992). Danbury (1999) and Danbury et al., (2000) investigated many methods to assess the degree of lameness in broiler chickens along with the one formulated by Kestin. In observational scan sampling, she defined broiler behaviour into 15 different activities that normal, sound broiler chickens engage in. These 15 activities were walk, run, stand idle, stand eat, stand drink, stand preen, stand ground peck, total lie, leg stretch, lie sleep, lie eat, lie preen, dust bath, lie ground peck and fight. There were 6 pens which housed between 10 to 255 birds in each pen. Their activities were recorded at the same time each day for 30 seconds and the observation period was for 60 minutes. The results were reported as percentages of time spent on each of those 15 activities during the day.

She also investigated an induced standing test. This method shows that how long could a bird stand when forced to stand. The longer the time the bird stands the better its leg health. Another test was the ramp test in which a ramp was designed to test the speed and manoeuvrability of lame birds when compared to sound birds.

Corr et al., (1998) used a pedograph to analyse the severity of lameness in broiler chickens. The pedograph was a rubber mat surface with multiple, protruding, small grid lines on one side. When covered with ink, it imprinted on the underlying sheet of paper as the foot passes over it.

It was based on the principle that if bird is lame or there is some problem in a leg, then the subject will be less willing to bear weight on it. The pressure exerted on the ground by this leg will be less and that pressure can be measured by the use of pedograph. He found that this method evaluates a bird's lameness more accurately than gait scoring but application of this method could be difficult at farm level. Again Corr et al., (2003) conducted a trial to evaluate ground reaction forces with the help of force plate. A standard force plate was embedded in the middle of the runway and the ground reaction force was measured when birds walked over that plate. The sound bird will exert more pressure on the force plate in comparison with the lame bird. They suggested that a standard force plate can objectively measure different ground reaction forces but clinical application of this technique was still doubtful.

Weeks et al., (2001) and Weeks et al., (2002) developed a new method to assess lameness called the latency to lie (LTL) test. It was based on the fact that broiler chickens have a strong disinclination to sit in water. In this test after segregating the lame birds or after handling them, they were given some time to adjust before the test, and this time was called settling time. Then after 15 minutes of settling time, broiler chickens were made to stand in shallow tepid water (submerging the foot only). Sound birds would stand in water for longer than the lame birds. They compared that method with gait scoring and found that some of the broilers with gait score 2 or 3 were able to stand in water for a longer duration and a few with the gait score 0 were not able to stand for a minute. They concluded that chances of error in the LTL test is much less than in gait scoring method but the former was more time consuming to apply at farm level. They suggested that LTL method could be an auditing tool for gait scoring or could be applicable as a research tool.

Berg and Sanotra, (2003) tried to modify the LTL test so that it could be adapted easily at farm level. Birds to be tested were placed in a plastic tub filled with water at 32˚C up to 3 cm. The settling time was omitted in this modified version. They found no significant difference in the results achieved by original LTL and modified LTL. This test took between 1 and 10 minutes per bird and the equipment required for this was not expensive, easily available and easy to disinfect. They concluded that the modified LTL test could be used as a method of validating gait score results at farm level.

Some researchers have also used a pedometer to assess the severity of lameness. Hocking (1999) determined severity of lameness in adult male turkeys by the change in the number of steps taken by turkeys without or without lameness.

1.3.5 Management of Broiler Lameness

Broiler lameness has intrigued many researchers worldwide and they have suggested various managerial, nutritional and genetic manipulations. They were somewhat successful in combating the problem but the major drawback was reduction in body weight and delay in achieving market age of broiler chickens (Robinson et al., 1992) (Su et al., 1999).

Robinson et al., (1992) and Edwards and Sorensen (1987) found that fasting the broiler chicken reduced the incidence of lameness. Su et al., (1999) found that meal feeding (in which feed was available ad libitum in each meal) was more efficient in reducing the leg problems but the effect was mainly due to reduced bodyweights. Bizeray et al., (2002) were successful in combating the leg problems by adopting sequential feeding in which chickens were fed with low lysine diet during one half and the normal diet during the remaining day. However there was no significant improvement in walking ability or leg health seen in broiler chickens subjected to periodic fasting (Walker, 1996). Also, Leterrier et al., (1998) suggested that reducing growth rate of broiler chickens by feeding them a low energy diet did not improve the skeletal development or cortical bone quality. They could not find any clear correlation between the bone mineral content and walking ability.

There are other managerial alterations through which good health of broiler chickens can be maintained. Rearing broiler chickens under fluorescent light decreased the incidence of angular deformities and total leg abnormalities but surprisingly, tibial dyschondroplasia (TD) was higher in the treatment group (Hulan and Proudfoot, 1987). Coenen et al., (1988) proposed that broiler chickens reared under continuous light spend most of the time sitting and show very low activity which could be due to an inability to maintain a diurnal rhythm and orientation which poses various problems to broilers like leg weakness and sudden death syndrome. The incidence of leg abnormalities can be reduced by adopting an abrupt increase in the day-length light programme, in which day-length was 6 hours between the 3rd and 21st day then increased

to 23 hours until the day of slaughter (Classen and Riddell, 1989). Sørensen et al., (1999) could not find any relationship between photoperiod and gait score. There was improvement in the gait score in birds reared in shorter days but these birds had lower body weights. The introduction of dark periods and decreasing stocking density increases the activity of the birds and also reduces the incidence of leg abnormalities (Sanotra et al., 2002).

Genetic manipulations can also reduce the leg problems in modern day broiler chickens. Genetic selection has achieved higher growth rates and improved the feed efficiency but according to a hypothesis supported by many authors, the leg problems have strong genetic correlation with body weight and the lameness in broiler chickens is a result of genetic selection for higher body weight and growth rates (Webster, 1995; Kestin et al., 2001; Julian, 1998; Mercer and Hill, 1984; Havenstein et al., 1994; Kestin et al., 1999; Emmerson, 1997). The feed restrictions, dietary dilutions and other managerial alterations may help in reducing the leg weakness syndrome but higher growth rates are certainly not the only factor (Hester et al., 1990).

There is experimental evidence that the lameness in broiler chickens is also associated with pain. Lame broilers prefer to consume feed containing analgesic carprofen as compared to normal feed (Danbury et al., 2000). S/C administration of carprofen increased the walking ability and the speed of lame birds in an experiment conducted by McGeown et al., (1999). They concluded that lame broiler chickens suffer from pain. The leg health and behaviour of fattening turkeys was also improved by treatment with butorphanol (Buchwalder and Huber-Eicher, 2005) which suggests that the fast growing turkeys show lameness associated with pain. The effect of various NSAIDs was evaluated by (Hocking et al., 2005). Intra-articular injection of sodium urate crystals was given to induce acute pain in domestic fowl. Carprofen, flunixin, ketoprofen were administered I/V and sodium salicylate was given orally at dose rates recommended for large animals. In their first experiment they could not observe any improvement. The experiments were repeated with higher dose rates for each analgesic via I/M injection. The behavioural improvement was clearly noticed in the treated birds as compared to the control group.

There is still a huge gap in the treatment of lame birds. Therefore in this study we studied the pharmacokinetics of various analgesics - morphine, butorphanol, aspirin and salicylic acid to

evaluate the dose rate and duration of analgesia, after which these analgesics were used in lame birds and the behavioural changes were recorded.

1.4 OBJECTIVES:

This study was designed with the following objectives:

1. To describe the pharmacokinetics and side effects of analgesic drugs; aspirin, salicylic acid, morphine and butorphanol in broiler chickens to enable the rational use of analgesics in broiler chickens.

2. To alleviate pain and improve the welfare of broiler chickens suffering from pain by administering above said analgesics.

3. To validate the use of healthy broiler chickens as a model of drug research and lame broiler chickens a chronic pain model for other wild and rare avian species.

This study will be a step forward towards enhancing the welfare of lame broiler chickens, which ultimately will increase production and profits in broiler farming. Internationally the broiler chicken industry is incurring huge losses due to lameness in broilers. Lameness hinders movement of the chicken, thus it is not capable of moving freely and is dominated by its sound compatriots. In severe cases, the affected bird is not able to consume feed and water and ultimately, may die due to starvation or dehydration. Lameness also impacts on the welfare of the birds as most of the time they live in pain.

In our research we are trying to develop assays for optimising dosage of analgesics in broiler chickens and then alleviating pain with their administration at set dosage.Analgesic studies on broiler chickens can also be used as an auditing tool for the industry standards on animal welfare.

The pharmacokinetic parameters obtained from the broiler chickens can be used as a basis for allometric scaling to predict the pharmacokinetics of these analgesics in various rare and endangered wild avian species found in New Zealand such as kiwis, kakapos and albatrosses. Allometric scaling should be used in caution, however the scaling between birds gives a smaller error compared to scaling between mammals and birds (Hunter et al., 2008). It will never be possible

to study the pharmacokinetics of every drug in each and every avian species. All the experiments were approved by Massey University Animal Ethics Commitee.

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CHAPTER TWO

Pharmacokinetics of opioids in broiler chickens

2.1: PHARMACOKINETICS OF MORPHINE IN BROILER CHICKENS

ABSTRACT

The pharmacokinetics of morphine was evaluated after intravenous administration in broiler chickens. Morphine sulphate (10 mg/mL) was injected intravenously at 2 mg/kg in 27 healthy broiler chickens. The pharamcokinetic parameters were calculated both by compartmental and noncompartmental approaches. The two compartment model best described the concentration time curve after I/V administration in chickens. The $T_{1/2\beta}$ (minutes), $Vd_{(central)}$ (mL/kg) and the clearance (mL/min/kg) were 69.3, 6.3 and 63.02, respectively. The elimination rate constant (k_{el}) and the micro rate constants k_{21}, k_{12} and k_{10} were 0.10 and 0.021, 0.039 and 0.046, respectively. The $T_{1/2kz}$, *Vd* and *Cl* after noncompartmental analysis approach were 68.1, 8.1 and 82.68, respectively. Morphine was well distributed and rapidly cleared in chickens. The plasma concentration of morphine was above the limit of quantification for 4 hours after 2 mg/kg I/V dose, but the therapeutic concentration, assuming that chickens are similar to man, was only maintained for 2 hours. If we assume similar effective plasma concentration as in mammals, then morphine could provide good analgesia of short duration in chickens too. The correlation of the plasma concentration and the analgesic effects in chickens will be dealt with in later chapters.

2.1.1 INTRODUCTION

Morphine is the standard analgesic drug and recommended by World Health Organisation for the treatment of acute and chronic pain in people (Glare and Walsh, 1991). A lot of work has been done on the pharmacokinetics of morphine in humans after I/V, I/M and oral route of administration. (Brunk andDelle, 1974) studied the metabolism of morphine after I/V, I/M, S/C, and oral routes of administration at the dose rate of 5.75 mg/sqm of body (0.15 mg/kg) in 6 healthy men. They found that I/V injection produced initial higher levels of morphine as compared to other routes but were followed by rapid distribution, metabolism and excretion as after 15 minutes the levels were significantly lower than the I/M route and similar to S/C route of administration. After 1 hour after I/V and I/M or S/C injection the levels were 21 μg and 35 μg, respectively. The biological half-life ranged from 2.1 to 2.6 hours.

Ten mg injection of morphine I/V in people had 2.9 hours elimination half-life, volume of distribution 3.2 L/kg, clearance 14.7 mL/min/kg (Stanskl et al., 1979) and the peak plasma concentration after I/M injection ranged from 51 to 62 ng/mL after 20 minutes of drug administration.

The terminal elimination half-life of morphine in postoperative patients under the influence of anaesthesia was 2.2+/- 1.1 h, plasma clearance of 21+/- 6.0 mL/min/kg and mean plasma concentration of 21+/-12 ng/mL with a Minimum Effective Concentration (MEC) at 16 +/- 9 ng/mL (Dahlstrom et al., 1982). Chauvin et al., (1982) studied the kinetics of morphine and duration of analgesia given at dose rate of 0.2 mg/kg given by I/M, I/T and extradural routes of administration in patients after surgery. Plasma concentrations after administration by the I/T route were significantly less than after extradural and I/M administration. The maximum plasma concentration and time to reach that level after I/M, extradural and I/T routes were 60, 0.43; 50.3, 0.23; 35 ng/mL, 1.65 h respectively. Morphine had slower rate of vascular absorption following I/T administration than I/M and extradural administration and analgesia is more prolonged after extradural administration.

Hoskin et al., (1989) made a comparison between the bioavailability of morphine after I/V (5mg) and three formulations for oral administration (10mg each); oral aqueous solution, con-

trolled release oral tablets and controlled release buccal tablets in healthy subjects. Morphine, and its major metabolites, morphine-3-glucoronide (M-3-G) and morphine-6-gluconoride (M-6-G) were measured by radioimmunoassay. Bioavailability after the three oral formulations was 23.9, 22.4 and 18.7%, respectively. The maximum plasma concentration was seen after 45 min, 2.5 and 6 hours which were 10.6, 3.7 and 2.5 ng/mL after oral aqueous solution, controlled release oral tablets and controlled release buccal tablets, respectively. The maximium concentration after I/V administration was 340.2 ng/mL and half-life was 1.9 hours with clearance of 23.8 mL/min/kg. There was no difference in the plasma concentration of M-6-G after either of two routes of administration (Glare and Walsh, 1991). M-6-G is a potent and well tolerated analgesic without many side-effects like nausea or vomition (Osborne et al., 1992)

The pharmacokinetics of morphine has also been described in dogs. In dogs it is the drug of choice for postoperative analgesia. Hug et al., (1981) studied the pharmacokinetics of morphine in anaesthetised dogs injected I/V at dose of 0.3 and 2.0 mg/kg. It is rapidly cleared from plasma (Hug et al., 1981) and it metabolism takes place by glucuronidation by both hepatic and extrahepatic glucuronyltransferases (Jacqz et al., 1986). M-6-G and M-3-G appeared in plasma after 1.5 minutes. Hug et al., (1981) also observed a lower volume of distribution with a higher dose rate of morphine probably becasue of lowered blood pressure caused by the 2.0 mg/kg dose rate of morphine. They fitted their data to a three-compartment model.

Dohoo et al., (1994) compared the pharmacokinetics of morphine after I/V and oral administration in dogs. Maximum serum concentrations were 128, 463, 229.6, 568, 21 and 15 ng/mL after administration of 0.5 mg/kg I/M and I/V; 0.8 mg/kg I/M and I/V ; 15 and 30 mg sustained release oral tablets of morphine. According to his observations, morphine was eliminated by first order metabolism best described by two-compartment model. There was no significant difference between the volumes of distribution, elimination half-life and plasma clearance between I/V and I/M routes of administration. Bioavailability after the parenteral route was the same while by the oral route it was 20%. He suggested that therapeutic concentration of morphine could be maintained by I/M adminisration every 12 hours. KuKanich et al., (2005a) suggested that I/V injection of morphine at dose rate of 0.5 mg/kg given every 2 hours maintained the plasma concentration required for analgesia. After slow I/V infusion of morphine (KuKan-

ich et al., 2005a; Guedes et al., 2007), the half life was lower compared to bolus injection, 0.87, 0.63 and 1.16 hours, respectively (Kukanich et al., 2005b).

Barnhart et al. (2000) desribed the pharmacokinetics of morphine after rectal, I/M and I/V route of administration. They described disposition of morphine by two-compartment model with terminal half-life of 94.6 minutes. The median plasma concentrations were 92, 185 and 51.3 ng/mL after 5 minutes of administration by I/V (0.5 mg/kg), I/M (1 mg/kg) and per rectum (5 mg/kg), respectively. Bioavailability of morphine after rectal route was significantly lower than I/M and I/V route. They concluded that administration of morphine by rectal route was of no clinical significance.

The disposition of morphine has also been studied in cats and horses. In cats morphine is cleared slower than in dogs, with similar elimination half life, 69 minutes (Kukanich et al., 2005b) and 76 minutes (Taylor et al., 2001) in dogs and cats, respectively. The pharmacokinetics of morphine in horses (Combie et al., 1983) is similar to humans. Levels of morphine in horses can be detected up to 48 hours in blood and 144 hours in urine. Combie et al., (1983) described three-compartment model for morphine disposition in horses with elimination half-life of 87.9 minutes.

As far as we know, no one has studied pharmacokinetics of morphine in any avian species, including broiler chickens. As stated earlier in chapter 1, there is lot of confusion about dose rate and analgesic effects of morphine in birds. Therefore, the rational approach would be to study the pharmacokinetics of morphine before the analgesic efficacy studies. This would tell us how long morphine remains in chicken's blood and its plasma concentration, which would be useful in designing the pharmacodynamic study.

The objective of this study was to describe the pharmacokinetics of morphine in broiler chickens, to ascertain the dose rate required for further pharmacodynamic studies.

2.1.2 EXPERIMENT ONE: Pharmacokinetics of morphine in broiler chickens (pilot study)

Study Design

This pilot experiment was carried out on eight broiler chickens with an average weight of 2 kg. These chickens were divided into four groups of two chickens each. A medial metatarsal vein of each broiler chicken was catheterised with a 22 G catheter under halothane anaesthesia. The birds were allowed to recover from the effect of anaesthesia before the injection of drugs. The birds were kept in groups under standard conditions of housing and management. They were fed *ad-lib* with commercially available broiler feed with a 24-hour supply of fresh potable water.

Drug Administration

Morphine was injected into the wing vein (vena basilica) at a dose of 0.5 mg/kg body weight using a 22 G needle.

Sample Collection

Four serial blood samples were collected from four different broiler chickens for each treatment, resulting in a total of 16 samples. The blood samples (2 mL each) were withdrawn from the catheterised vein of each broiler chicken in a heparinised vial at 0, 5, 10, 20 min (from 1st group), 0.5, 1, 2, 3 hours, (2nd group) 4, 6, 8, 10 hours (3rd group), 12, 24, 36 and 48 hours (4th group) after the injection of morphine. The amount of blood withdrawn from each bird was 8 mL, which is approximately 4% of the total blood volume of a 2 kg chicken. The vials were kept chilled immediately after collection and centrifuged at 2000 rpm for 10 minutes. Plasma was pipetted out and kept at –20 °C until the day of analysis.

Reagents and Standard Solutions

Morphine sulphate injection was purchased from Mayne Pharmaceuticals Australia. The same solution of morphine as injected was used as a standard for the analysis and did not contain any antioxidant or preservative. Laboratory reagents used were methanol and acetonitrile (Merck, New Zealand). Phosphate buffer (0.1M) pH 6.6 was prepared by mixing 187.5 mL of 0.2 M di-sodium phosphate and 312.5 mL of 0.2 M sodium hypophosphate; the volume was increased to 1 L with the addition of Milli-Q water (Milli-q PFplus system, Millipore Cooperation, USA). Na_2HPO_4 (0.2M) solution was prepared by mixing 14.15 g of Na_2HPO_4 salt in 500 mL Milli-Q water and 0.2 M $NaHPO_4$ solution was prepared by mixing 11.99 g of $NaHPO_4$ salt in 500 mL

of Milli-Q water.

Sample Analysis

The plasma samples were analysed by High Performance Liquid Chromatography (HPLC), using a LC-10AD HPLC pump, SIL-10AD auto-injector, DGU-14A de-gasser, SPD-M10A Diode Array Detector (Shimadzu Japan) and the chromatographs were analysed by the Shimadzu client server version 7.3.

The separation of morphine was achieved with a Phenomenex C18A (150 X 4.6 mm i.d, 5µm particle size) column at 30°C. The mobile phase consisted of 0.1 M phosphate buffer pH 6.6: methanol: acetonitrile (80:10:10) pH 7.1 with flow rate of 0.7 mL/min under isocratic conditions. The DAD detector was set at 210 nm wavelength (Wright and Smith, 1998; Gerostamoulos and Drummer, 1996) The Limits of Quantification and the Limits of Detection were 20 and 10 ng/mL, respectively. The specificity of the method was confirmed by the absence of any peaks at the same retention time in the blank plasma after following the same extraction method (figure 8).

Sample Preparation

The plasma samples were prepared by solid phase extraction using Strata X (Phenomenex) polysorbent cartridges and the manufacturer's recommended method with slight alterations. 300 µL of plasma was diluted with 500 µL of Milli-Q water and loaded into the Strata X cartridges preconditioned with 1 mL of methanol followed by 1 mL of Milli-Q water. The first wash used 1 mL of water followed by drying for 3 minutes and the second wash with 40% methanol followed by drying for 3 minutes. Elution of the compound of interest was with 100% methanol. The samples were dried under a gentle stream of air at 20°C. The dried samples were reconstituted in 200 µL of mobile phase and the injection volume was 50 µL. Each sample was injected three times in the HPLC machine. The recovery of morphine after the solid phase extraction was determined by comparing the area of the peaks for 781.25, 390.62, 195.31 and 97.65 ng/mL morphine concentrations made in the mobile phase with the areas for the same concentrations spiked in the blank plasma. The recovery after the sample preparation was 95.95%.

The standard curve for morphine was made by spiking the morphine standard with blank plasma and the resultant concentrations were 781.25, 390.62, 195.31 and 97.65 ng/mL, respectively. The unknown concentrations were calculated from the standard curve by linear regression using GraphPad Prism. A standard curve was constructed before and after every run of samples.

Results

The plasma concentration of morphine after 5 minutes (figure 9) following injection was 398 ng/mL, and it dropped to 41 ng/mL after 10 minutes (figure 10). It was not possible to conduct an accurate pharmacokinetic analysis with only two data points, as the minimum number of data points required is five. From these results, we found that the chickens eliminate morphine much faster than mammals and the mammalian dose rate of 0.5 mg/kg lasted only for 10 minutes. Therefore, we decided to inject morphine at a much higher dose rate, and frequency of sampling was increased for our second experiment, to get more data points in the concentration time curve.

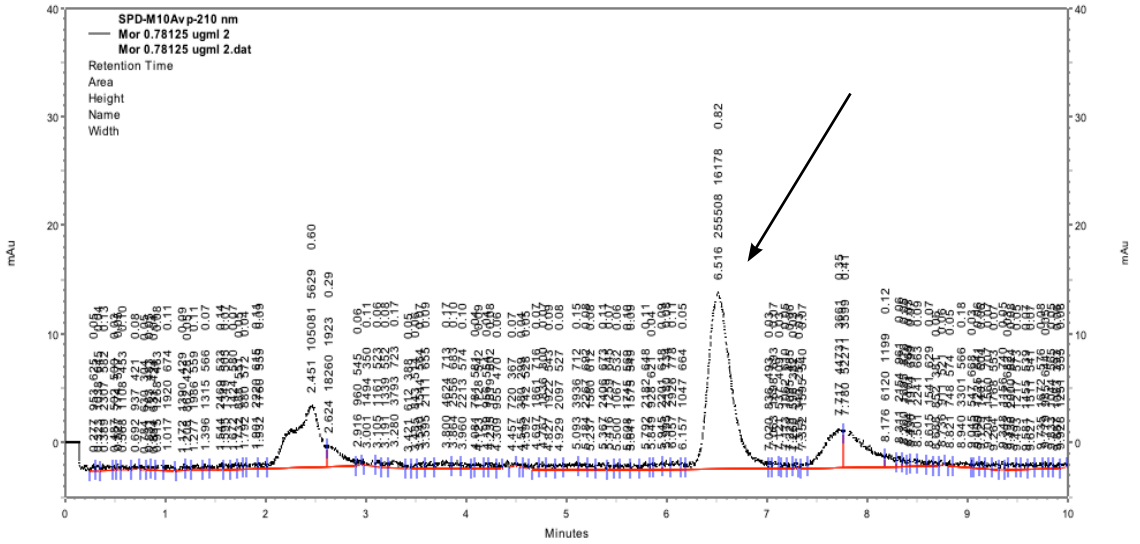

Figure 7: Chromatograph showing morphine standard solution 781.25 ng/mL in mobile phase

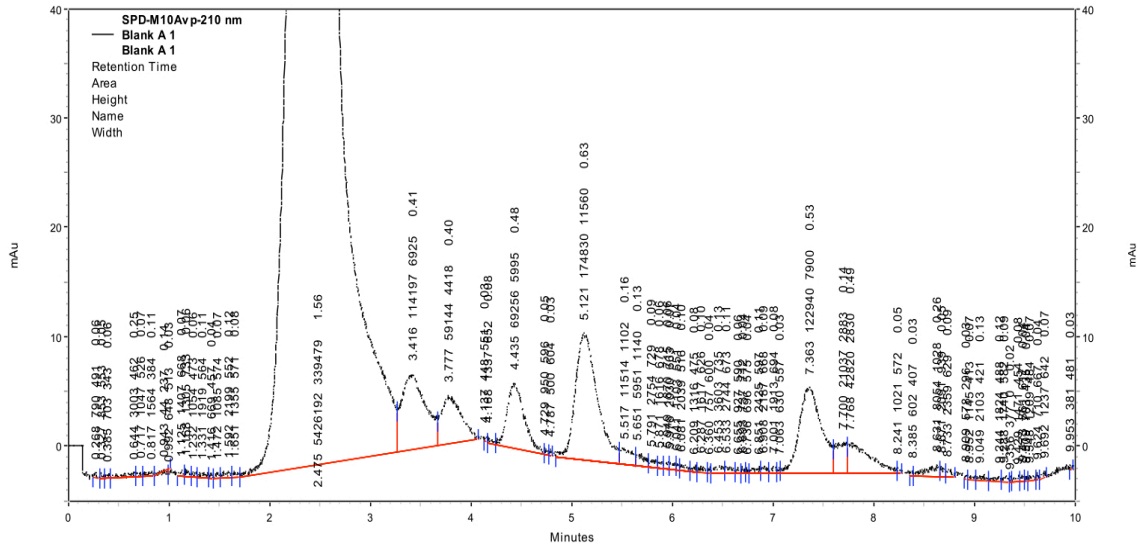

Figure 8: Chromatograph showing blank plasma after following similar sample preparation procedure for extraction of morphine as other plasma samples

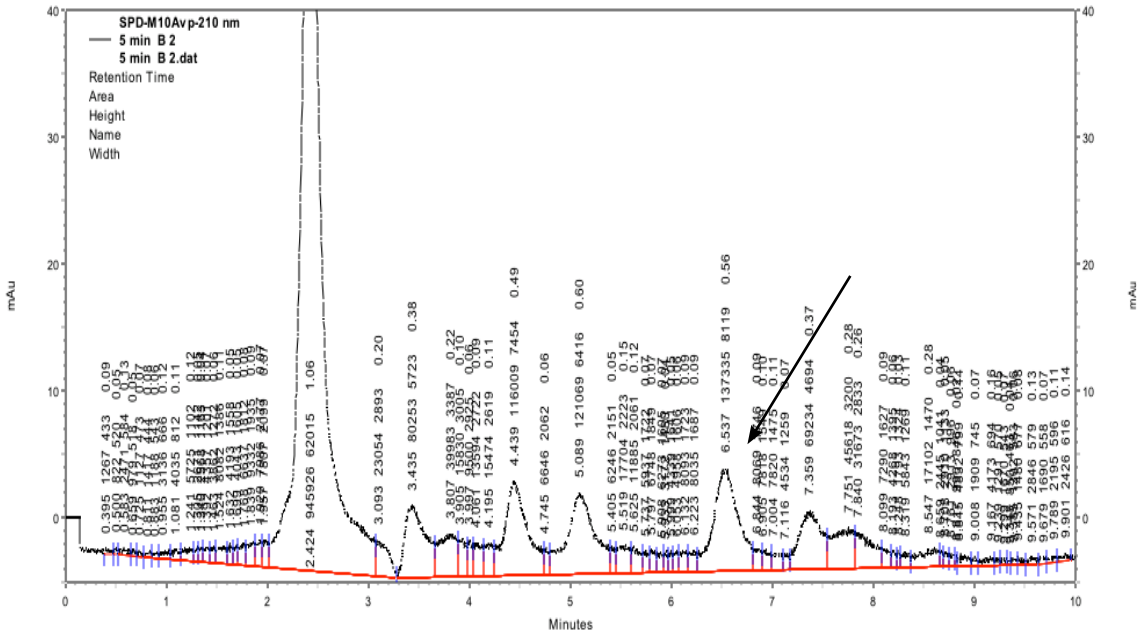

Figure 9: Chromatograph showing morphine peak after 5 minutes of intravenous injection at 0.5 mg/kg dose rate.

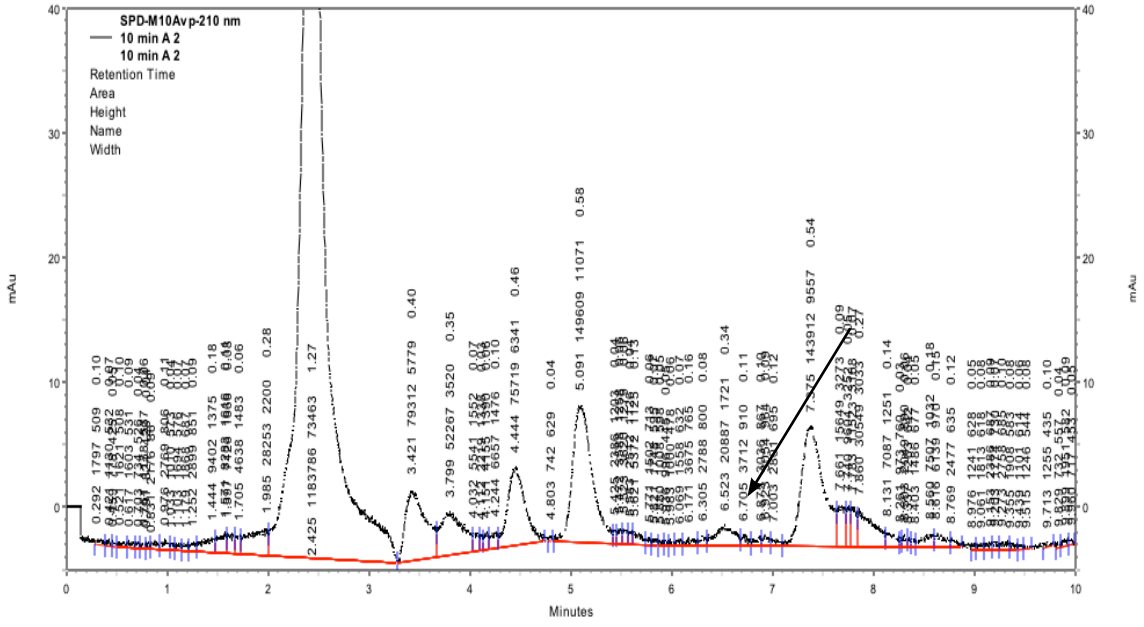

Figure 10: Chromatograph showing morphine peak after 10 minutes of intravenous injection at 0.5 mg/kg dose rate.

2.1.3 EXPERIMENT TWO: Pharmacokinetics of morphine in broiler chickens

Study Design

This study was conducted on 27 healthy broiler chickens with an average body weight of 2 kg divided into three groups of nine chickens in each. A medial metatarsal vein of each broiler chicken was catheterised with a 22 G catheter under halothane anaesthesia. The birds were allowed to recover from the effect of anaesthesia before the injection of drugs. The birds were kept in similar conditions as in the pilot study.

Drug Administration

Morphine sulphate (10 mg/mL, Mayne Pharmaceuticals, Australia) was injected intravenously into the contralateral vein at the dose rate of 2 mg/kg body weight using a 23 G needle.

Sample Collection

Four serial blood samples of 2 mL each were taken at 0, 1, 3 and 5 minutes from group 1; 10, 15, 20 and 30 minutes from group 2; 1, 2, 4 and 6 hours from group 3 respectively. The samples were treated in the same way as in the pilot study.

Sample Analysis

The plasma samples were analysed following the same High Performance Liquid chromatography method as in pilot study.

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated using standard equations in a spreadsheet. These included half life of the terminal phase ($T_{1/2\,\lambda z}$), area under the curve extrapolated from time zero to infinity ($AUC_{0-\infty}$), area under the moment curve extrapolated from time zero to infinity ($AUMC_{0-\infty}$), volume of distribution (*Vd* mL/kg), clearance (*Cl* mL/min/kg) and mean residence time (*MRT* min).

The noncompartmental pharmacokinetics were calculated from the mean of pooled data. The

samples from each of the three groups at various time points were pooled to give one mean concentration time curve. (Gagnon and Peterson, 1998; Cheung et al., 2005). All the compartmental parameters were calculated by the the equations described in chapter 1.

Results

The chromatographs obtained from the HPLC for morphine standard, blank plasma and the 1 minute time point are shown in figure 11, 12, 13, respectively. Morphine eluted at 5.7 minutes and there was no peak at that time in the blank plasma. This shows that there was no interference of any sort in the morphine assay.

The linear and semi-log concentration time curve are shown in figures 14 and 15, respectively. The pharmacokinetic profile for morphine in chickens is best described by a two compartment model. Both the compartmental and noncompartmental parameters are shown in table 2.

The maximum concentration achieved after a 2 mg/kg I/V dose of morphine in chickens was 2234 ng/mL. It declined rapidly in the first 15 minutes. 82.25% of the drug was cleared in that time span. Though the concentration was detected till 4 hours after the injection, it remained above the MEC levels (for humans) for only 2 hours. It had a much higher volume of distribution (8.1 L/kg) than the blood volume and the distribution half life was only 7 minutes. These results suggest that morphine rapidly distributed to the tissues. Its clearance was also very fast resulting in a short elimination half-life.

Discussion

The maximum plasma concentration seen in chickens is much higher than in other species, as it was injected at 4 times higher dose rate than the usual maximal dose for humans. The volume of distribution of morphine was higher in chickens compared to other mammals (table 3). In man and other mammals, morphine is well distributed in the tissues (Lugo and Kern, 2002) and this is also true for chickens. The elimination half-life of morphine after a single intravenous bolus dose in man is 174 min (Stanskl et al., 1979), which is higher than other animals: dogs 94.9 min (Barnhart et al., 2000) 69.6 min (Kukanich et al., 2005b), cats 76.3 min (Taylor et al., 2001) and horses 87.9 min (Combie et al., 1983). In chickens, the elimination half-life (68.1

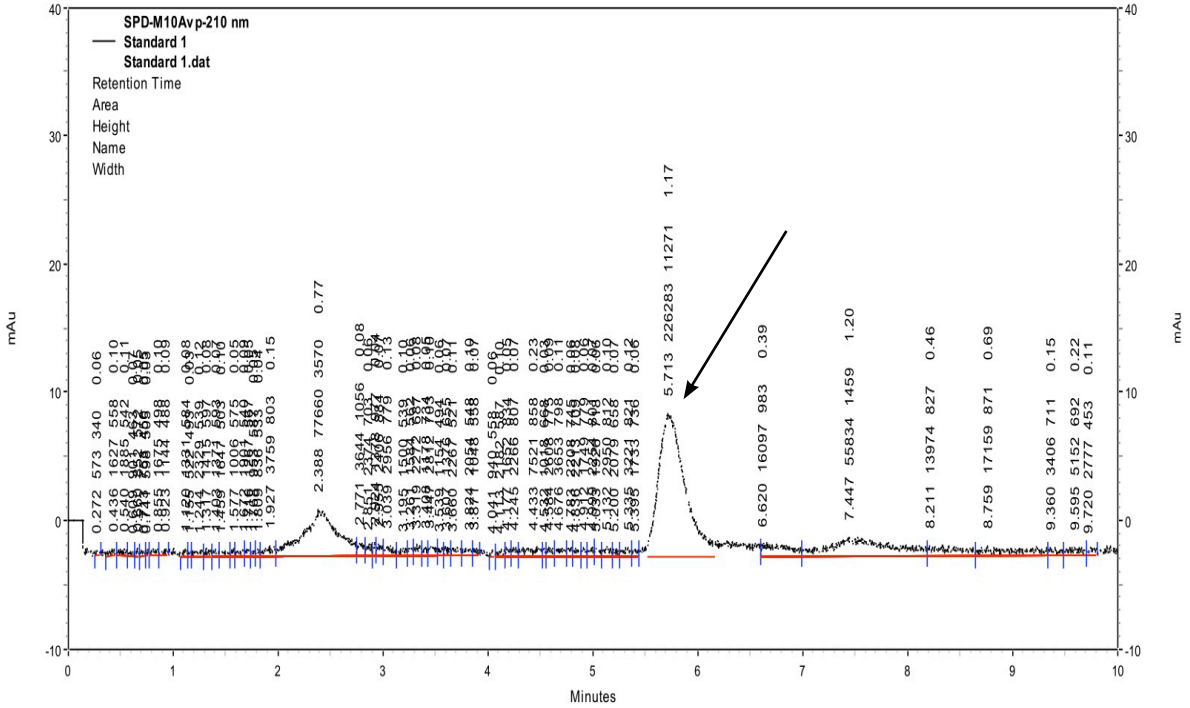

Figure 11: Chromatograph showing morphine standard solution 390.62 ng/mL, in mobile phase

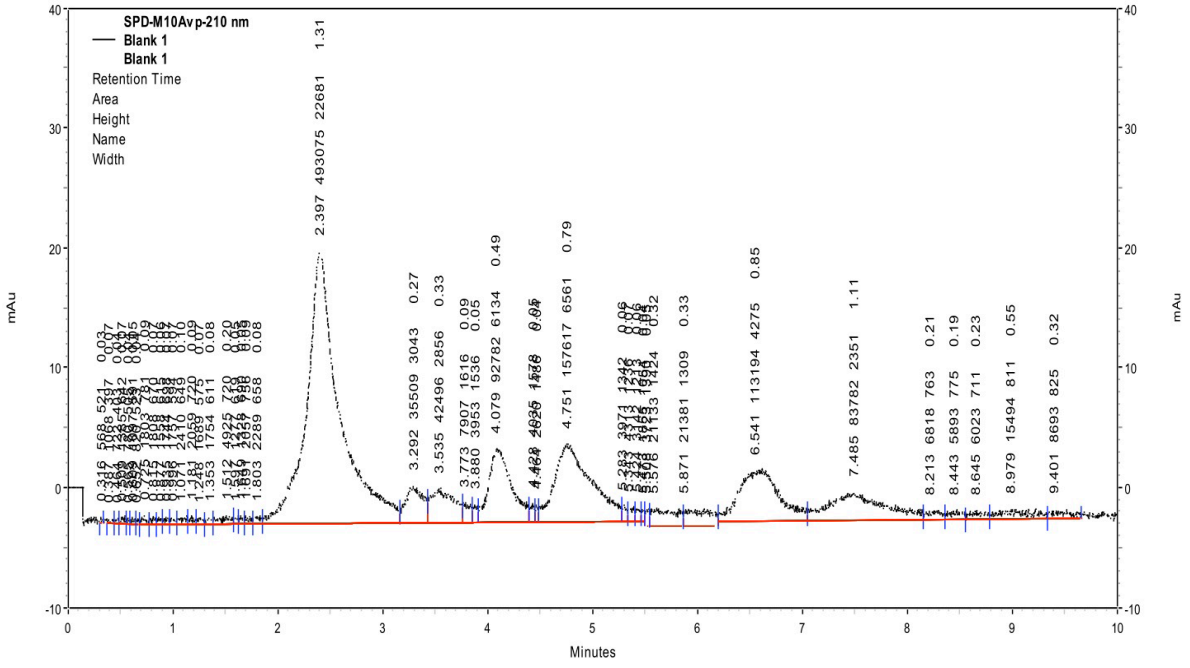

Figure 12: Chromatograph showing blank plasma after following similar sample preparation procedure for extraction of morphine as other plasma samples

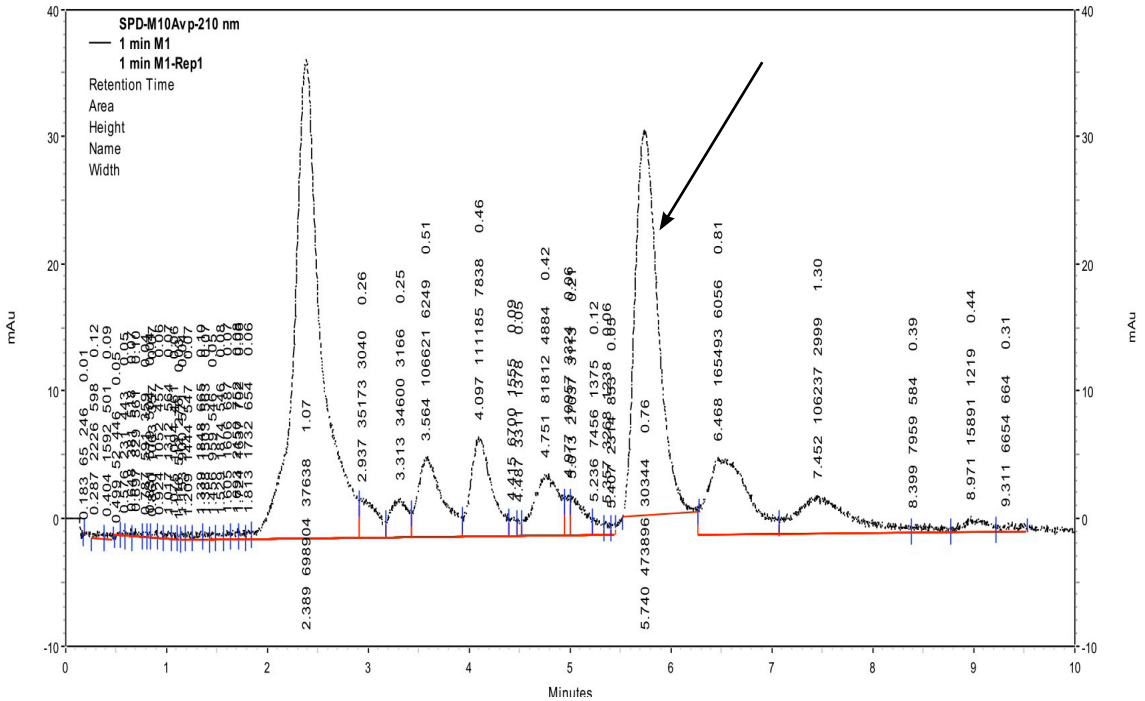

Figure 13: Chromatograph showing morphine peak after 1 minute of intravenous injection at 2 mg/kg.

min) is less than other species but some studies in dogs have shown shorter half-life of morphine, 38 minutes (Guedes et al., 2007), 52 minutes (KuKanich et al., 2005a) after intravenous infusion. In our study, we also observed that the chickens cleared morphine at a higher rate than man, cats, horses and dogs but Barnhart et al., (2000), found morphine clearance higher in dogs (85.2 mL/min/Kg) as compared to chickens in our study. This observation is consistent with the kinetic studies of other drugs in chickens depicting faster clearance as compared to mammals; this could be due to chicken's higher metabolic rate and body temperature (Baert and De Backer, 2002; Graham et al., 2005).

The potential side-effects reported for the use of morphine in chickens are hyperalgesia (Sufka and Hughes, 1990) and motor-incoordination (Rager and Gallup, 1986). In people, the major side-effects observed are respiratory depression and sedation (Lugo and Kern, 2002). In this study, only minor sedation was noticed which is due to its action on μ receptors. The activation of μ receptors block the calcium influx and activates the inward rectifying potassium channels (section 1.1.1). These events cause hyperpolarisation which is related to sedation and analgesia. The other side-effects reported after morphine administration were not observed.

The HPLC method used here for analysis of morphine in chicken plasma was adapted from Wright and Smith (1998) and Gerostamoulos and Drummer (1996). The limit of quantification with this method was comparable with the other published methods using the same diode array detectors. The limit of quantification for morphine was 20 and 25 ng/mL for the methods developed by Brandsteterova et al. (2002) and Bourquin et al. (1997). The methods using electrochemical detection, had much lower detection limits ranging from 0.1 to 5 ng/mL (Liaw et al, 1998, Taylor et al. 2001, Liu et al. 1997). The use of diode array detector with fewer reagents makes this method an inexpensive and easy to follow method in pharmaceutical laboratories.

The blood sampling method followed here was earlier used and validated by Graham et al., (2005). This method was used for ethical reasons to avoid excessive blood loss due to sampling. This experimental design did not allow us to calculate individual bird pharmacokinetic parameters. The pharmacokinetic parameters were calculated on the naive pooled data. The mean concentration from each time point was used to plot a mean concentration time curve, which was

used to calculate the pharmacokinetic parameters both by compartmental and noncompartmental pharmacokinetic analysis. This curve shown in Figure 14 also gives an indication of the variability between different birds. Although the conventional approach is usually used in clinical pharmacokinetics, variations on our study design have always been used in situations where it is not possible to obtain sufficient samples to construct a curve for an individual animal, for instance drug residues in meat.

This pharmacokinetic study concludes that clearance of morphine is much higher than in mammals: all of the drug was cleared in 10 minutes even though the limit of detection of our method was 10 ng/mL. Therefore, to achieve therapeutic plasma concentratio of morphine similar to those in man, morphine should be injected at 2 mg/kg at least. This is supported by Concannon et al., (1995), who found that morphine at 3 mg/kg reduced the MAC required for anaesthesia in chickens. In our study, at 2 mg/kg, the plasma concentration of morphine in chickens was above the minimum effective concentration for humans, for approximately 2 hours. Thus it may provide good analgesia although this needs to be evaluated further.

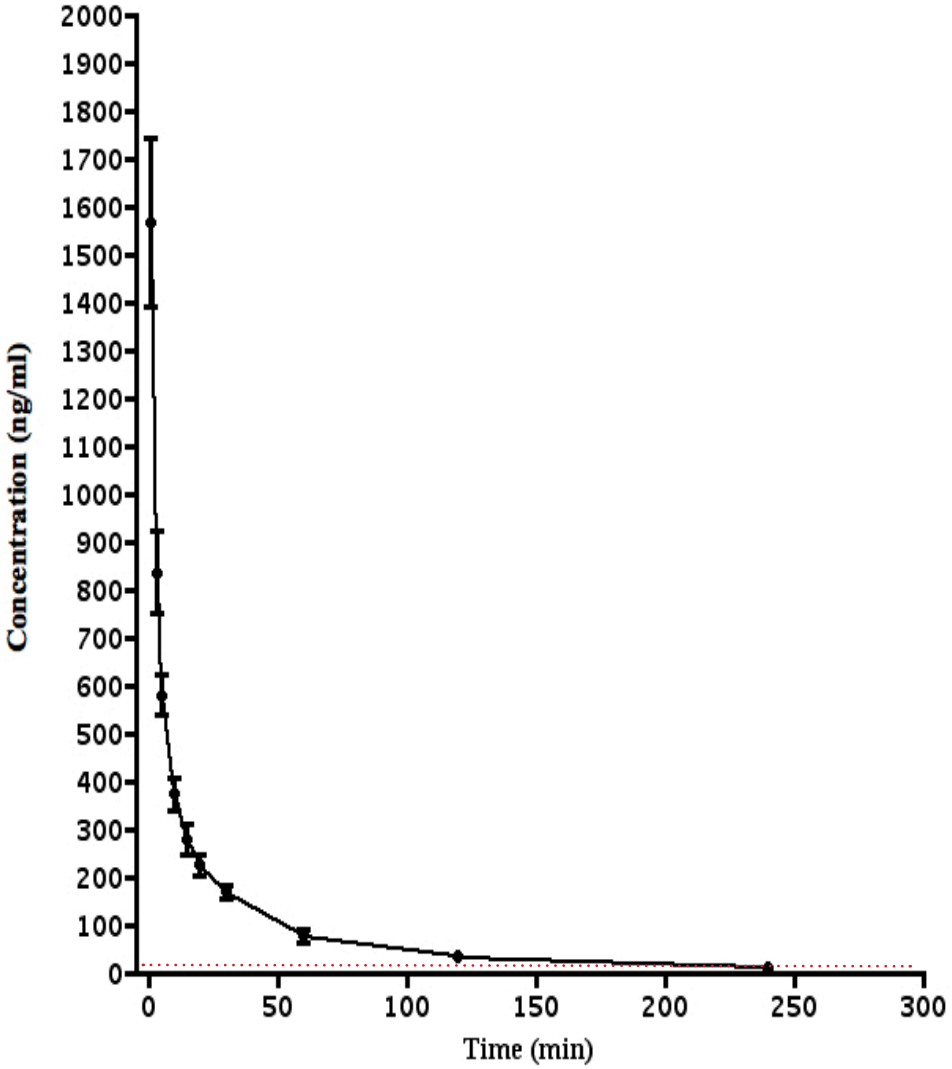

Figure 14: Concentration time curve for morphine after intravenous administration at 2 mg/kg in broiler chickens. Each data point represents mean of 9 chickens, and a total of 27 chickens were used for the whole study. The red dotted line indicates the minimum effective plasma concentration of morphine required to maintain analgesia in humans.

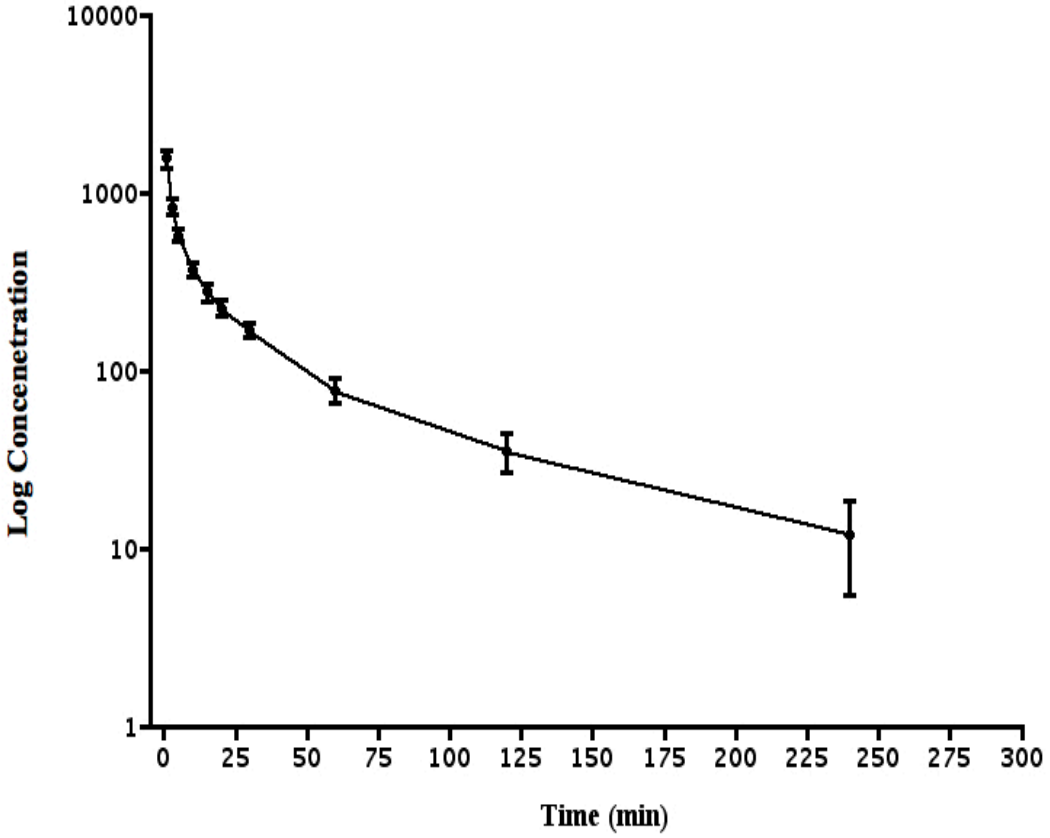

Figure 15: Semi-log plot of concentration time curve for morphine after intravenous administration at 2 mg/kg in broiler chickens. Each data point represents mean of 9 chickens, and a total of 27 chickens were used for the whole study.

Parameters	Units	Mean
Noncompartmental		
$AUC_{(0-\infty)}$	ng.min/mL	24187.4
$AUC_{(0-t)}$	ng.min/mL	22999.9
$AUMC_{(0-\infty)}$	$ng.min^2/mL$	1365612
Vd_{area}	mL/Kg	8125.8
Cl	mL/min/Kg	82.68
$T_{1/2\lambda z}$	minutes	68.1
$MRT_{\lambda z}$	minutes	98.3
Compartmental		
A_1	ng/mL	909.77
A_2	ng/mL	134.22
α	1/min	0.096
β	1/min	0.010
k_{21}	1/min	0.021
k_{12}	1/min	0.039
k_{10}	1/min	0.046
$T_{1/2\alpha}$	minutes	7.21
$T_{1/2\beta}$	minutes	69.3
$Vd_{(central)}$	mL/Kg	1915.72
Vd_{ss}	mL/Kg	5459.80
$Vd_{(peripheral)}$	mL/Kg	3544.08
Cl	mL/min/Kg	54.59

A_1 and A_2 are mathematical constants, α distribution rate constant, β elimination rate constant, k_{21}, k_{12}, k_{10} are the microconstants, $T_{1/2\alpha}$ distribution half-life, $T_{1/2\beta}$ elimination half-life, $T_{1/2\lambda z}$ half-life of terminal phase.

Table 2: Compartmental and noncompartmental analysis of morphine in broiler chickens.
Morphine was injected at 2 mg/kg intravenously. These pharmacokinetic parameters were calculated from the mean of nine concentration time curves from the mean pool samples.

Species	Dose (mg/kg)	$T_{1/2}$ (min)	*Vd* (L/kg)	*Cl* (mL/min/kg)	Authors
Chickens	2	68.1	8.12	82.6	This study
Humans	10*	174	3.2	14.7	(Stanskl et al., 1979)
Dogs	0.5	94.9	7.2	85.2	(Barnhart et al., 2000)
	0.5	69.6	5.62	62.46	(Kukanich., 2005b)
Horses	0.1	87.9	---	---	(Combie et al., 1983)
Cats	0.2	76.3	2.6	24.1	(Taylor et al., 2001)

Table 3: Comparative pharmacokinetics of morphine in various species.

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2.2 PHARMACOKINETICS OF BUTORPHANOL IN BROILER CHICKENS

ABSTRACT

The pharmacokinetics of butorphanol in broiler chickens was investigated in this study. Butorphanol tartrate was injected intravenously at 2 mg/kg in 18 healthy broiler chickens. A simple and highly sensitive HPLC-DAD method was developed to analyse butorphanol in chicken plasma samples. The plasma samples were prepared by a solid phase extraction (SPE) method using Phenomenex Strata X Reversed Phase SPE cartridges. The extraction efficiencies were more than 100%. The analytical column used was Phenomenex C-18(2) (150 X 4.6 mm I.d, 5μm particle size). The mobile phase consisted of phosphate buffer: acetonitrile (80:20) at 1mL/min flow rate. The DAD detector was set at 202 nm wavelength. The lower limit of quantification (LOQ) was 3.9 ng/mL and 15.62 ng/mL in mobile phase and plasma, respectively. The correlation coefficient for the standard curve was 0.9985 and 0.9995 in mobile phase and plasma, respectively. The assay was precise and reproducible with excellent within-day and between-day variations as low as 0.02%. The higher precision and recoveries along with the simplicity of this method makes it suitable for the study of pharmacokinetics of butorphanol in chickens and other avian species.

The pharmacokinetic parameters were calculated both by compartmental and noncompartmental methods. The $T_{1/2\lambda}$(minutes), *Cl* (mL/min/kg) and *Vd* (mL/kg) were 71.3, 67.59 and 6.9, respectively. In compartmental analysis, the terminal half life $T_{1/2\beta}$ (min), $Vd_{(central)}$ (mL/kg) and *Cl* (mL/min/kg) was 69.3, 74.64 and 7.4, respectively. The concentration time curve fitted a two compartmental model well. The micro rate constants k_{21}, k_{12} and k_{10} were 0.034, 0.050 and 0.029, respectively. Butorphanol was well distributed in chickens with rapid clearance and was detectable for only two hours in broiler chickens. Butorphanol could provide analgesia for short duration after I/V administration, which needs to be evaluated further.

2.2.1 INTRODUCTION

2.2.1a PHARMACOKINETICS OF BUTORPHANOL IN BROILER CHICKENS

Butorphanol tartrate is a μ receptor antagonist and κ receptor agonist, in contrast to morphine, which is a μ receptor agonist, but structurally these two opioid drugs are quite similar. Clinical trials on humans, horses and dogs have shown that butorphanol can be an effective and safe analgesic (Orsini, 1988), but dysphoria is common in mammals and has limited its use in people. It is widely used clinically in birds but the scientific justification is limited.

Pharmacokinetic studies on humans have demonstrated that the plasma levels were 1.0 to 1.5 ng/mL, 15 to 30 minutes after I/V administration at a dose of 1 mg/kg (Gaver et al., 1980b; Pittman et al., 1980) and metabolised extensively in liver by N-dealkylation and hydroxylation. Elimination was mostly through the kidney but a small fraction was eliminated in the bile also.

In New Zealand white rabbits, at the dose of 0.5 mg/kg, administered S/C and I/V, the peak plasma concentration was 60 to 78 ng/mL after 25 minutes (Portnoy and Hustead, 1992). They described a two-compartment model for disposition of butorphanol in rabbits. The elimination half-life was 1.64 hours. The volume of distribution and clearance was 10.755 L/kg and 75.458 mL/kg/min, respectively.

In dogs, Pfeffer et al. (1980) described the pharmacokinetics of butorphanol after I/M and S/C administration at 0.25 mg/kg body weight. Serum levels of butorphanol were analysed by gas chromatography and the mean peak serum concentration was 29 ng/mL at mean times of 28 and 40 minutes after S/C and I/M injection. There was no significant difference in the pharmacokinetic parameters between S/C and I/M routes of administration. The mean serum half-life, clearance and volume of distribution were 1.62 hours, 3.45 L/kg/hr and 7.96 L/kg, respectively.

In a pharmacokinetic trial of butorphanol in horses (Sellon et al., 2001), butorphanol tartrate was injected as single I/V injection at the dose rate of 0.1 to 0.13 mg/kg and a continuous I/V infusion with loading dose of 17.8 μg/kg and infusion dose of 23.7 μg/kg/hour for 24 hours. The plasma levels of the drug were analysed by HPLC with electrochemical detection. The peak

mean plasma concentration of butorphanol after single I/V dose was 24.8 ng/mL and with continuous I/V infusion was 26.3 ng/mL all the time during infusion. The mean systemic clearance, volume of distribution and terminal half life after single I/V and continuous I/V infusion were 21; 18.5 mL/kg/min, 1.0; 1.1 L/kg, and 44; 34 minutes, respectively.

In llamas (*Lama glama*), the mean peak plasma concentration of butorphanol after I/V and I/M injection were 94.8 and 34.3 ng/mL (Carroll et al., 2001). There was a significant difference in the elimination half-life after I/V (15.9 minutes) and I/M (66.8 minutes). The significantly shorter half-life after I/V administration limits the clinical use of butorphanol for analgesia in lamas.

There is a single study reporting pharmacokinetics of butorphanol in red tailed hawks (*Buteo jamaicensis*) and great horned owls (*Bubo virginianus*) (Riggs et al., 2008). The best fit for butorphanol plasma concentrations was a two (red tailed hawks) and one compartmental model (great horned owls) after 0.5 mg/kg, I/V and I/M administration. The elimination half-life was 0.94 and 1.79 hours in red tailed hawks and great horned owls, respectively. The clearance (L/h/kg) and volume of distribution (L/kg) at steady state was 3.4 and 2.8, for red tailed hawks and 1.5 and 2.06 for great horned owls, respectively. After I/M administration, the C_{max} (ng/ml), T_{max} (h), $T_{1/2}$ (h) was 154.4, 0.150, 0.94 and 229.9, 0.181, 1.84 for red tailed hawks and great horned owls, respectively. The clearance of great horned owls was significantly lower than red tailed hawks with higher volume of distributions, in both species. Owing to the shorter half-life, it should be administered more frequently to maintain the therapeutic levels, though there was no pharmacodynamic data given in this study.

As there is no published pharmacokinetic study of butorphanol in chickens, it was required to evaluate the pharmacokinetics of butorphanol in broiler chickens. This study would further help in determining the dose rate and regimen for the pharmacodynamic experiments. The pharmacokinetic parameters obtained from chickens could also be used to formulate the dosing regimen of butorphanol in endangered bird species. Exptrapolating pharmacokinetic parameters from one bird species to another has been shown to be more accurate than using mammalian data (Hunter et al., 2008). The data generated from this study should allow more rational

use of an analgesic drug in birds in which it will never be possible to carry out pharmacokinetic studies, such as critically endangered avian species. In our wild life ward, we get certain species eg kakapos, for which no animal ethics committee will grant permission to conduct any experiment as there are only 122 kakapos left. Therefore, it very important to use chickens or other easily available avian species as a model of drug research to extrapolate the pharmacokinetic parameters as accurately as possible. As already stated in the introduction, extrapolation from mammalian to avian species generates higher percent error as compared to the within avian to avian extrapolation.

2.2.1b HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR BUTORPHANOL ANALYSIS

There are various methods published for the analysis of butorphanol in plasma using HPLC. Chang and co-workers (Chang et al., 1999), used HPLC with a diode array detector (DAD) for the analysis of stability of butorphanol in aqueous and oil based suspensions. They used a μBondpak Phenyl column (3.9 x 300mm) coupled with DAD detector at 280 nm wavelength. The limit of detection (LOD) for this method was only 5 μg/mL, which is enough for detecting butorphanol concentration in its formulations. In order to analyse butorphanol concentration in plasma, they used a more sensitive electrochemical detection method with an ODS-AQ YMC column (250 x 4.6 mm). The LOD was 1 ng/mL and the linearity varied from 1-533 ng/mL. Sellon et al. (2001) determined butorphanol concentrations in horse plasma using HPLC with ED. The column used in this study was Zorbax SB C8 (4.6 x 150 mm). The limit of quantification (LOQ) in plasma was 7.8 ng/mL. The precision and accuracy varied from 6.2 to 9.2 and 7.0 to 8.8%, respectively. Boulton et al. (2002) validated a LC/MS method for butorphanol analysis in human plasma. This method was highly sensitive with LOQ of only 137 pg/mL and the within and between days precision ranged from 3.9 to 5.4 and 2.7 to 6.3%, respectively. Though these methods are sensitive enough to detect low levels of butorphanol in plasma, they use LC/MS and electrochemical detectors, which are expensive and delicate compared to a DAD. The DAD is the most commonly used detector in pharmaceutical research (Stoev and Michailova, 2004). The plasma samples for these detection methods were prepared either by liquid-liquid extraction (Chang et al., 1999) or solid phase extraction (Sellon et al., 2001; Boulton et al., 2002). The percentage recovery was reported only by Boulton and co-workers at 84.4%. The amount of plasma used for these methods was 0.5 and 1 mL for Chang and Sellon, and Boulton, respectively. The minimum volume of a sample required for analysis is higher which makes it impossible to follow these methods in case of smaller avian species where sample volume is a limitation.

This study aimed to develop a simple, inexpensive and sensitive HPLC method for analysis of butorphanol in chicken plasma samples, which could be further applied to study the pharmacokinetics of butorphanol in chickens and other avian species.

2.2.2 EXPERIMENT THREE: A simple and sensitive method for the detection of butorphanol in plasma samples by High Performance Liquid Chromatography with diode array detection.

Reagent and Solutions

Butorphanol injection 10 mg/ml (Lloyd Laboratories, New Zealand) was used as the standard as it does not contain any antioxidant or preservative. The working standard solution was prepared every day in mobile phase. Laboratory reagents used were methanol and acetonitrile (Sigma-Aldrich, New Zealand). Phosphate buffer (0.1 M) was prepared by mixing 187.5 mL of 0.2 M di-sodium phosphate and 312.5 mL of 0.2 M sodium hypophosphate with the volume made to 1 L by Milli-Q water (Milli-Q PFplus system, Millipore Cooperation, USA). Na_2HPO_4 (0.2 M) solution was prepared by mixing 14.15 g of Na_2HPO_4 salt in 500 mL Milli-Q water and 0.2 M $NaHPO_4$ solution was prepared by mixing 11.99 g of $NaHPO_4$ salt in 500 mL of Milli-Q water.

Chromatographic Conditions

The HPLC system consisted of LC-20AD pumps, SIL-20AC HT auto-injector, SPD-M20A diode array detector, CTO-20A column oven, DGU-20A3 Degasser (Shimadzu Japan). The chromatographs were analysed in LC Solutions software (Shimadzu Japan). The analytical column used was a Phenomenex C18(2) (150 x 4.6 mm, 5 μm particle size). The mobile phase consisted of 0.1 M phosphate buffer pH 4.8: acetonitrile (80:20) with flow rate of 1.0 mL/min. The separation was achieved under isocratic conditions at 30ºC. The injection volume was 50 μL and the DAD was set at 202 nm wavelength.

Sample Preparation

The plasma samples were prepared by solid phase extraction using Phenomenex Strata X reversed phase SPE cartridges. The standard solution used to spike the plasma samples was prepared in Milli-Q water. 300 μL of plasma was mixed with 300 μL of the standard solution and 300 μL of concentrated HCl was added and vortex mixed for 1 minute. Then this solution was centrifuged at 3000 rpm for 10 minutes. The supernatant was separated and loaded into the

SPE cartridge preconditioned with one volume of methanol followed by one volume of water. The first wash used 3 mL of water and dried for 2 minutes followed by a second wash with 2 mL of 40% methanol and again the cartridge was dried for 2 minutes. The elution was made with 100% methanol . The sample was dried under a gentle stream of air at 20˚C and was re-constituted with 200 µL of mobile phase. The injection volume was 50 µL and each sample was injected three times in the HPLC system.

Data Analysis

In the chromatograms peak height, area and width were analysed using the software LC solutions (Shimadzu). The standard calibration curve was also processed in the same software.

Validation Protocol

- Lower limit of quantification (LLQ)

The LLQ in mobile phase was measured by using low concentrations of butorphanol (15.6, 7.8, 3.9 and 1.95 ng/mL) diluted in the mobile phase. After determining the LLQ for the mobile phase the same low concentrations were added to drug-free plasma. The lower limit of quantification was set at signal to noise ratio of 10.

- Intra-day and inter-day accuracy and precision

The inter-day accuracy and precision was determined by running standards in mobile phase and in the spiked plasma at four different concentrations (125, 62.5, 31.2 and 15.6 ng/mL) every day for three consecutive days. The intra-day accuracy and precision was done by analysing these four concentrations on each day for three days.

- Linearity

Linearity of the method was performed to check whether the detector response to the drug was linear by running eight different concentrations (500 to 3.9 ng/mL) in the mobile phase and same in plasma. The data thus obtained was subjected to the linear regression in LC solutions software.

- Recovery

The recovery of butorphanol after the solid phase extraction was determined by comparing the area of the peaks for 125, 62.5, 31.2 and 15.6 ng/mL butorphanol concentrations made in the mobile phase with the areas for the same concentrations spiked in the blank plasma.

- Specificity

The blank plasma from 10 different broiler chickens was extracted and analysed to check the specificity of the SPE and HPLC method.

Results

The retention time for butorphanol was 13.8 minutes (figure 16). At this time, there was no peak in plasma interfering with the drug peak (figure 17). The lower limit of quantification (LLQ) of this method was 15.6 ng/mL in plasma (figure 18) and 3.9 ng/mL in the mobile phase (figure 19). The significant difference in the LLQs was due to the higher baseline noise in the plasma as compared to the mobile phase and also the linearity at concentrations lower than 15.62 ng/mL was very poor. The chromatograph obtained at these low concentrations showed good peak resolution.

The detector response was linear from 3.9 to 500 ng/mL in mobile phase and 15.62 to 500 ng/mL in plasma. The correlation coefficients were 0.9985 and 0.9995 for the standard curves in mobile phase and plasma, respectively. The inter-day and intra-day variation for this method in plasma (tables 4 and 5) ranged from 4.23 to 8.80% and 0.51 to 4.04 %, respectively, while in mobile phase (tables 6 and 7) ranged from 2.85 to 7.24 % and 0.02 to 2.70 %, respectively. The highest variation was seen in the lowest concentration, 15.6 ng/mL in plasma and 3.9 ng/mL in mobile phase. The recovery of the SPE method was 98.65, 105, 107.8 and 97.37% for 125, 62.5, 31.2 and 15.6 ng/mL (table 8), respectively. The relative standard deviation (RSD) for the recovery studies ranged from 4.23 to 8.8%. The mean overall recovery of butorphanol for this method of SPE was 102.08%.

Specificity of the method was confirmed by absence of any interfering peak with the butorphanol peak in blank plasma from 10 different broiler chickens.

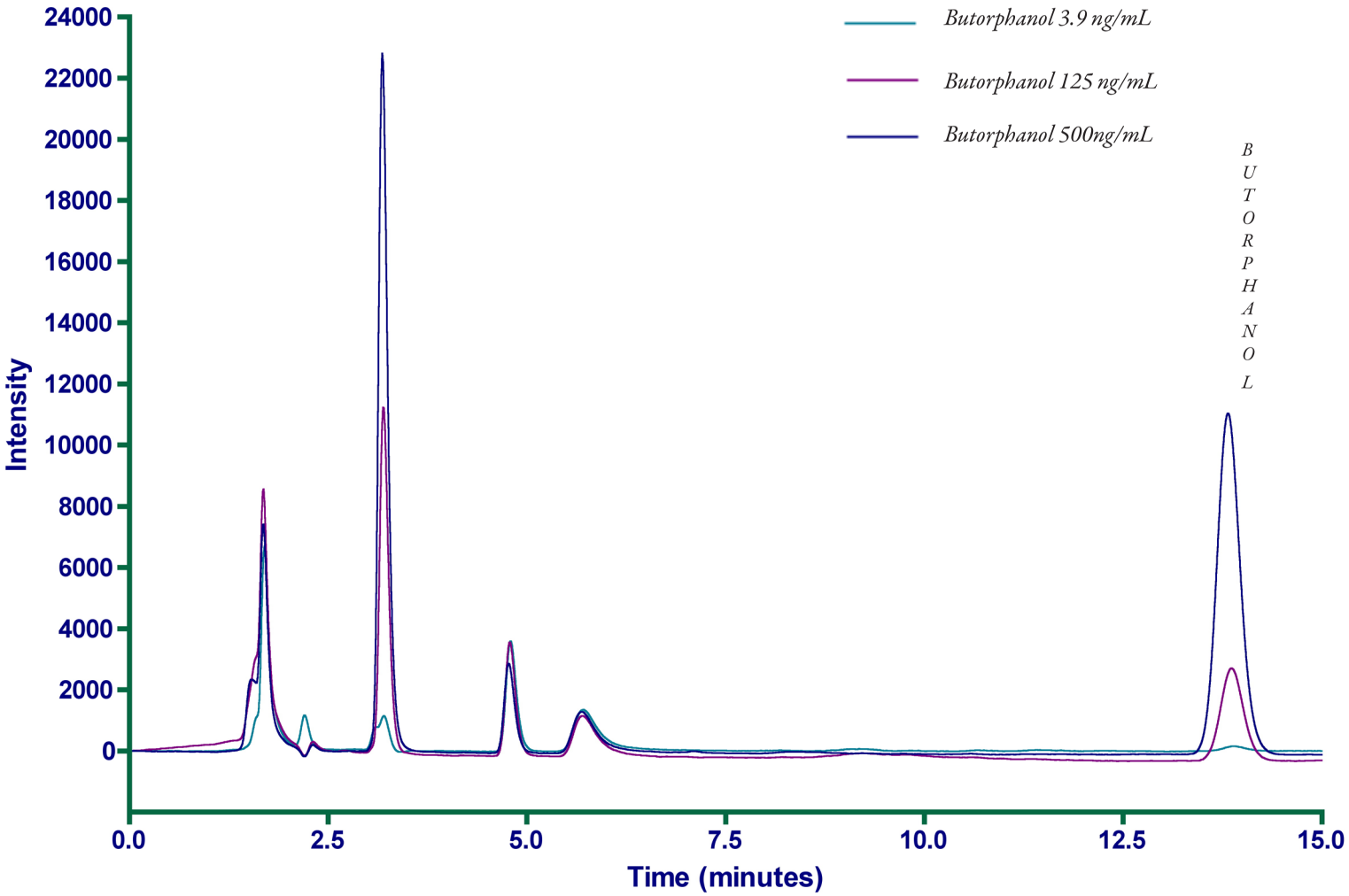

Figure 16: Butorphanol standard at three concentrations 3.9 ng/mL LLOQ , 125 ng/mL and 500 ng/mL, in mobile phase

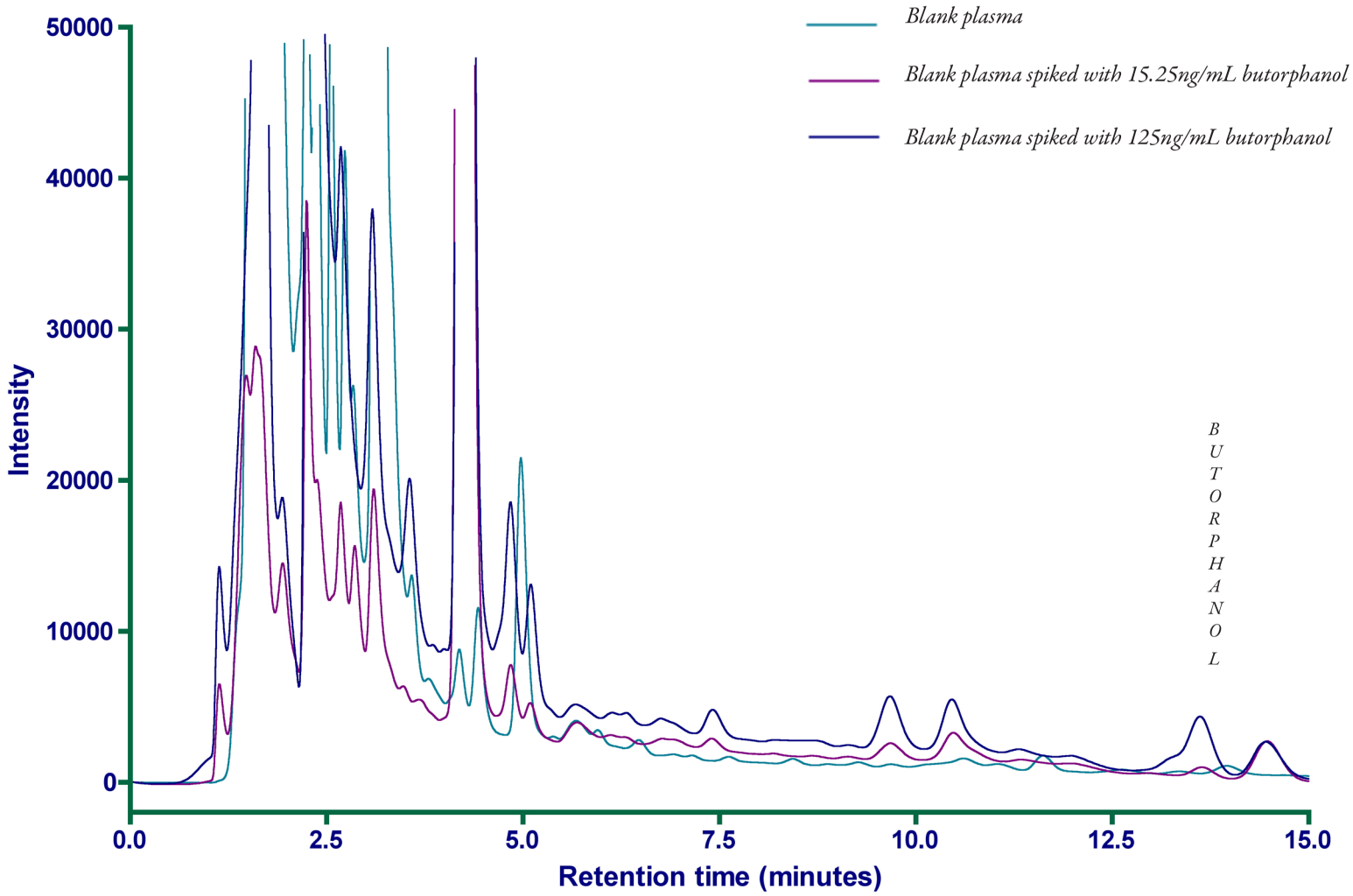

Figure 17: Butoprhanol standard at two different concentrations spiked in blank plasma (a), 15.625 ng/mL (b) and 500 ng/mL (c)

Discussion

The method developed here, was simple, sensitive and inexpensive. The use of a diode array detector makes it an inexpensive method. The DAD is the most commonly used detector in pharmaceutical laboratories. The SPE method described in this current assay uses fewer reagents and solutions, making it simple and easier to follow. The higher recoveries, good precision and accuracy makes this method suitable for application in the pharmacokinetics study of butorphanol in avian species and other animals.

The precision and accuracy of our method is similar to the published LC MS methods, <6.5% (Chang et al., 1999), 6.2 to 9.2% (Sellon et al., 2001) and 2.7 to 6.8% (Boulton et al., 2002). Also the correlation coefficient for the standard curve in plasma was similar to the LCMS method described by Boulton. Though the LLQ for these methods are lower than for our method, with small volume samples we were able to achieve LLQ well below the minimum effective concentration (MEC) using a standard HPLC machine with DAD detection. In our method, the sample volume required for analysis was only 300 μL. The lower sample volume required makes it possible to study the pharmacokinetics of butorphanol in small animals where blood volume to be withdrawn is a limiting factor. The recovery of butorphanol after SPE is better than other described methods at around 80% (Sellon et al., 2001; Boulton et al., 2002). The extraction of drug in the published methods was carried out in alkaline pH as against the acidic pH in our method. Addition of concentrated HCl disrupted the plasma protein and drug binding (Chamberlain, 1986), thus giving higher recoveries. This makes it possible to detect the lower levels of butorphanol with no drug loss during sample preparation. The reversed phase solid phase extraction procedure after the protein precipitation by HCl gave cleaner chromatographs, without any interfering peaks.

Concentration spiked (ng/mL)	125	62.5	31.25	15.625
A1	119.93	61.82	30.39	13.89
A2	121.57	63.11	31.29	13.75
A3	120.28	62.79	29.98	13.83
B1	130.37	66.32	35.79	14.52
B2	130.63	68.59	35.04	15.68
B3	133.53	68.35	35.04	14.79
C1	117.57	69.59	34.07	16.95
C2	118.56	65.40	35.51	16.59
C3	117.46	64.67	35.53	16.89
Mean	123.32	65.63	33.69	15.21
%RSD	5.13	4.23	7.21	8.80
SD	6.38	2.78	2.43	1.34

Table 4: Inter day variation of HPLC method for analysis of butorphanol in plasma. Blank plasma spiked with 125, 62.5, 31.25 and 15.625 ng/mL of Butorphanol for three days. A, B and C represents day 1, 2 and 3 respectively. Three samples were tested each day, represented by the subscript numbers. The mean, percent relative standard deviation (RSD) and standard deviation (SD) were calculated from the actual concentration measured.

Concentration spiked (ng/mL)	125	62.5	31.25	15.625
A_1	119.93	61.82	30.39	13.89
A_2	121.57	63.11	31.29	13.75
A_3	120.28	62.79	29.98	13.83
Mean	120.59	62.57	30.55	13.82
%RSD	0.71	1.05	2.16	0.52
SD	0.86	0.66	0.66	0.07
B_1	130.37	66.32	35.79	14.52
B_2	130.63	68.59	35.04	15.68
B_3	133.53	68.35	35.04	14.79
Mean	130.37	67.75	35.49	15.00
%RSD	1.33	1.83	1.09	4.04
SD	1.75	1.24	0.39	0.60
C_1	117.57	69.59	34.07	16.95
C_2	118.56	65.40	35.51	16.59
C_3	117.46	64.67	35.53	16.89
Mean	117.86	66.55	35.03	16.81
%RSD	0.51	3.98	2.36	1.15
SD	0.60	2.65	0.83	0.19

Table 5: Intra day variation of HPLC method for analysis of butorphanol in plasma. Blank plasma spiked with 125, 62.5, 31.25 and 15.625 ng/mL of Butorphanol for three days. A, B and C represents day 1, 2 and 3 respectively. Three samples were tested each day, represented by the subscript numbers. The mean, percent relative standard deviation (RSD) and standard deviation (SD) were calculated from the actual concentration measured.

Concentration (ng/mL)	125		62.5		31.25		15.625	
	Area	Height	Area	Height	Area	Height	Area	Height
A_1	105100	5146	55196	2710	26974	1331	13227	662
A_2	105097	5159	54533	2697	27407	1347	13357	666
A_3	----	----	54575	2697	26618	1322	12847	660
B1	101772	4998	52049	2563	27652	1374	14053	719
B2	101760	4979	51894	2556	27676	1377	13609	696
B3	101869	4992	51764	2557	28385	1393	14362	714
C1	110392	5409	55511	2725	29964	1505	15463	779
C2	110309	5404	55443	2724	29820	1499	15997	786
C3	110162	5406	54972	2700	29933	1495	15322	778
Mean	105807.6	5186.6	53993.0	2658.7	28269.8	1404.7	14248.5	717.7
%RSD	3.73	3.74	2.97	2.85	4.66	5.31	7.82	7.24
SD	3953.79	194.13	1604.53	75.81	1319.99	74.62	1115.07	52.02

Table 6: Inter day variation of HPLC method for analysis of butoprharnol in mobile phase. Four different concentrations of butorphanol were made in mobile phase. A, B and C represents day 1. 2 and 3 respectively. Three samples were tested each day represented by subscript numbers. The area and height for each peak was used to calculate the mean, percent relative standard deviation (RSD) and standard deviation (SD).

Concentration (ng/mL)	125		62.5		31.25		15.625	
	Area	Height	Area	Height	Area	Height	Area	Height
A_1	105100	5146	55196	2710	26974	1331	13227	662
A_2	105097	5159	54533	2697	27407	1347	13357	666
A_3	-----	-----	54575	2697	26618	1322	12847	660
Mean	105098.5	5152.5	54768.0	2701.3	26999.6	1333.3	13143.6	662.6
%RSD	0.02	0.17	0.60	0.27	1.40	0.94	2.01	0.46
SD	2.12	9.19	371.25	7.50	395.12	12.66	265.01	3.05
B_1	101772	4998	52049	2563	27652	1374	14053	719
B_2	101760	4979	51894	2556	27676	1377	13609	696
B_3	101869	4992	51764	2557	28385	1393	14362	714
Mean	101800.3	4989.6	51902.3	2558.6	27904.3	1381.3	14008.0	709.6
%RSD	0.05	0.19	0.27	0.14	1.49	0.73	2.70	1.70
SD	59.76	9.71	142.68	3.78	416.44	10.21	378.51	12.09
C_1	110392	5409	55511	2725	29964	1505	15463	779
C_2	110309	5404	55443	2724	29820	1499	15997	786
C_3	110162	5406	54972	2700	29933	1495	15322	778
Mean	110287.6	5406.3	55308.6	2716.3	29905.6	1499.6	15594.0	781.0
%RSD	0.10	0.04	0.53	0.52	0.25	0.33	2.28	0.55
SD	116.47	2.51	293.53	14.15	75.79	5.03	356.05	4.35

Table 7: Intra day variation of HPLC method for analysis of butoprharnol in mobile phase. Four different concentrations of butorphanol were made in mobile phase. A, B and C represents day 1. 2 and 3 respectively. Three samples were tested each day represented by subscript numbers. The area and height for each peak was used to calculate the mean, percent relative standard deviation (RSD) and standard deviation (SD).

Concentration spiked (ng/mL)	Concentration measured (ng/mL)	% Recovery	Standard Deviation	% RSD
125	123.32	98.65	6.38	5.13
62.5	65.63	105.00	2.78	4.23
31.25	33.69	107.80	2.43	7.21
15.62	15.21	97.37	1.34	8.80

Table 8: Recovery of butorphanol after sample preparation by solid phase extraction (n=9)

2.2.3 EXPERIMENT FOUR: Pharmacokinetics of butorphanol after intravenous administration in broiler chickens (pilot study)

Study Design

This pilot experiment was carried out on eight broiler chickens with an average weight of 2 kg. These chickens were divided into four groups of two chickens each. A medial metatarsal vein of each broiler chicken was catheterised with a 22 G catheter under halothane anaesthesia. The birds were allowed to recover from the effect of anaesthesia before the injection of drugs. The birds were kept in groups under standard conditions of housing and management. They were fed *ad-lib.* with commercially available broiler feed with a 24 hour supply of fresh drinking water.

Drug Administration

Butorphanol tartrate (10 mg/mL, Llyod Laboratories, New Zealand) was injected I/V into the wing vein (*vena basilica*) at 0.5 mg/kg body weight using a 22 G needle.

Sample Collection

Four serial blood samples were collected from four different broiler chickens receiving each treatment, resulting in a total of 16 samples. The blood samples (2 mL) were withdrawn from the catheterised vein of each broiler chicken in a heparinised vial at 0, 5, 10, 20 min (from 1st group), 0.5, 1, 2, 3 hours, (2nd group) 4, 6, 8, 10 hours (3rd group), 12, 24, 36 and 48 hours (4th group) after the injection of butorphanol. The total amount of blood withdrawn from each bird was 8 mL, which is 4% of the total blood volume of a 2 kg chicken. The vials were kept chilled immediately after collection and centrifuged at 2000 rpm for 10 minutes. Plasma was pipetted out and kept at –70 °C until the day of analysis.

Sample Analysis

The plasma samples were analysed by High Performance Liquid Chromatography (HPLC). The HPLC assay and sample preparation technique for butorphanol analysis has already been described in section 2.2.2.

Results

Butorphanol was detected until 5 minutes post injection and the concentration at this time was 56 ng/mL.

The results of the pilot study of butorphanol pharmacokinetics were similar to the morphine study. Both drugs were eliminated quickly by chickens. Therefore, we decided to inject butorphanol at much higher dose rate, and frequency of sampling was increased in the second experiment to get more data points for the concentration time curve.

2.2.4 EXPERIMENT FIVE: Pharmacokinetics of butorphanol after intravenous administration in broiler chickens.

Study Design

This study was conducted on 18 healthy broiler chickens with an average body weight of 2 kg divided into three groups of six chickens in each. A medial metatarsal vein of each broiler chicken was catheterised with a 22 G catheter under halothane anaesthesia. The birds were allowed to recover from the effect of anaesthesia before the injection of drugs. The birds were kept in similar conditions as in the pilot study.

Drug Administration

Butorphanol tartrate (10 mg/mL, Lloyd Laboratories, New Zealand) was injected intravenously into the contralateral medial metatarsal vein at the dose rate of 2 mg/kg body weight using a 23 G needle.

Sample Collection

Four serial blood samples of 2 mL each were taken at 0, 1, 3 and 5 minutes from group 1; 10, 15, 20 and 30 minutes from group 2; 1, 2, 4 and 6 hours from group 3 respectively. The samples were treated in the same way as described in the pilot study.

Sample Analysis

The plasma samples were analysed following the same High Performance Liquid Chromatography method as in the pilot study.

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated using standard equations in a spreadsheet. These included half life of the terminal phase ($T_{1/2\,\lambda z}$), area under the curve extrapolated from time zero to infinity ($AUC_{0-\infty}$), area under the moment curve extrapolated from time zero to infinity ($AUMC_{0-\infty}$), volume of distribution (*Vd* mL/kg), clearance (*Cl* mL/min/kg) and mean residence time (*MRT* min).

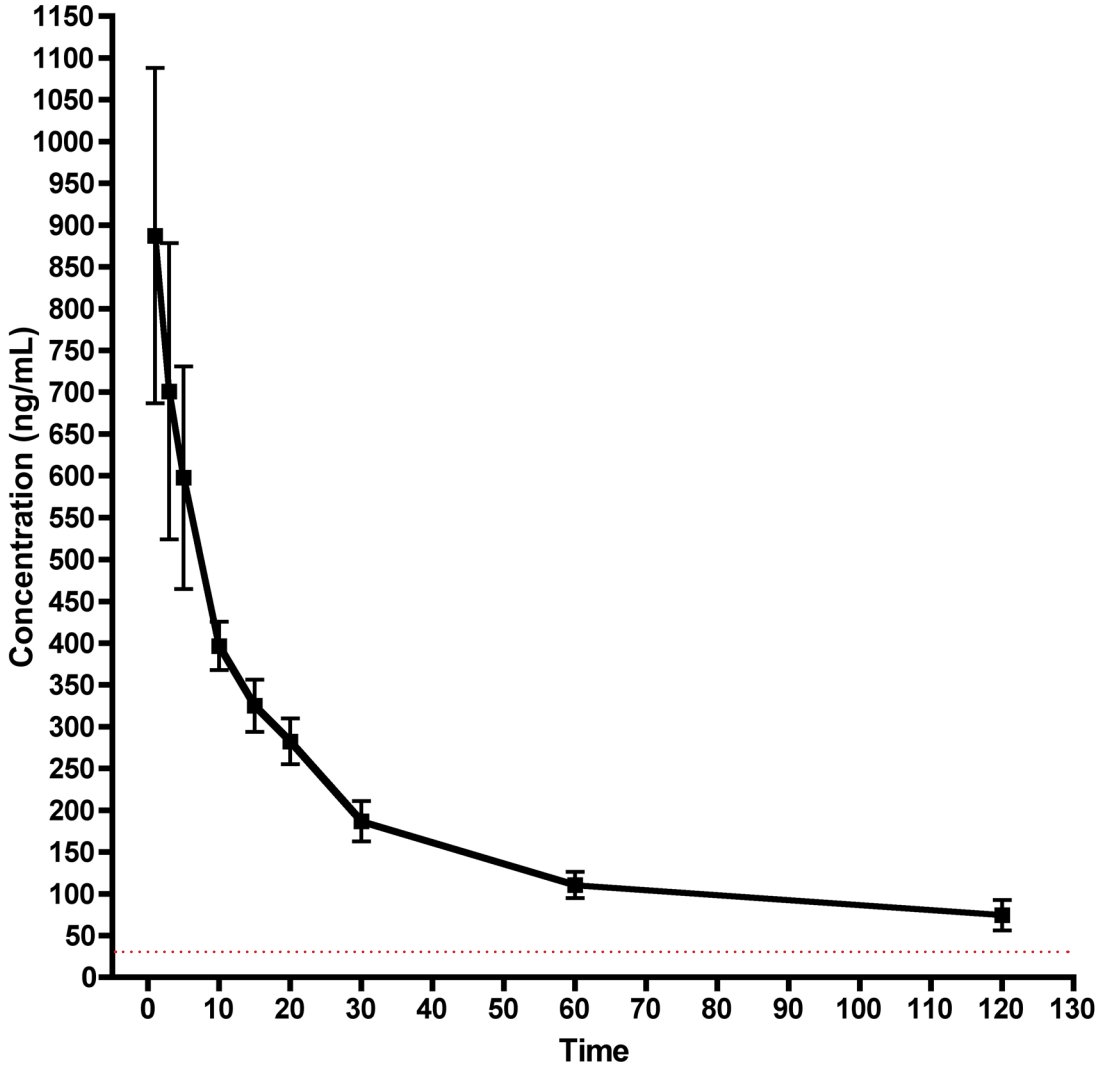

Figure 18: Plasma concentration time curve for butorphanol after intravenous administration at 2 mg/kg dose rate in broiler chickens. Each data point represents mean of 6 chickens, and a total of 18 chickens were used for the whole study. Red dotted line represents the minimum effective plasma concentration required to maintain analgesia in horses.

The noncompartmental pharmacokinetics were calculated from the mean of pooled data. The samples from each of the three groups at various time points were pooled to give one mean concentration time curve (Gagnon and Peterson, 1998) (Cheung et al., 2005). All the compartmental parameters were calculated by the equations described in chapter 1.

Results

The plasma concentration versus time curve and its semi-log plot are shown in figures 18 and 19, respectively. The two compartmental model best fitted the butorphanol plasma concentrations in chickens. Butorphanol was detectable only for 2 hours after a 2 mg/kg I/V dose in chickens. The concentration at 2 hours time point was 78. 37 ng/mL, though the LOD of our method was 15.625 ng/mL. The maximum concentration achieved was 876.1 ng/mL. 63.36% of the drug was cleared from plasma in first 15 minutes, showing the rapid distribution of butorphanol in chickens. The volume of distribution was high at 6.9 L/kg. The clearance was 82.68 mL/min/kg, half life was 71.3 minutes and mean resident time was 112.5 minutes. The pharmacokinetic parameters calculated after compartmental and noncompartmental analysis are shown in table 9.

Discussion

Butorphanol is an opiate having mixed actions. It antagonises the actions of μ opioid receptors while it acts as a κ receptor agonist (Orsini, 1988). Since birds have a higher number of κ opioid receptors compared to μ and δ (Reiner et al., 1989), butorphanol is regarded as the drug of choice for treatment of pain in avian species (Paul-Murphy and Fialkowski 2001). Injection of butorphanol at 0.5 mg/kg S/C alleviated pain in lame turkeys and improved their walking ability (Buchwalder and Huber-Eicher, 2005). Paul-Murphy and her group have demonstrated the analgesic efficacy of butorphanol in various avian species (Paul-Murphy et al., 1999; Sladky et al., 2006; Paul-Murphy et al., 2009) They have also analysed the serum concentrations of butorphanol in parrots (Sladky et al., 2006) but used ELISA for analysis, which is not a very sensitive nor specific method for butorphanol analysis. Also, their study was not designed to calculate the pharmacokinetics. They found good correlation between the serum concentrations and butorphanol analgesia. I/M injection of butorphanol at 1 or 2 mg/kg had analgesic effects

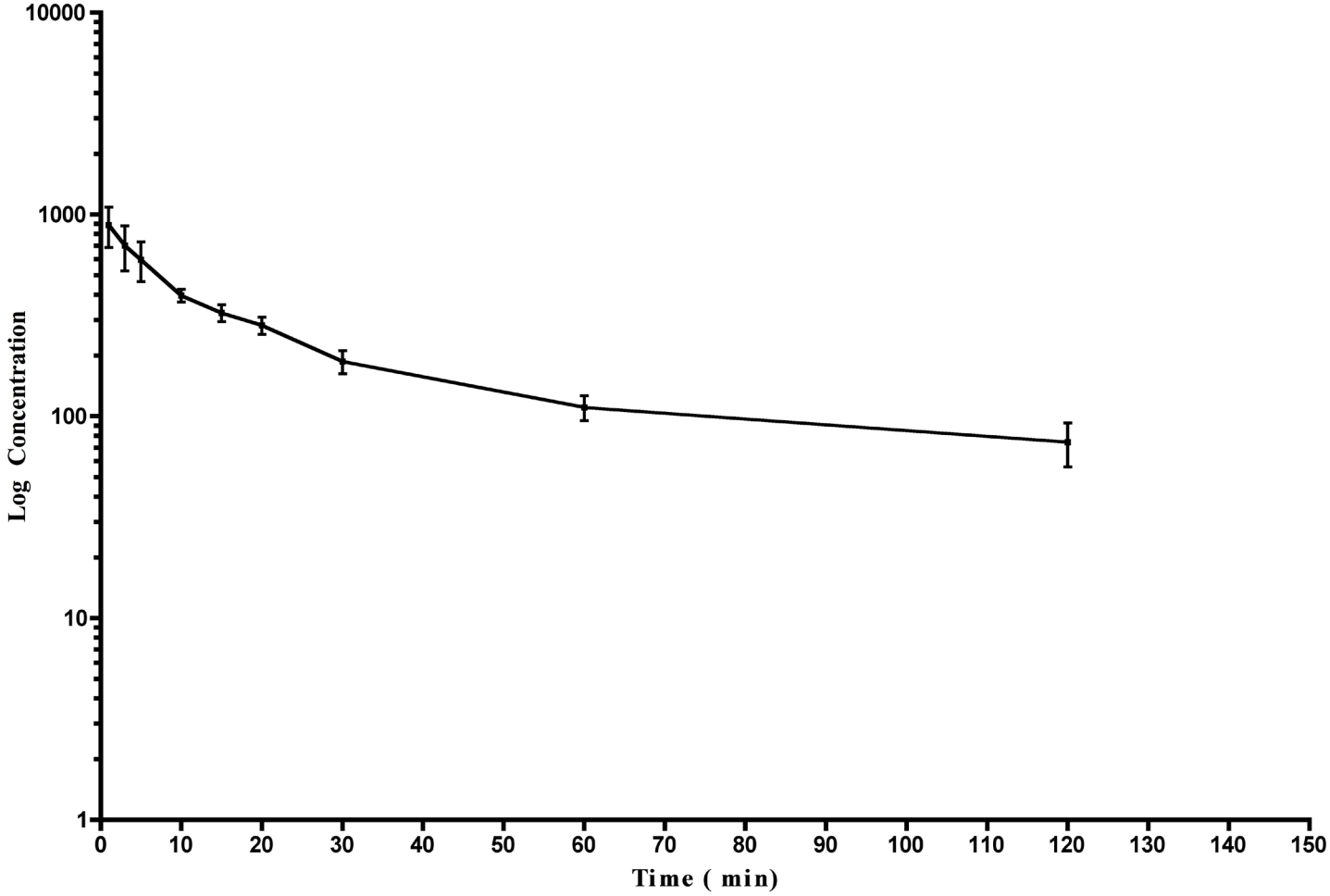

Figure 19: Semi-log plot of concentration time curve for butorphanol after intravenous administration at 2 mg/kg dose rate in broiler chickens. Each data point represents mean of 6 chickens, and a total of 18 chickens were used for the whole study.

in African grey parrots but that effect did not last longer than 2 hours (Paul-Murphy et al., 1999). The previous studies depicting the analgesic effects of butorphanol and the lack of extensive pharmacokinetic studies prompted us to conduct the pharmacokinetics trial before testing the efficacy, which will be discussed in later chapters.

The comparison of our results to various pharmacokinetic parameters in different species published to date is given in table 10. The results of pharmacokinetics studies on chickens revealed that butorphanol has extensive tissue distribution, which is typical for opiates. High volumes of distribution for butorphanol and other opioids like morphine were seen in chickens, in our first experiment, and are comparable to the results of studies in other animals such as horses (Sellon et al., 2001) and humans (Gaver et al., 1980a). Higher volume of distributions were also seen in hawks and owls for butorphanol (Riggs et al., 2008). It seems that, like mammals, birds also have high tissue distribution for opiates. The clearance of butorphanol was also higher in our study, which is similar to the clearance in hawks and owls. High clearance was also seen in the pharmacokinetic studies of variety of drugs in birds (Baert and De Backer, 2002; Graham et al., 2005), suggested to be due to the higher metabolic rates in birds as compared to mammals. The half life of butorphanol was shorter in chickens than in humans, dogs, cow, rabbits and owls while in hawks, lamas and horses it was much shorter than chickens. The clearance of butorphanol was much higher in chickens than previously described for any species (table 10).

The pharmacokinetic parameters from our study and those of Riggs et al. (2008) in red tailed hawks and great horned owls shows that butorphanol is well distributed and rapidly cleared from the circulation in birds. However, significant differences occur between bird species in the pharmacokinetics of butorphanol. The *AUC* after 0.5 mg/kg I/V in hawks and owls was 156.2 and 377.5 ng.h/mL, respectively, while in chickens it was 493.16 ng.h/mL after a 2 mg/kg I/V dose. When the chickens were injected at 0.5 mg/kg I/V. dose rate, (in our pilot study) the butorphanol was detectable only for less than 10 minutes. Riggs et al. (2008) in their study have found that the analgesic levels of butorphanol similar to those in humans were maintained for 4 hours, which is highly unlikely in case of chickens. Sellong et al. (2001), found that the therapeutic plasma concentration of butorphanol in horses ranged from 20-30 ng/mL. These analgesic levels were maintained in broiler chickens after a dose of 2 mg/kg for only two hours.

This prediction required confirmation in a pharmacodynamic study, which will be discussed in later chapters.

Parameters	Units	Mean
Noncompartmental		
$AUC_{(0-\infty)}$	ng.min/mL	29589.9
$AUC_{(0-t)}$	ng.min/mL	21939.5
$AUMC_{(0-\infty)}$	ng.min^2/mL	2486266.7
Vd_{area}	mL/Kg	6952.5
Cl	mL/min/Kg	67.59
$T_{1/2\lambda z}$	minutes	71.3
$MRT_{\lambda z}$	minutes	112.5
Compartmental		
A_1	ng/mL	648.18
A_2	ng/mL	227.94
α	1/min	0.103
β	1/min	0.010
k_{21}	1/min	0.034
k_{12}	1/min	0.050
k_{10}	1/min	0.029
$T_{1/2\alpha}$	minutes	6.72
$T_{1/2\beta}$	minutes	69.3
$Vd_{(central)}$	mL/Kg	2282.79
Vd_{ss}	mL/Kg	5638.4
$Vd_{(peripheral)}$	mL/Kg	3355.70
Cl	mL/min/Kg	56.38

A_1 and A_2 are mathematical constants, α distribution rate constant, β elimination rate constant, k_{21}, k_{12}, k_{10} are the microconstants, $T_{1/2\alpha}$ distribution half-life, $T_{1/2\beta}$ elimination half-life, $T_{1/2\lambda z}$ half-life of terminal phase.

Table 9: Compartmental and noncompartmental pharmacokinetic parameters for butorphanol in broiler chickens after intravenous administration at 2 mg/kg dose rate. These pharmacokinetic parameters were calculated from the mean of six concentration time curves from the mean pool samples.

Species	Dose (mg/kg)	$T_{1/2}$ (min)	*Vd* (L/kg)	*Cl* (mL/min/kg)	Authors
Chickens	2	71.3	6.93	67.45	This study
Humans	1 mg (total dose)	262.2	1.7*	7.6*	(Shyu et al., 1994)
Dogs	0.05	172.12	27.58	137.87	(Vaughan 2003)
Horses	0.1	44.4	1.03	21	(Sellon et al., 2001)
Lamas	0.1	15.9	0.822	50	(Carroll et al., 2001)
Cows	0.25	82	1.14	34.6	(Court et al., 1992)
Rabbits	0.5	98.4	10.76	75.46	(Portnoy and Hustead.,1992)
Hawks	0.5	56.4	2.89	58.03	(Riggs et al., 2008)
Owls	0.5	107.4	2.06	26.10	

Table 10: Comparative Pharmacokinetics of Butorphanol after intravenous administration in various species

* Calculations based on average human body weight as 65 kg

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CHAPTER THREE

Pharmacokinetics of nonsteroidal antiinflammatory drugs in broiler chickens

3.1 PHARMACOKINETICS OF ASPIRIN AND SALICYLIC ACID IN BROILER CHICKENS

ABSTRACT

This experiment was designed to describe the pharmacokinetics of aspirin and salicylic acid in broiler chickens. The drugs were injected I/V at 50 mg/kg in 18 healthy broiler chickens. The pharmacokinetic parameters were calculated both by compartmental and noncompartmental analysis on mean pooled data. Concentration time curves for aspirin fitted best a single compartment open model. The half life of aspirin was 12 minutes and it was hydrolysed to salicylic acid within 1 hour of injection. Salicylic acid concentration increased gradually after aspirin administration and its half life was 4 hours after aspirin administration.

The decline in plasma concentration for salicylic acid after its I/V administration at 50 mg/kg was best described by a two compartment open model. The $T_{1/2}$ terminal was 2.9 hours, *Vd area* was 251.7 mL/kg and *Cl* was 60.6 mL/min/Kg. The micro rate constants k_{21}, k_{12} and k_{10} were 0.318, 0.007 and 0.263, respectively. Aspirin and salicylic acid had a lower volume of distribution compared to opioids in chickens. Salicylic acid remained above the minimum effective concentration in mammals for four hours in plasma and thus may be an effective analgesic agent in broiler chickens.

3.1. INTRODUCTION

Aspirin (acetyl salicylic acid) is one of the most popular and widely used traditional NSAIDs in the world. It was first discovered by the German chemist Felix Hoffman (Vane and Botting, 2003). He chemically synthesised aspirin from salicylic acid by acetylation. Salicylic acid's use as an analgesic drug goes back to 400 BC when Hippocrates used to recommend chewing willow tree bark for pain relief (Andermann, 1996). Willow bark is rich in salicin and salicylic acid is its major metabolite, along with salicyluric acid and gentisic acid as minor metabolites (Schmid et al., 2001). Though aspirin and salicylic acid were widely used as pain relief agents for many years, the mechanism of action was first proposed by Vane in 1971. He proposed that aspirin inhibits the action of COX enzymes and thus inhibiting the formation of various inflammatory mediators. The detailed explanation for mechanism of action of NSAIDs has already been given in chapter 1, section 1.1.2.

In people, aspirin is readily absorbed after oral administration due to the acidic pH of the stomach (Needs and Brooks 1985). It rapidly undergoes first pass metabolism and is hydrolysed to salicylic acid in the liver by a nonspecific esterase. Salicylic acid is the major metabolite of aspirin (Rowland and Riegelman, 1969; Hutt et al., 1986). The elimination half life of aspirin is only 20 minutes and within one hour most aspirin administered orally or I/V hydrolyses to salicylic acid (Rowland et al., 1967). Only 68% of aspirin reaches systemic circulation as aspirin. Salicylic acid thus formed has a much longer half life as compared to its precursor; 3.5 to 4.5 hours in man (Rowland and Riegelman 1969). Metabolism of salicylic acid takes place by glucuronidation to salicyluric acid, salicyl acyl glucuronide and gentisic acid (Patel et al., 1990). Salicylates extensively bind to plasma proteins, ranging from 50 to 70% bound (Davis, 1980). The plasma concentrations of salicylates that should be maintained for analgesia are between 50 to 300 μg/mL and beyond the upper limit it is considered to be toxic (Davis, 1980).

The pharmacokinetics of aspirin has been extensively studied in various animals. Morton and Knottenbelt, (1989) studied the pharmacokinetics of aspirin in dogs after 900 mg total dose intravenously and 50 mg /kg orally. Salicylates followed first order kinetics. The plasma half life was 5.5 hours after I/V injection and 23.9 hours after oral administration. The volume of

distribution was 0.5 L/kg and oral bioavailability 71.4%. They recommended a total dose of 900 mg in dogs, as the therapeutic levels were maintained throughout their study period. However, there is problem with using "total doses" in animals whose size varies.

Gingerich et al., (1975) studied the disposition of aspirin in cattle after single I/V and oral doses and repeated oral dosing schedules in cattle. In the single dose study, sodium salicylate was given at the dose rate of 50 mg/kg I/V and 100 mg/kg orally, while in multiple dose schedule 50 and 100 mg/kg oral aspirin tablets were given twice daily for 5 days. After I/V administration, a rapid elimination was seen and the half life was 32 minutes with 0.24 L/kg volume of distribution. After oral administration, peak plasma concentration was seen after 2-4 hours and the elimination half life was 3.71 hours. The bioavailability of aspirin was 70%. The multiple dosing schedules of aspirin maintained the plasma concentration between 11 to 25 µg/mL and 45 to 65 µg/mL after 50 and 100 mg/kg oral dose, respectively. The authors recommended 100 mg/kg oral dose twice a day for 5 days for providing sufficient analgesia and antiinflammatory effect of aspirin. The major metabolite of salicylic acid in cattle was salicyluric acid (Short et al. 1990). In rabbits the majority of salicylic acid is excreted unchanged and only a small amount of salicyluric acid was detected (Short et al. 1991).

Parton et al. (2000), studied the pharmacokinetics and effect of salicylate on the gastrointestinal mucosa of healthy cats. After an I/V dose of 20 mg/kg, the therapeutic levels of salicylic acid were maintained for 24 hours. The elimination half life was much higher (22.2 hours) as compared to other mammalian species. The lower clearance in cats could be due to lack of enzymes required for glucuronidation (Davis and Westfall 1972). One in five cats had submucosal bleeding and gastric lesions with aspirin administration (Parton et al. 2000).

In horses after I/V administration at 20 mg/kg, aspirin was only detected for 6 hours and the salicylic acid levels peaked after 1 hour at 124.18 µg/mL (Broome et al. 2003). The elimination half life was 0.53 hours. The clearance (mL/h/kg) and volume of distribution (L/kg) was 472 and 0.36, respectively. The plasma concentration of salicylic acid was detectable at 36 hours of administration of aspirin.

The only previous study on the pharmacokinetics of salicylic acid in chickens was conducted by Baert and De Backer (2002). Salicylic acid was injected I/V in chickens at 50 mg/kg dose

rate. The elimination half life was 4.04 hours, the volume of distribution 0.39 L/kg and clearance 70 mL/h/kg. The pharmacokinetics was best described by a one compartment model and the therapeutic levels were maintained for 5 hours after I/V injection. They found that chickens cleared salicylic acid more slowly than ruminants and salicyluric acid, which is one of major metabolites of salicylic acid in humans (Patel et al. 1990), was not found in plasma.

This current study was designed to describe the pharmacokinetics of aspirin and salicylic acid in broiler chickens. Although salicylic acid kinetics has been reported in chickens, there is no evidence of aspirin kinetics, in chickens or any other avian species. The pharmacokinetic comparison of aspirin and its metabolite salicylic acid allows prediction of the plasma concentration and rate of hydrolysis of aspirin in the body after its administration. The pharmacokinetic information could be used to formulate a recommended dosing regimen for aspirin and salicylic acid in chickens, which could be further extrapolated to other avian species.

3.1.2 EXPERIMENT SIX: Pharmacokinetics of aspirin in broiler chickens

Study Design

This study was conducted on 18 broiler chickens with an average weight of 2 kg. These chickens were divided into three groups of six chickens each. A medial metatarsal vein of each broiler chicken was catheterised with a 22 G catheter under halothane anaesthesia. The birds were allowed to recover from the effect of anaesthesia before the injection of drugs. They were kept in groups under standard conditions and were fed *ad lib.* with commercially available broiler feed and a 24 hour supply of fresh drinking water.

Drug Administration

Aspirin for injection was made by dissolving 100 mg of acetylsalicylic acid in 200 µL of dimethyl sulphoxide and the resulting solution was diluted to 1 mL with 50% solution of polyethylene glycol-300 in water. The injection was filtered through 0.2 µm syringe filters and immediately administered to the chickens. The stability of aspirin in the injection solvent was checked by analysing the amount of aspirin hydrolysed to salicylic acid after 1, 2 and 3 hours of making the solution (figure 20). Only 0.085% of aspirin was converted to salicylic acid after three hours of injection preparation. Aspirin solution (100 mg/mL) was injected I/V into the chickens using medial metatarsal vein at the dose rate of 50 mg/kg.

Sample Collection

Four serial blood samples of 2 mL were taken at 0, 5, 10, and 30 minutes from the first six birds; 1, 2, 4, 8 hours from the next six and 12, 16, 24 and 32 hours from from the final six. The blood samples were collected in the heparinised vials. The total amount of blood withdrawn from each bird was 8 mL, which is 4% of the total blood volume of a 2 kg chicken. The vials were kept chilled immediately after collection and subjected to centrifugation at 2000 rpm for 10 minutes. Plasma was pipetted out and flash frozen in dry ice. The plasma samples were stored at –70 °C until the day of analysis.

Reagents and Standard Solutions

Acetylsalicylic acid (Aspirin) and Salicylic Acid standard powder were purchased from Sigma-Aldrich (Germany). The standard solution was made daily in acetonitrile (Merck, New Zealand) for aspirin, while water (Milli-q PFplus system, Millipore Cooperation, USA) for salicylic acid. Phosphate buffer (0.1 M pH 6.6) was prepared by mixing 187.5 mL of 0.2 M di-sodium phosphates and 312.5 mL of 0.2 M sodium hypophosphate; the volume was increased to 1 L with the addition of Milli-q wate. Na_2HPO_4 (0.2 M) solution was prepared by mixing 14.15g of Na_2HPO_4 salt in 500 mL water and 0.2 M $NaHPO_4$ solution was prepared by mixing 11.99 g of $NaHPO_4$ salt in 500 mL of water.

Sample Analysis

The plasma samples were analysed by High Performance Liquid Chromataography (HPLC) with Diode Array Detection (DAD). The HPLC system consisted of LC-20AD pumps, SIL-20AC HT auto-injector, Diode array detector SPD-M20A, CTO-20A column oven, DGU-20A3 Degasser (Shimadzu Japan). The chromatographs were analysed in LC solutions (Shimadzu, Japan).

The separation of aspirin and salicylic acid was achieved with a Phenomenex C18A (150 X 4.6 mm i.d, 5µm particle size) column at 32°C. The mobile phase consisted of 10 mM phosphate buffer pH 5.8: acetonitrile:acetic acid (71:28:1) with flow rate of 1 mL/min under isocratic conditions. The detector was set at 254 nm wavelength (Broome et al. 2003; Pirola et al. 1998). The lowest limit of detection for this method was 100 ng/mL for aspirin and 50 ng/mL for salicylic acid. The specificity of the method was confirmed by the absence of any peaks at the same retention time in the blank plasma after following the same extraction method .

Sample Preparation

Aspirin and salicylic acid were extracted from plasma samples by a liquid-liquid extraction procedure. 300 µL plasma vortex mixed with 10% acetic acid in acetonitrile and centrifuged at 3000 rpm for 15 minutes. The supernatant was separated and dried under gentle stream of compressed air at 20°C. The dried samples were mixed thoroughly in 200 µL of mobile phase on a vortex mixer and centrifuged at 4000 rpm for 10 minutes. The 50 µL of this supernatant was injected into the HPLC system. The recovery of the drugs after sample preparation was calculated by comparing area of the peaks for four different concentrations in blank plasma

and in mobile phase. The average recoveries for aspirin and salicylic acid after sample preparation were 98.46 and 89.57%, respectively. The standard curve for aspirin and salicylic acid were made by spiking blank plasma with known amounts of drug. The r square value for the standard curve was 0.993 for aspirin and 0.999 for salicylic acid.

The method was linear from 0.05 to 500 µg/mL both for aspirin and salicylic acid. The unknown concentrations were calculated by linear regression using Prism 4 for Macintosh (GraphPad Software, Inc).

Results

The curve showing the stability of aspirin in injection solvent is shown in figure 20. 0.02, 0.064 and 0.085% of aspirin was hydrolysed to salicylic acid after 1, 2 and 3 hours of dissolution in injection solvent. Therefore, negligible amounts of salicylic acid, if any, were injected along with aspirin in the experimental chickens.

The chromatographs for blank plasma, aspirin after 5 and 30 minutes of injection are shown in figures 21, 22 and 23, respectively. The absence any interfering peaks in the blank plasma at the same retention time as of aspirin shows the specificity of this method for aspirin and salicylic acid. The concentration time curve and the semi-log plot for these are shown in figures 24 and 25. Aspirin was hydrolysed to salicylic acid within 1 hour of I/V injection. Pharmacokinetic parameters after compartmental and noncompartmental pharmacokinetic analysis of aspirin in broiler chickens is shown in table 11. The pharmacokinetic data fitted best a single compartment open model. The half-life of aspirin was 12 minutes in chickens. Salicylic acid concentration increased gradually as the aspirin concentration was decreasing. The peak concentration for salicylic acid (93.75 µg/mL) was achieved after 1 hour of aspirin injection and at this time, plasma concentration of aspirin was lowest. The pharmacokinetic parameters of salicylic acid after administration of aspirin are given in table 13. The half life of salicylic acid after aspirin injection was 4 hours. The volume of distribution was 0.4 mL/kg and clearance was 68.67 mL/hr/kg. The salicylic acid was detectable in plasma until 16 hours after injection.

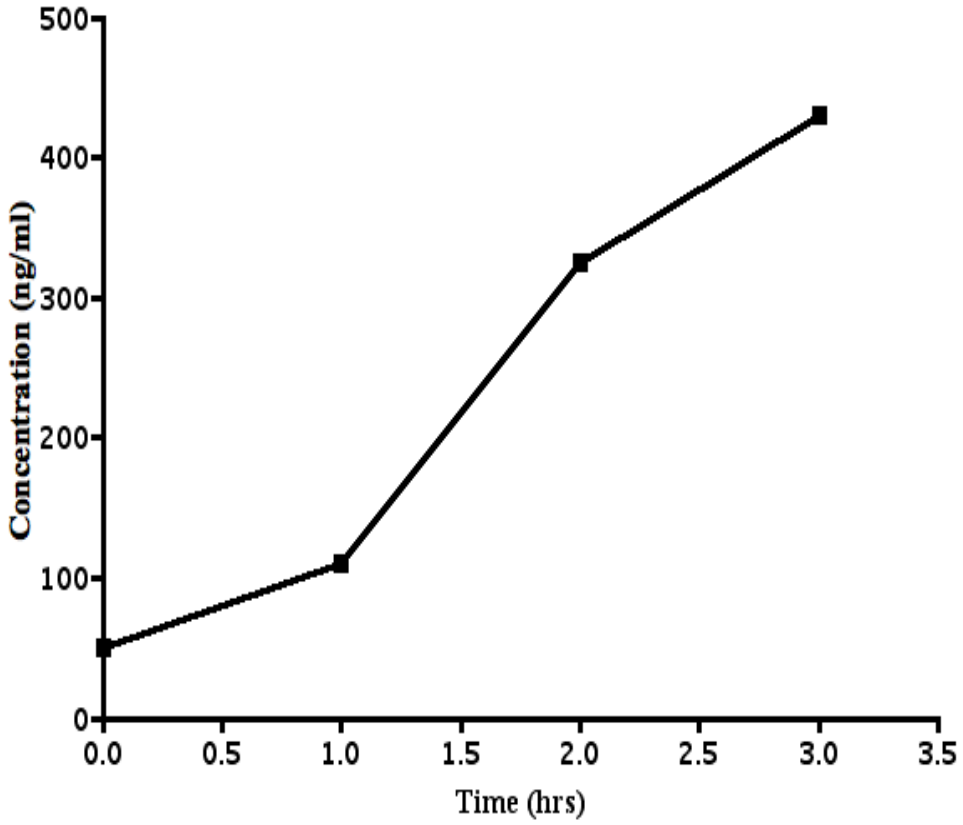

Figure 20: Stability of aspirin in its excipient. This graph shows the concentration of salicylic acid in the excipient after 1, 2 and 3 hours of dissolving aspirin in it.

100 mg Aspirin dissolved in 200 µL of Dimethyl Sulphoxide (DMSO) and the resulting solution was diluted to 1 mL with 50% solution of Polyethylene glycol (PEG)-300

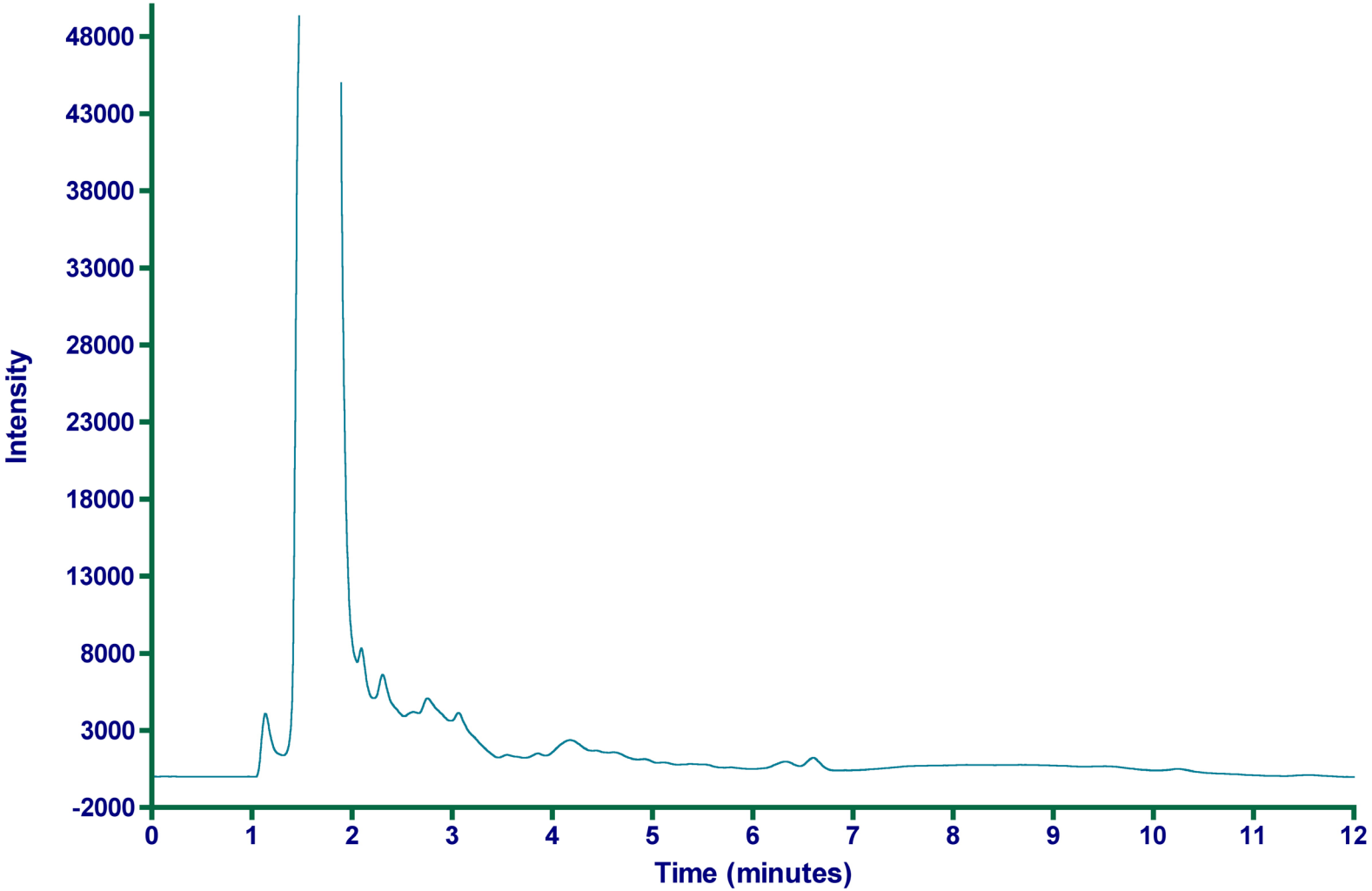

Figure 21: Chromatograph showing blank plasma after following similar sample preparation procedure for extraction of aspirin as other plasma samples

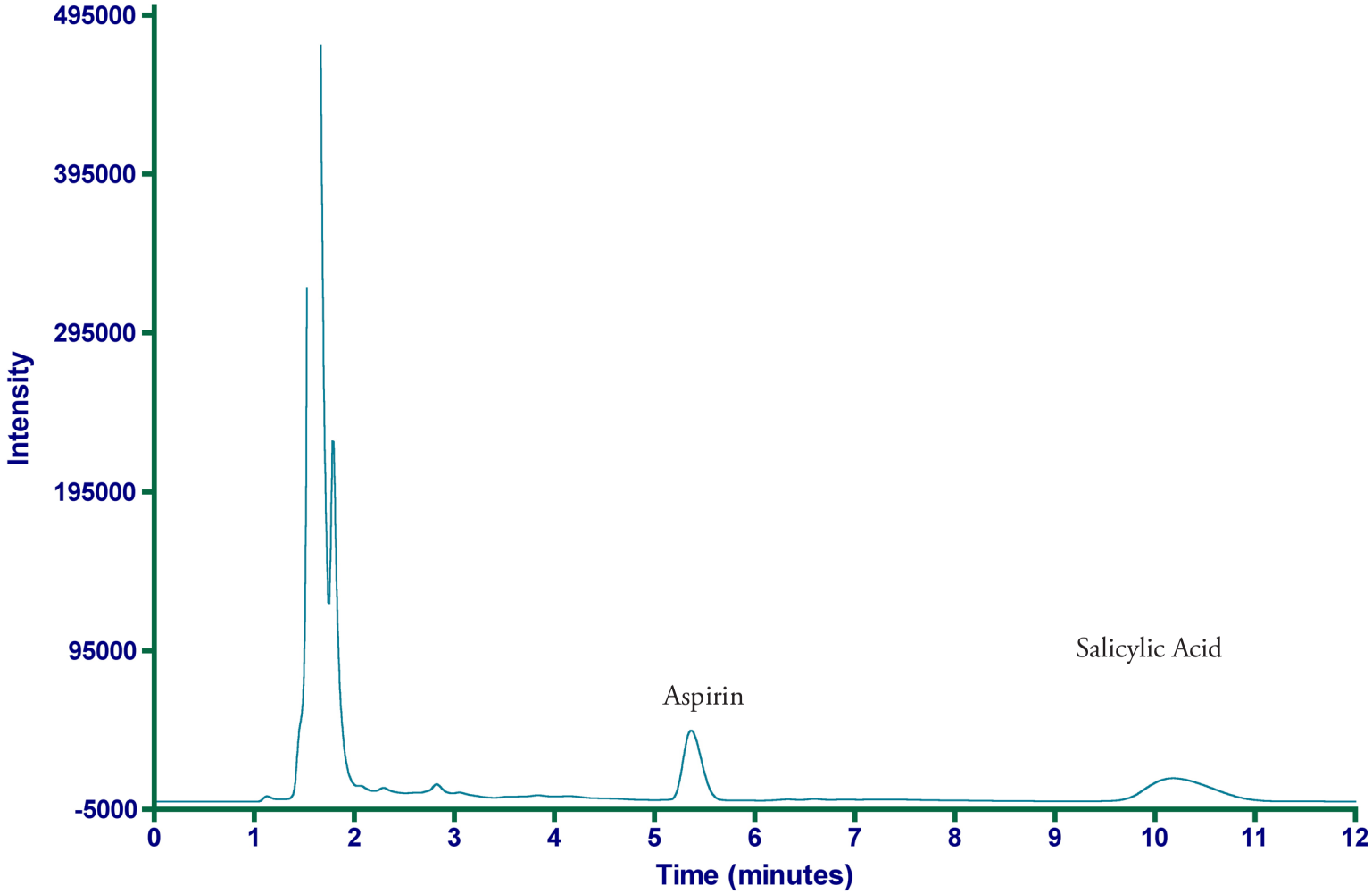

Figure 22: Chromatograph showing Aspirin peak and Salicylic acid peak after 5 minutes of aspirin injection at 50 mg/kg.

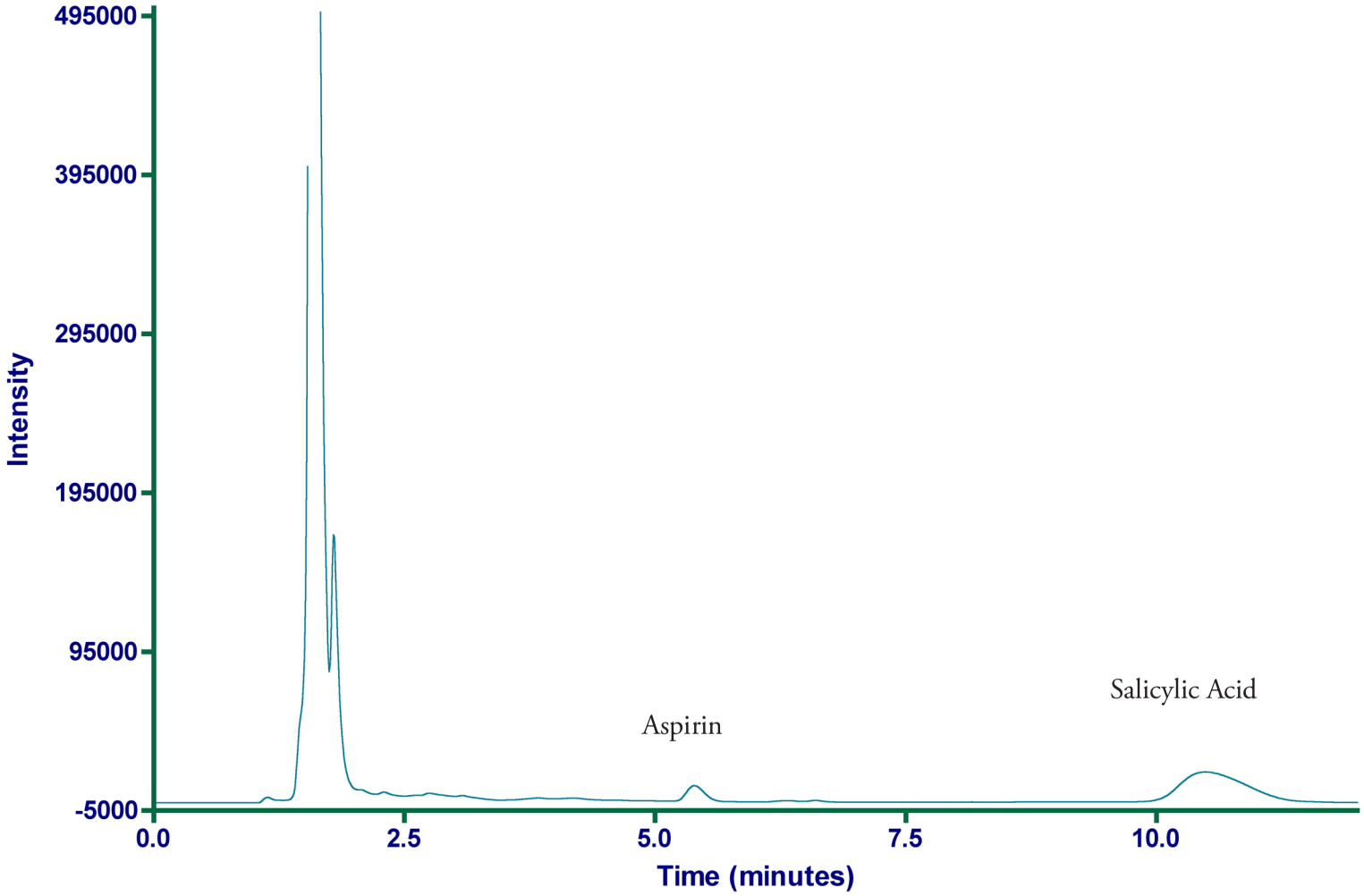

Figure 23: Chromatograph showing Aspirin peak and Salicylic acid peak after 30 minutes of aspirin injection at 50 mg/kg.

Discussion

After I/V administration, aspirin rapidly hydrolysed to salicylic acid, which is its major metabolite in mammals (Hutt et al., 1986). The time to achieve the peak plasma concentration (tmax) for salicylic acid coincided with the time at which the aspirin plasma concentration was at its lowest. Aspirin has a shorter half life in all the species studied excluding cats (table 12). Within 2 hours of aspirin administration, most of the aspirin had hydrolysed to salicylic acid. Chickens have higher volume of distribution and faster clearance as compared to other species. This trend was also seen in the pharmacokinetic study of morphine and butorphanol in chickens (Singh et al., 2010). Aspirin has a much shorter half life in chickens; only 12.6 minutes, which renders it unsuitable as analgesic after I/V administration. Also the plasma concentration of aspirin was much below the therapeutic levels seen in mammals. The maximum plasma concentration achieved in chickens was 46.72 µg/mL. If the therapeutic effects of aspirin are due to salicylic acid, which is debatable as not many comparative studies between the two salts have been published (Levy, 1979), aspirin could be analgesic in chickens as the duration for which salicylic acid remained within the mammalian therapeutic levels was from 1 until 4 hours after aspirin injection. However, I/V administration is not recommended, because aspirin is highly insoluble in water and it rapidly hydrolyses to salicylic acid in aqueous solution.

The major side effect of NSAIDs is gastric irritation and ulcers (Vane et al., 1998). No birds showed clinical signs attributable to ulcers although all the birds were injected only once with aspirin and the side effects only become obvious after long term use of aspirin. Also, no postmortem examination was conducted on the chickens after end of the study to confirm the presence of gastric ulcers, if any.

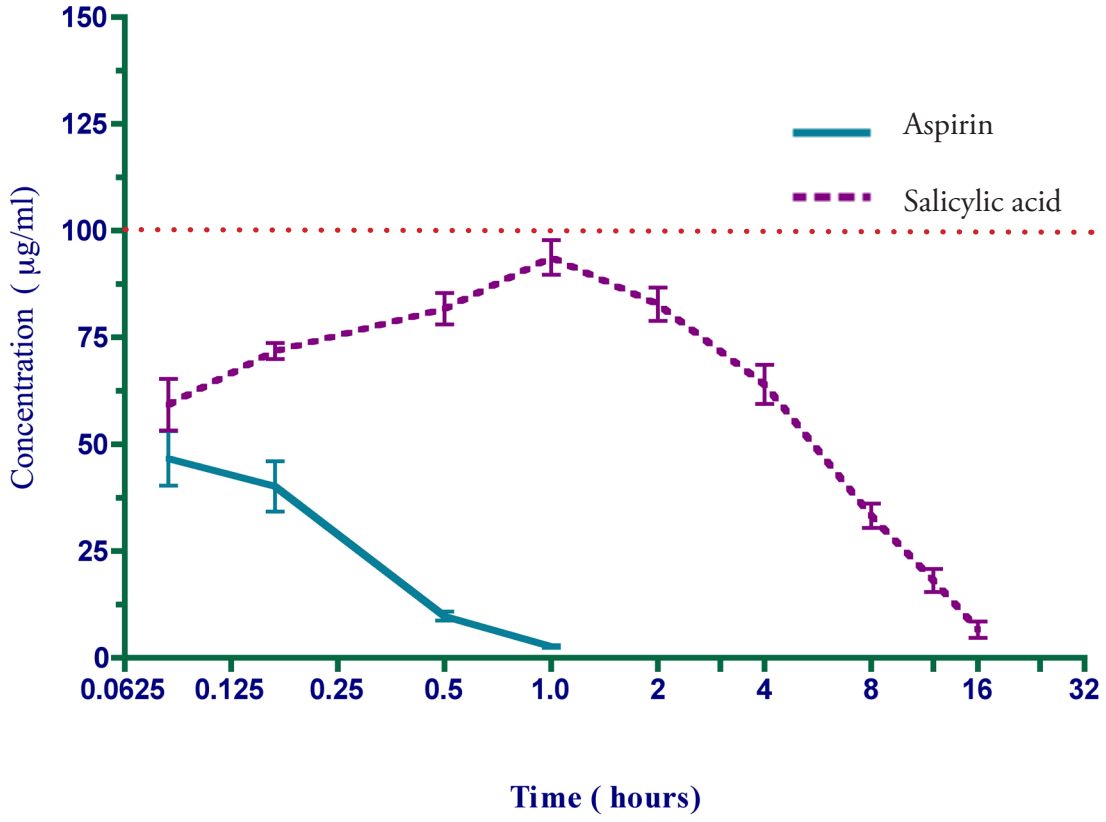

Figure 24: Concentration time curve for Aspirin and its metabolite Salicylic acid after intravenous administration of aspirin at 50 mg/kg in broiler chickens. Each data point represents mean of 6 chickens, and a total of 18 chickens were used for the whole study. The red dotted line indicates the minimum effective plasma concentration of salicylates required to maintain analgesia in humans

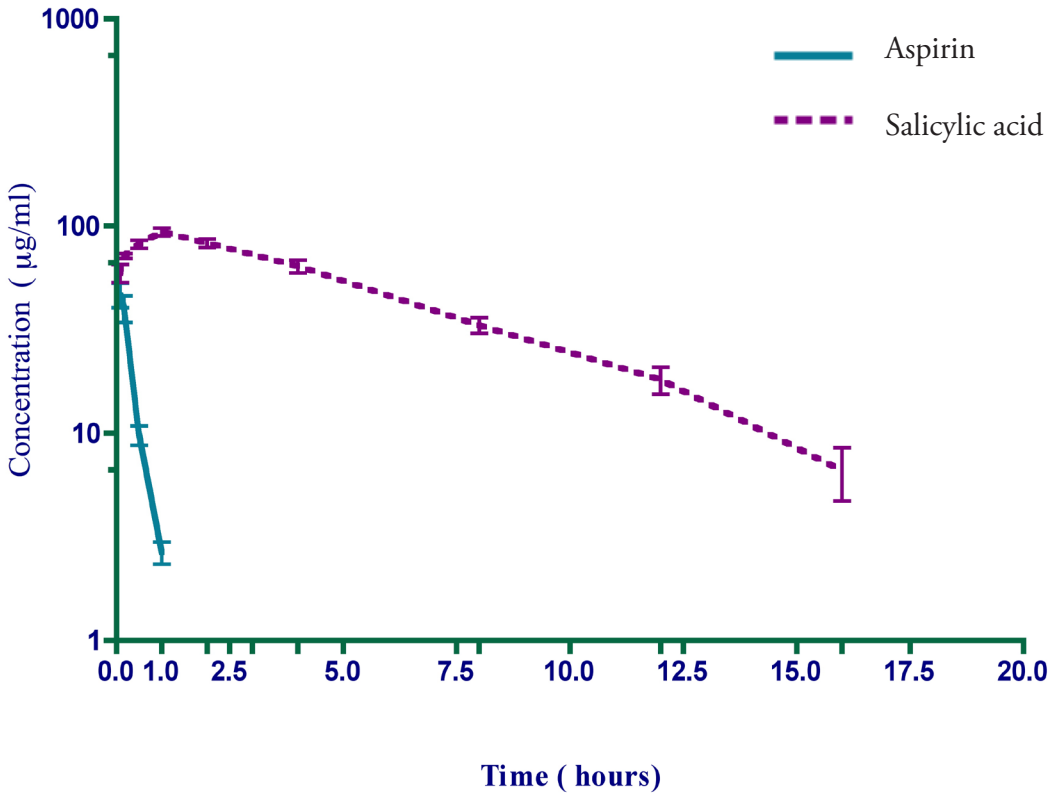

Figure 25: Semi log plot of concentration time curve for Aspirin and its metabolite Salicylic acid after intravenous administration of aspirin at 50 mg/kg in broiler chickens. Each data point represents mean of 6 chickens, and a total of 18 chickens were used for the whole study.

Parameters	Units	Mean
Noncompartmental		
$AUC_{(0-\infty)}$	μg.hr/mL	19.5
$AUC_{(0-t)}$	μg.hr/mL	20.4
$AUMC_{(0-\infty)}$	$\mu g.hr^2/mL$	5.5
Vd_{area}	mL/Kg	754.5
Cl	mL/min/Kg	2456.9
$T_{1/2\lambda z}$	hours	0.2
$MRT_{\lambda z}$	hours	0.3
Compartmental		
C_0	μg/mL	60.6
β	1/hour	3.214
$T_{1/2\beta}$	hours	0.216
$Vd_{(central)}$	mL/Kg	825.08
Cl	mL/hr/Kg	2651.8

β elimination rate constant, $T_{1/2\beta}$ elimination half-life, $T_{1/2\lambda z}$ half-life of terminal phase.

Table 11: Compartmental and Noncompartmental pharmacokinetics analysis of aspirin in broiler chickens after 50 mg/kg intravenous injection. These pharmacokinetic parameters were calculated from the mean of six concentration time curves from the mean pool samples.

Species	Dose (mg/kg)	$T_{1/2}$ (hours)	Vd (L/kg)	Cl (mL/hr/kg)	Authors
Chickens	50	0.21	0.75	2456.9	This study
Horses	20	0.53	0.36	472	(Broome., et al. 2003)
Dogs	900*	5.5	0.50	70	(Morton and Knotten-belt 1989)
Cats	20	22.5	0.17	5.1	(Parton., et al. 2000)
Cows	26	0.51	0.199	263.9	(Whittem., et al. 1996)

Table 12: Comparative Pharmacokinetics of Aspirin after intravenous administration in various species

3.1.3 EXPERIMENT SEVEN: Pharmacokinetics of salicylic acid in broiler chickens

Study Design

This study was conducted on 18 broiler chickens with an average weight of 2 kg. These chickens were divided into three groups of six chickens each. A medial metatarsal vein of each broiler chicken had a 22 G catheter placed under halothane anaesthesia. The birds were allowed to recover from the effect of anaesthesia before the injection of drugs. The birds were kept in groups under standard housing conditions and were fed *ad lib*. with commercially available broiler feed with a 24-hour supply of fresh drinking water.

Drug Administration

Salicylic acid injection was made by dissolving 100 mg of sodium salicylate in 1 mL of distilled water. The injection was filtered through 0.2 µm syringe filters and immediately administered in the chickens. The salicylic acid injection (100 mg/mL) was made into the contralateral vein at the dose rate of 50 mg/kg using a 23 G needle.

Sample Collection

Four serial blood samples of 2 mL each were taken at 0, 5, 10, and 30 minutes from the first six birds; 1, 2, 4, 8 hours from the second six and at 12, 16, 24 and 32 hours from the third six. The blood samples were collected in the heparinised vials. The total amount of blood withdrawn from each bird was 8 mL, which is 4% of the total blood volume of a 2 kg chicken. The vials were kept chilled immediately after collection and subjected to centrifugation at 3000 rpm for 5 minutes. Plasma was pipetted out and kept at –70 °C until the day of analysis.

Reagents and Standard Solutions

Salicylic acid (sodium salt) standard powder was purchased from Sigma-Aldrich (Germany). The standard solution was made daily in water (Milli-q PFplus system, Millipore Cooperation, USA). Phosphate buffer (0.1 M) pH 6.6 was prepared by mixing 187.5 mL of 0.2 M di-sodium phosphate and 312.5 mL of 0.2 M sodium hypophosphate; the volume was increased to 1 L

with the addition of Milli-q water. Na_2HPO_4 (0.2 M) solution was prepared by mixing 14.15 g of Na_2HPO_4 salt in 500 mL Milli-q water and $NaHPO_4$ (0.2 M) solution was prepared by mixing 11.99 g of $NaHPO_4$ salt in 500 mL of Milli-q water.

Sample Analysis

Sample preparation and the HPLC protocol were similar to aspirin as described in experiment 7.

Results

The chromatographs for blank plasma and salicylic acid after 5 and 30 minutes of injection are shown in figures 21, 26 and 27, respectively. The same HPLC analysis method worked well for salicylic acid as there was no interfering peak at the elution time for salicylic acid. The concentration time curve and semi-log plot for these are shown in figures 28 and 29, respectively. After I/V administration, the salicylic acid followed a two compartment model with 1.99 hours as absorption and 2.63 hours as elimination half lives. The pharmacokinetic parameters calculated both by compartmental and noncompartmental analysis are given in table 13. The volume of distribution was 0.25 L/kg, terminal elimination half life 2.9 hours and clearance 60.6 mL/hr/kg. Salicylic acid was detectable in circulation until 16 hours after I/V bolus injection at 50 mg/kg. Plasma concentration at 16 hours was 3.3 µg/mL.

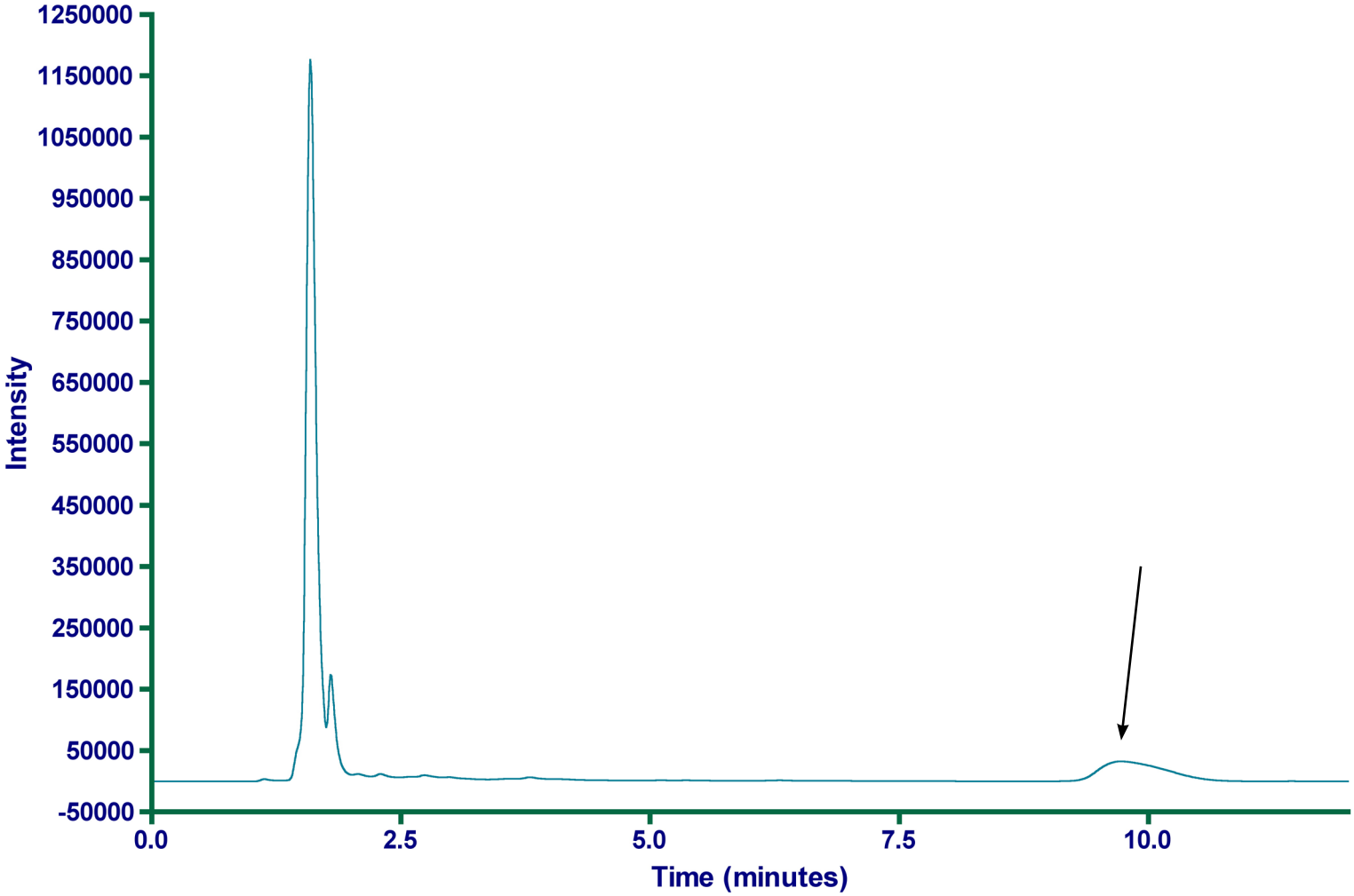

Figure 26: Chromatograph showing Salicylic Acid peak 5 minutes after intravenous administration of salicylic acid at 50 mg/kg in broiler chickens

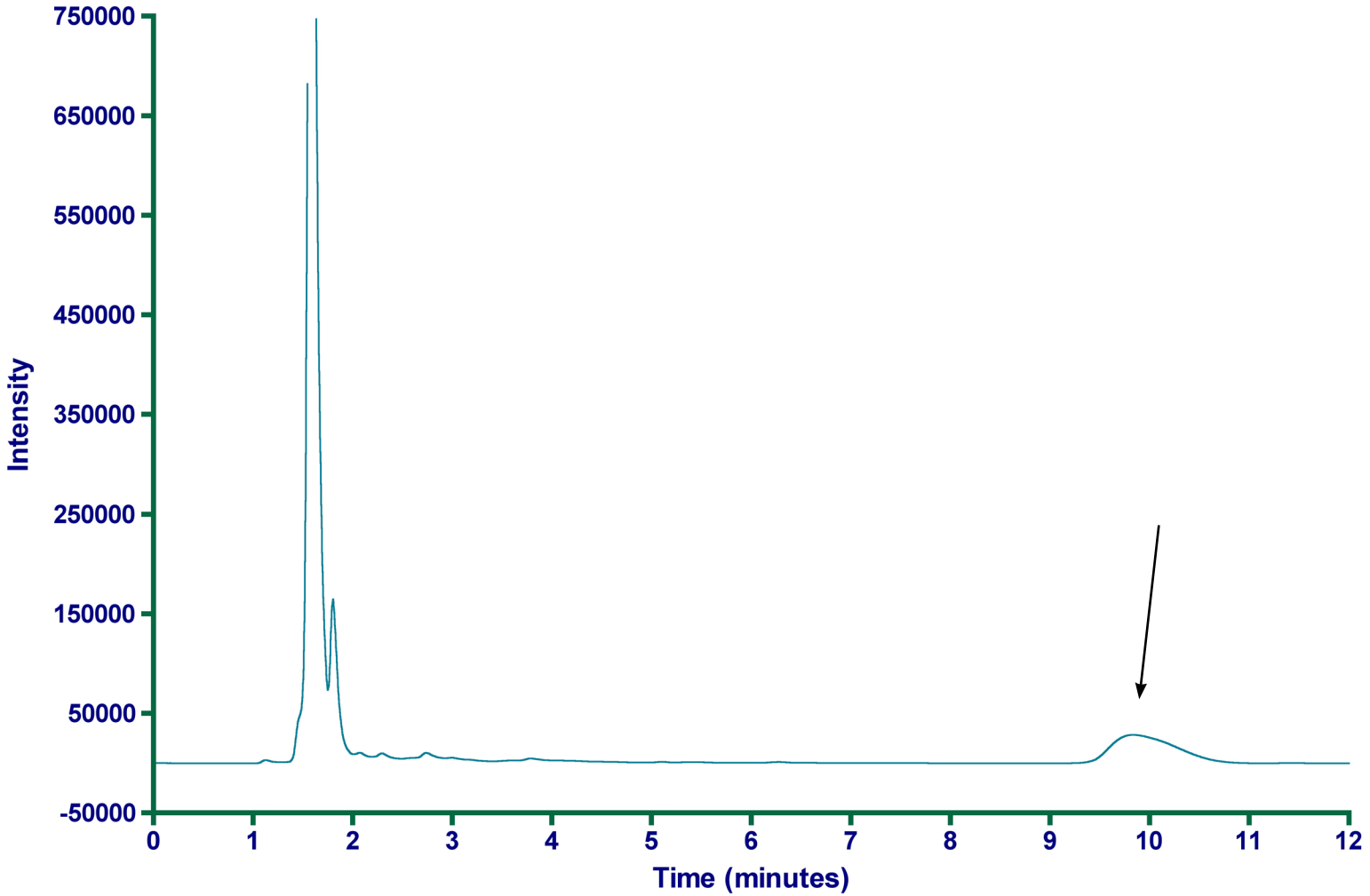

Figure 27: Chromatograph showing Salicylic Acid peak 30 minutes after intravenous administration of salicylic acid at 50 mg/kg in broiler chickens

Discussion

Salicylic acid occurs naturally in willow bark and has been used since 400 BC for treatment of various conditions in man and animals (Andermann 1996). In contrast to aspirin, it is highly soluble in water, which makes it easier for use by injection. Pharmacokinetic parameters obtained after I/V injection of salicylic acid in chickens were similar to the previous study by Baert and Backer, (2002) (table 14). Broiler chickens had a low volume of distribution which is typical for all the NSAIDs (Davis, 1980). NSAIDs in general have low tissue distribution compared to opioids. Ostrichs had a similar volume of distribution as chickens but had shorter half life due to faster clearance (Baert et al., 2002). Goats also had higher plasma clearance for salicylic acid, which could be due to diffusion of drugs into body compartments such as rumen (Davis and Westfall, 1972). Consequently, ostrich and goats had shorter half lives as compared to chickens. Cattle also had shorter half life than chickens which could be due to faster clearance but plasma clearance was not reported (Gingerich et al., 1975). In general, salicylates have higher elimination rates in herbivores as compared to carnivores (Davis and Westfall, 1972), as would be expected in animals which have evolved eating salicylates from plants. Dogs and cats had much slower clearance as compared to other species, resulting in longer half lives. Chickens metabolise salicylic acid faster than other species, but in our study we did not conduct any metabolic study to detect the various metabolites of salicylic acid which in other species are salicyluric acid and gentisic acid.

The analgesic and antipyretic actions of salicylic acid are due to inhibition of COX-1 and COX-2 enzymes (Vane, 1971). COX-1 is the constitutive enzyme and is responsible for most normal physiological functions (Vane et al., 1998). It is believed that major side effects such as gastric irritation and ulcers associated with use of NSAIDs are mainly due to inhibition of COX-1 enzyme (Vane et al., 1998). In our study we did not notice any such effects. This may be due to single use of salicylic acid in chickens or to the effects being too mild to cause obvious behavioural changes. We did not conduct any post-mortem examinations to look for sub-clinical ulceration.

Salicylic acid could be used as an analgesic agent in chickens at 50 mg/kg I/V bolus dose, based on plasma concentrations. At this dose rate, the plasma concentrations achieved were

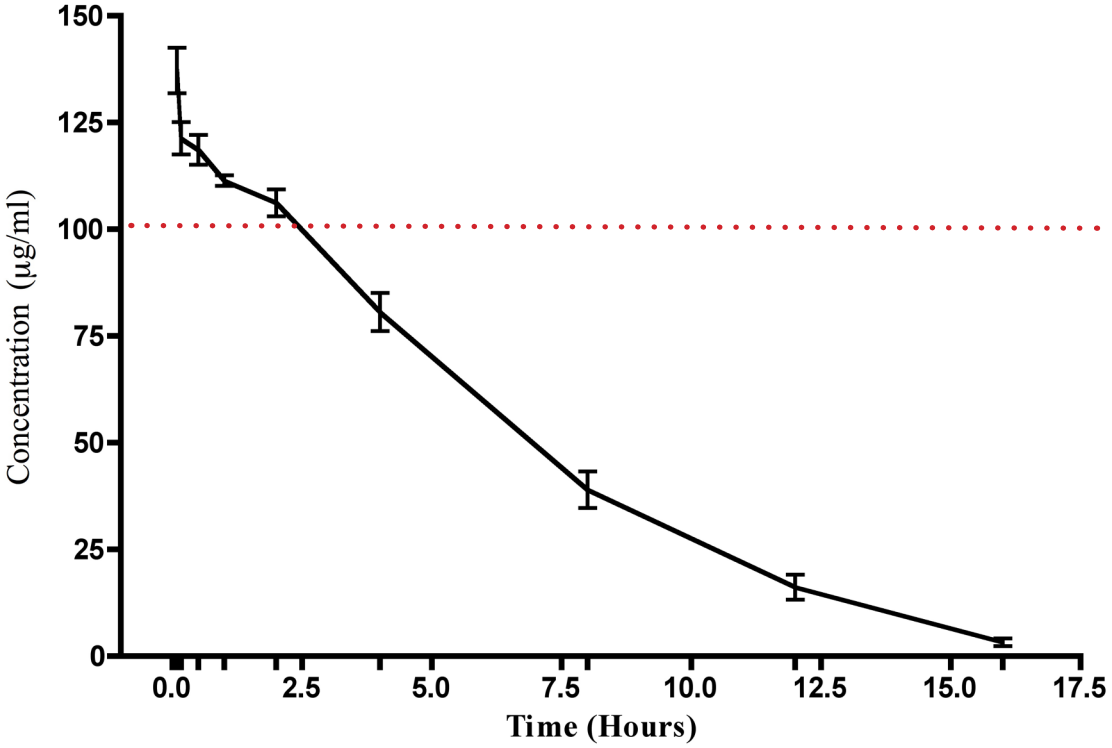

Figure 28: Concentration time curve for Salicylic acid after intravenous administration at 50 mg/kg in broiler chickens. Each data point represents mean of 6 chickens, and a total of 18 chickens were used for the whole study. The red dotted line indicates the minimum effective plasma concentration of salicylic acid required to maintain analgesia in humans

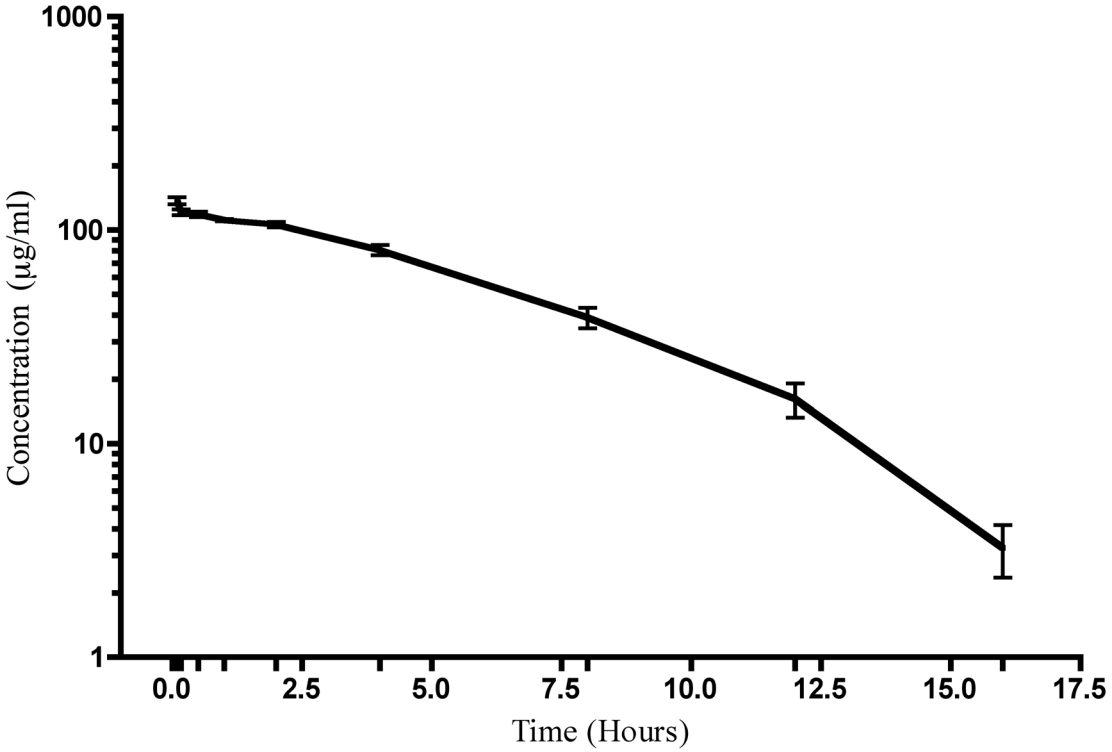

Figure 29: Semi log plot of concentration time curve for Salicylic acid after intravenous administration at 50 mg/kg in broiler chickens. Each data point represents mean of 6 chickens, and a total of 18 chickens were used for the whole study.

much higher than the minimum therapeutic concentrations reported for other mammals and the levels remained within this therapeutic range for about four hours. Thus, for the pharmacodynamic study, we decided to inject salicylic acid at this dose rate in broiler chickens suffering from pain caused by lameness.

Parameters	Units	Mean
Noncompartmental		
$AUC_{(0-\infty)}$	µg.hr/mL	823.9
$AUC_{(0-t)}$	µg.hr/mL	810.4
$AUMC_{(0-\infty)}$	ng.hr^2/mL	3802.0
Vd_{area}	mL/Kg	251.7
Cl	mL/hr/Kg	60.68
$T_{1/2\lambda z}$	hours	2.9
$MRT_{\lambda z}$	hours	4.6
Compartmental		
A_1	µg/mL	213.165
A_2	µg/mL	81.190
α	1/hr	0.348
β	1/hr	0.241
k_{21}	1/hr	0.318
k_{12}	1/hr	0.007
k_{10}	1/hr	0.263
$T_{1/2\alpha}$	hour	1.99
$T_{1/2\beta}$	hour	2.63
$Vd_{(central)}$	mL/Kg	169.86
Vd_{ss}	mL/Kg	173.25
$Vd_{(peripheral)}$	mL/Kg	3.39
Cl	mL/hr/Kg	41.75

A_1 and A_2 are mathematical constants, α distribution rate constant, β elimination rate constant, k_{21}, k_{12}, k_{10} are the microconstants, $T_{1/2\alpha}$ distribution half-life, $T_{1/2\beta}$ elimination half-life, $T_{1/2\lambda z}$ half-life of terminal phase.

Table 13: Compartmental and Noncompartmental pharmacokinetics analysis of Salicylic Acid in Broiler Chickens after 50 mg/kg intravenous injection. These pharmacokinetic parameters were calculated from the mean of six concentration time curves from the mean pool samples.

Species	Dose (mg/kg)	$T_{1/2}$ (hours)	*Vd* (L/kg)	*Cl* (mL/hr/kg)	Authors
Chickens	50	2.9	0.251	60.68	This study
	50	4.04	0.390	70	Baert and Backer 2002
Rabbits	44	4.3	0.249	43.2	Short 1991
Cattle	50	0.53			Gingerich and Baggot 1975
Ostrich	25	1.32	0.36	190	Baert and Backer 2002
Goat	44	0.8	0.129	114.03	Davis and Westfall 1972
Dog	44	8.6	0.189	15.03	
Cat	44	37.6	0.209	3.70	

Table 14: Comparative Pharmacokinetics of Salicylic Acid after intravenous administration in various species

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CHAPTER FOUR

Alleviation of pain in lame broiler chickens

ABSTRACT

The analgesic effects of morphine, butorphanol and salicylic acid were studied in lame broiler chickens. Two studies were conducted to observe the behavioural changes in lame broiler chickens after intravenous injection of morphine and butorphanol at 2 mg/kg and salicylic acid at 50 mg/kg. Lame and sound broiler chickens were randomly selected from a flock based on gait scoring. These birds were subjected to an obstacle course and latency to lie test either after injection of drugs or normal saline. One group of lame and sound birds each were not injected with anything but were handled in the same way as the others. Three observation were made, one before injection, after 30 minutes and two hours. There was no improvement seen in walking ability of lame chickens after injection of morphine. There was reduction in walking pace and standing time of sound chickens. Morphine caused sedation and drowsiness both in lame and sound chickens. There was a significant difference in time taken to finish the obstacle course by lame broiler chickens after the injection of butorphanol and salicylic acid but the difference in standing time was not significant, though the trend suggested analgesia. These two drugs are effective in alleviating pain in chickens but the effect lasted only two hours. Using the pharmacokinetic data from the previous experiments, the minimum effective concentration for butorphanol was 50-80 ng/mL for salicylic acid 50-110 ng/mL.

4.1.1 INTRODUCTION

Lameness can be defined as the inability to accomplish normal locomotion and deviation from normal gait. Broiler lameness is one of the most serious problems affecting the broiler industry worldwide. It poses a large welfare problem to broiler chickens, if they live in continuous pain. Due to impaired locomotion they find difficulty in reaching the drinker and feeder, therefore unable to fulfil their basic needs of eating and drinking. They are also incapable of exhibiting normal locomotive behaviour (Kestin et al., 1992; Sanotra et al., 2001). The lame birds are also dominated by their sound compatriots and end up dying because of dehydration and starvation (Butterworth et al., 2002). There is much evidence showing that lame broiler chickens suffer from pain. According to a study conducted by Weeks et al., (2000), lame chickens spent significantly more time lying (86%) than their sound compatriots. Sound birds chose a standing posture for eating whereas the lame ones were seen eating while lying down. Sound birds fed over 50 times in 24 hours, while the number of visits to the feeder for a broiler with gait score 3 was reduced to 30 times. Dust bathing is quite a common behaviour in broiler chickens as it was seen everyday and almost in every chicken (Vestergaard and Sanotra, 1999). Lame birds spent a significantly less time in standing preening, standing idle and dust bathing. Vestergaard and Sanotra, (1999) found there was a significant decrease in time spent on dust bathing by lame chickens. Dust bathing involves forceful movements with legs and wings, which might be difficult for broilers in pain or having leg abnormalities. Lack of dust bathing was also associated with an increase in blood corticosterone levels in affected birds (Vestergaard et al., 1997). Thus at least three (and possibly all) of the five freedoms are likely to be compromised in lame broilers.

Analgesic drugs can have an effect. The walking ability of lame chickens was improved with the injection of carprofen, a NSAID (McGeown et al., 1999). Lame birds preferred to consume feed mixed with carprofen as compared to the healthy birds (Danbury et al., 2000). The leg health and behaviour of fattening turkeys was also improved by the treatment with butorphanol (Buchwalder and Huber-Eicher, 2005) which suggests that the fast growing turkeys show lameness associated with pain. The effect of various NSAIDs was evaluated by (Hocking et al., 2005). Intra-articulate injection of sodium urate crystals was given to induce acute pain in do-

mestic fowl. Carprofen, flunixin, ketoprofen were administered I/V and sodium salicylate was given orally at dose rates recommended for large animals. In their first experiment they could not observe any improvement. The experiments were repeated with higher dose rates for each analgesic, administered via I/M injection. The behavioural improvement was clearly noticed in the treated birds as compared to the control group in case of carprofen and ketoprofen. Salicylic acid and flunixin at higher dose rates induced panting and piloerection.

It is difficult to assess the behaviour of broiler chickens as they spend most of their day in sleeping and eating (Murphy and Preston, 1988). Therefore, to evaluate efficacy of analgesic drugs in alleviating pain in lame broiler chickens, we used two previously validated tests to measure of the degree of lameness in broiler chickens suffering from leg related problems. Firstly, we used gait scoring (Kestin et al. 1992). In this method, birds were visually examined and rated on the scale of 0 to 5, 5 being the lamest and 0 perfectly sound. Briefly, a gait score zero bird will walk normally. Gait score 1 and 2 describes the birds having uneven gait, but there is no obvious lesion and the pace and agility is not compromised. Gait score 3 bird will have obvious leg abnormality. Limping can be detected easily, and the walking ability of the bird is reduced. The birds with severe defects will fall into gait score 4 category. These birds will walk only when encouraged. The lesion will be more severe as compared to gait score 3 birds. In case of gait score 5, birds will walk only with some assistance from wings or may crawl. Gait score 4 and 5 birds are considered to be in unacceptable distress and are usually euthanised. The initial categorisation of lame and sound birds were based on this method. Birds with gait scores from 0 to 2 were considered sound and 3 or greater were lame. The other two tests to evaluate the analgesia were obstacle course test (OC) (McGeown et al., 1999) and latency to lie test (LTL) (Berg and Sanotra, 2003).

This experiment was designed to evaluate the efficacy of analgesic drugs in broiler chickens. We chose lame broiler chickens as a model of chronic pain because it has been generally accepted that the lame broiler chicken suffers from pain.

4.1.2 EXPERIMENT EIGHT: Effect of intravenous injection of analgesic drugs on walking ability of lame broiler chickens

Study Design

Two double-blinded randomised studies were conducted to evaluate the analgesic efficacy of morphine, butorphanol and salicylic acid in lame broiler chickens. Broiler chickens were classified as lame and sound on the basis of gait score.

In study one, 12 broiler chickens were assigned to each group namely; lame morphine, lame butorphanol, lame salicylic acid, lame placebo, sound morphine, sound butorphanol, sound salicylic acid and sound placebo. The birds in the analgesic treatment group were injected intravenously with either morphine (2 mg/kg), butorphanol (2 mg/kg) or salicylic acid (50 mg/kg). The placebo birds were injected with the same volume of normal saline. The person conducting the test was unaware of the allocation of broiler chickens to groups.

In study two, in addition to the groups in study one, control lame and sound groups were also included. In these groups, the birds were not injected with anything, but were handled in the same way as the other groups. The rest of the study design was same as in study one.

Some lame birds selected for the study became more lame before testing. If they could not walk or stand (GS 4 or 5), they were removed from the analysis. Thus, in some groups the final number of birds was less than 12.

All groups were subjected to following tests for accessing the lameness and analgesia:

Obstacle Course (OC):

This test was based on the assumpion that lame broiler chickens walk slowly and will take more time to finish the course compared to their sound compatriots. A 150 mm high and 550 mm wide wooden block was placed at the middle of 2 m long and 550 mm wide course. The feeder was kept at one end of the course and broiler chickens had to walk towards the feeder in such a way that they had to clear the obstacle in order to reach the feeder. They were fasted for 4 hours before the test. Another broiler chicken was allowed to feed at the same time the test bird

was at the other end of the course to encourage the test bird to clear the obstacle. Time (seconds) taken to complete the course was recorded. The cut-off time for this test was 5 minutes and the birds who could not finish the course were excluded from the study.

Latency to Lie (LTL):

This test is based on the fact that the broiler chickens have strong disinclination to sit in water. Sound chickens will be able to stand for a longer time than lame birds. The birds were made to stand in a trough having shallow tepid water, just submerging their toes. The cut-off time for this test was 10 minutes, as no birds were able to stand after that time. The standing time (seconds) in the trough was recorded. The birds who could not stand in water were excluded from the study.

All the birds were subjected to these tests three times. The first recording was made before the administration of drugs. The subsequent recordings were made 30 minutes and 2 hours after injection or handling. These observation times were based on the pharmacokinetic studies conducted previously. All the drugs used in the study remained above the minimum effective concentration (as for mammals) for about 2 hours after the injection (chapter 2 and 3).

Drugs and Administration

Butorphanol tartate injection 10 mg/mL (Lloyds Pharma, New Zealand) and morphine sulphate injection 10 mg/mL (Mayne Pharma, Australia) were injected I/V at 2 mg/kg. The salicylic acid (100 mg/mL) injection solution was made in up normal saline and filtered through 0.45 μm, 4 mm syringe filters (Phenomenex, New Zealand) then given at 50 mg/kg. The drugs were injected into the metatarsal vein of 12 lame and 12 sound broiler chickens.

Data Analysis

The before and after injection mean values obtained from the groups were compared. The data was subjected to Friedman test (one-way ANOVA) for P values and if there were significant differences ($P<0.05$) then Dunn's multiple column comparison was made to know the significant difference between the treatments. Prism 4 for Macintosh (GraphPad Software, Inc) was used for statistical analysis. The graphs were also plotted using Prism.

Results

Study one: Analgesic efficacy of butorphanol, morphine and salicylic acid in lame broiler chickens.

Butorphanol

Obstacle course: Two lame chickens were not able to walk or stand, and were excluded from the study. Results for OC are shown in table 15. Butorphanol injection reduced the time taken by the lame birds by 58 seconds at 30 minutes after injection (34 seconds) compared to time taken before the injection of butorphanol (178 seconds), but this difference was not statistically significant (P=0.0924). After 2 hours, this difference was reduced to 34 seconds only. The non-significant time reduction was also seen in the case of sound birds (P=0.0895), but that reduction was relatively smaller than the lame birds.

Latency to lie Test: the latency to lie test gave similar results to the obstacle course. The results for LTL are shown in table 16. The lame birds were able to stand for a longer time after injection (405 secs after 30 minutes and 383 secs after 2 hours of injection) as compared to before the injection (234 secs), but this difference was statistically non-significant (P=0.3675). In the case of sound birds, there was non-significant difference (P=0.569) among the various observations.

Morphine

Obstacle course: Four lame broiler chickens were not able to stand, so they were excluded from the analysis. Results are shown in table 17 . Morphine injection increased the time to finish the course by lame broiler chickens (figure 30). This difference was statistically significant with (P = 0.047). When the Dunn's multiple comparison test was applied, there was no significant difference within the observations. At 30 minutes after morphine injection, lame broiler chickens took 17 seconds more to finish the course and after 2 hours they took 31 seconds less than the time taken before the injection (113 secs).

In sound broiler chickens, there was significant difference between the observations at the three time points (figure 31) (P = 0.003). Two hours after injection, sound broiler chickens took a significantly less time to finish the course compared to 30 minutes after injection. There was

	SOUND			LAME		
	Before injection (sec)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	35	30	26	178	34	37
2	14	26	14	242	120	67
3	13	4	5	80	22	20
4	9	8	10	191	21	18
5	19	11	13	300	100	300
6	14	20	10	135	95	87
7	18	23	17	49	300	300
8	27	11	10	56	64	220
9	16	25	34	62	15	17
10	19	14	9	134	72	18
11	19	15	10	DNF	DNF	DNF
12	16	15	15	DNF	DNF	DNF
MEAN	**18.25**	**16.83**	**14.42**	**142.70**	**84.30**	**108.40**
SEM	**1.98**	**2.29**	**2.34**	**26.89**	**26.66**	**37.37**

Table 15: Study one. Obstacle Course observations. before, 30 minutes and 2 hours after intravenous injection of butorphanol at 2mg/kg dose rate in sound and lame broiler chickens. (DNF-did not finish)

	SOUND			LAME		
	Before injection (sec)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	600	600	600	56	104	76
2	600	600	600	118	57	19
3	600	600	600	281	600	600
4	600	600	600	83	600	600
5	306	600	600	600	600	600
6	600	600	600	130	284	80
7	600	600	600	103	60	62
8	456	276	470	600	600	600
9	490	276	470	60	550	600
10	600	600	600	315	600	600
11	600	600	600	DNS	DNS	DNS
12	202	241	493	DNS	DNS	DNS
MEAN	**521.20**	**541.70**	**580.30**	**234.60**	**405.50**	**383.70**
SEM	**39.23**	**38.27**	**13.39**	**66.91**	**78.64**	**88.45**

Table 16: Study one. Latency to Lie test observations. Before, 30 minutes and 2 hours after intravenous injection of butorphanol at 2mg/kg dose rate in sound and lame broiler chickens. (DNS-did not stand)

	SOUND			LAME		
	Before injection (sec)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	24	20	13	273	130	208
2	32	22	13	40	150	35
3	5	9	5	44	16	7
4	7	22	7	150	45	14
5	28	7	7	134	300	300
6	32	146	24	157	300	300
7	28	57	10	54	62	26
8	32	39	17	54	40	22
9	21	42	24	DNF	DNF	DNF
10	4	40	11	DNF	DNF	DNF
11	36	57	55	DNF	DNF	DNF
12	31	100	19	DNF	DNF	DNF
MEAN	**23.33[a]**	**46.75[ab]**	**17.08[ac]**	**113.20***	**130.40***	**82.13***
SEM	**3.33**	**11.67**	**3.89**	**28.78**	**40.34**	**38.72**

Table 17: Study one. Obstacle Course observations. Before, 30 minutes and 2 hours after intravenous injection of morphine at 2mg/kg dose rate in sound and lame broiler chickens. (DNF-did not finish)

Significant difference is shown by the superscript alphabets (P value = 0.0032)
** No significant difference seen during Dunn's post test multiple column comparison (P value = 0.0469)*

	SOUND			LAME		
	Before injection (sec)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	600	600	600	600	600	271
2	40	98	221	393	600	600
3	600	130	600	516	127	600
4	216	273	600	105	460	600
5	600	530	600	196	176	334
6	600	168	600	174	134	600
7	149	138	267	34	452	275
8	20	600	600	204	62	383
9	600	600	600	DNS	DNS	DNS
10	20	120	422	DNS	DNS	DNS
11	245	152	600	DNS	DNS	DNS
12	600	600	201	DNS	DNS	DNS
MEAN	**357.50***	**334.10***	**492.60***	**277.80**	**326.40**	**457.90**
SEM	**75.76**	**65.66**	**48.22**	**71.47**	**79.35**	**55.12**

Table 18: Study one. Latency to lie test observations. Before, 30 minutes and 2 hours after intravenous injection of morphine at 2mg/kg dose rate in sound and lame broiler chickens. (DNS-did not stand)

** No significant difference seen during Dunn's post test multiple column comparison (P value = 0.0408)*

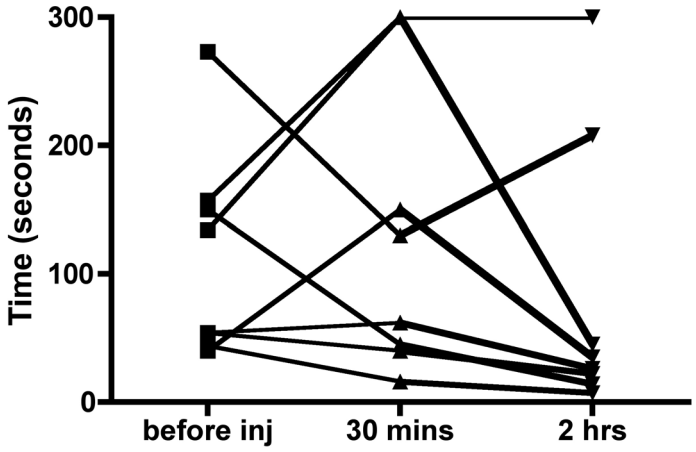


Figure 30: Lame broiler chickens during study one in Obstacle course test after injection of morphine at 2 mg/kg. There was significant increase in time to finish after the injection. P=0.0469. Lines represent individual birds.

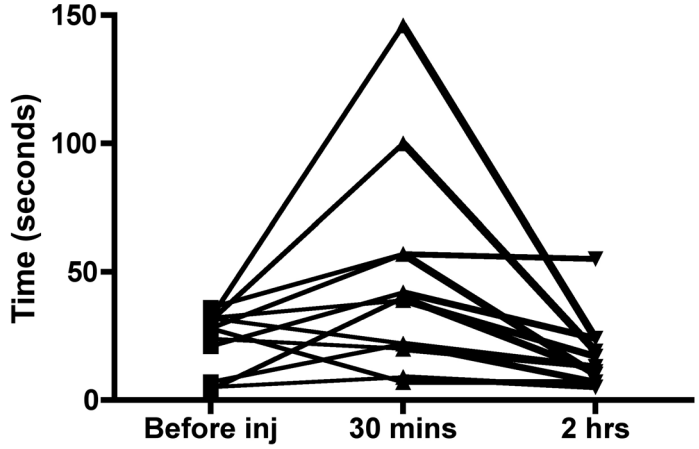


Figure 31: Sound broiler chickens during study one in Obstacle course test after injection of morphine at 2 mg/kg. There was significant increase in time to finish after the injection. P= 0.0032

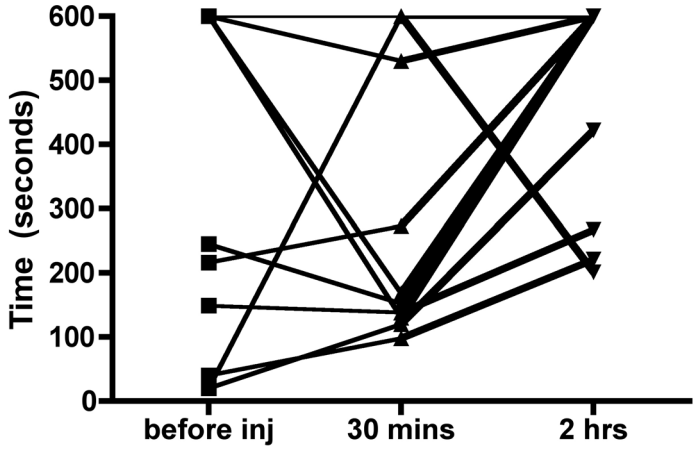


Figure 32: Sound broiler chickens during study one in Latency to Lie test after injection of morphine at 2 mg/kg. There was significant increase in standing time after the injection. P= 0.408. Lines represent individual birds.

an increase in the time taken at 30 minutes after the injection compared to before injection (23 secs), but this difference was non-significant.

Latency to lie Test: Treatment of lame broiler chickens with I/V morphine injection significantly increased the standing time (table 18, figure 32) (P = 0.04). The post-analysis Dunn's multiple comparison test did not show any significant difference within the three observation time points. The lame broiler chickens were able to stand for 48 and 180 seconds more after 30 minutes and 2 hours after the injection, compared to before the injection of morphine (277 secs).

There was no significant difference between the standing time of sound broiler chickens after the morphine injection (P=0.05). These birds stood for 23 seconds less after 30 minutes compared to before the injection (357 secs), but this difference was non-significant. At the 2 hours time point, sound broiler chickens stood for135 seconds more as compared to before injection, but this difference was non-significant.

Salicylic Acid

Obstacle course: One sound chicken wandered around and did not finish the course though it was walking normally. The results are shown in table 19. The injection of salicylic acid significantly reduced the time taken by lame broiler chickens both after 30 minutes and 2 hours (Figure 33) (P = 0.0002). Lame broiler chickens took 45 and 23 seconds less time compared to the time taken before the injection of salicylic acid (99 secs). There was no significant difference between the time taken by sound broiler chickens before and after the treatment (P=0.84).

Latency to lie Test: There was no significant difference in the standing time for lame broiler chickens before and after the injection of salicylic acid (P=0.4) (table 20). There was slight increase in standing time by 38 and 110 seconds at 30 minutes and 2 hours after injection compared to before the injection (315 secs).

There was also no significant difference seen in the standing time for sound broiler chickens before and after the treatment with salicylic acid (P=0.8).

	SOUND			LAME		
	Before injection (sec)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	19	10	26	90	50	82
2	33	17	12	82	16	26
3	8	8	56	80	52	36
4	14	17	7	102	51	28
5	9	14	24	47	35	40
6	8	53	22	40	16	19
7	5	25	17	300	220	230
8	27	12	18	139	57	25
9	10	20	16	54	33	34
10	30	19	16	100	30	34
11	24	10	28	62	34	300
12	DNF	DNF	DNF	DNF	DNF	DNF
MEAN	**17.00**	**18.64**	**22.00**	**99.64[a]**	**54.00[b]**	**76.45[bc]**
SEM	**3.02**	**3.76**	**3.87**	**21.81**	**17.13**	**28.96**

Table 19: Study one. Obstacle Course observations. Before, 30 minutes and 2 hours after intravenous injection of Salicylic acid at 50 mg/kg dose rate in sound and lame broiler chickens. (DNF-did not finish)
Significant difference is shown by the superscript alphabets (P value = 0.0002)

	SOUND			LAME		
	Before injection (sec)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	600	365	431	105	105	600
2	502	600	600	194	188	133
3	600	139	263	600	600	600
4	600	600	600	600	38	600
5	600	600	600	42	60	60
6	600	600	600	600	600	600
7	600	600	600	226	600	600
8	600	600	600	262	230	513
9	600	600	600	38	600	600
10	600	600	600	600	600	54
11	600	600	600	205	268	323
12	600	600	600	DNS	DNS	DNS
MEAN	**555.20**	**542.00**	**315.60**	**315.60**	**353.50**	**425.70**
SEM	**36.83**	**41.50**	**48.29**	**71.21**	**73.93**	**71.22**

Table 20: Study one. Latency to lie test observations. Before, 30 minutes and 2 hours after intravenous injection of Salicylic acid at 50 mg/kg dose rate in sound and lame broiler chickens. (DNS- did not stand)

Placebo

Only 10 lame birds were able to finish the course and stand in the trough. Two sound birds did not finish the course and wandered around. Therefore, they were excluded from the analysis of the obstacle course test. The results for placebo group for OC and LTL are shown in tables 21 and 22.

Obstacle course: There was no significant difference in the time taken to clear the course after injection of normal saline both in lame (P=0.07)and sound broiler chickens (P=0.97).

There was a slight increase at 30 minutes and decrease at 2 hours in the time taken to finish the course in lame broiler chickens compared to the first observation (98 secs). However, this difference was non- significant. In sound birds, there was 4 second non-significant decrease in the time taken.

Latency to lie test: There was also no significant difference in either the lame (P=0.6) or sound broiler chickens (P=0.58) after injection of normal saline.

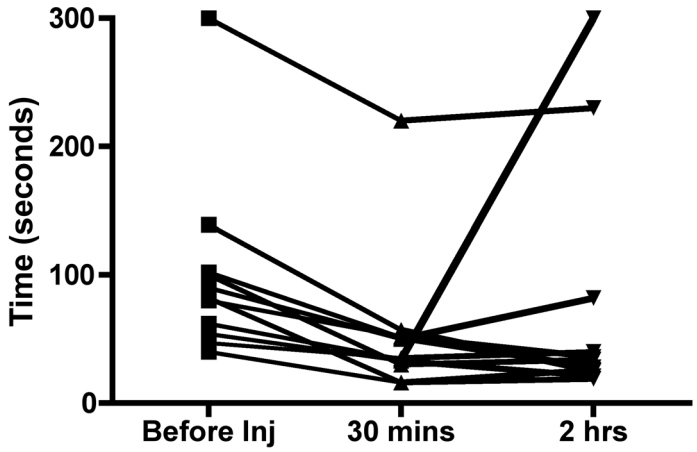


Figure 33: Lame broiler chickens during study one in Obstacle course test after injection of Salicylic acid at 50 mg/kg. There was significant decrease in time to finish after the injection. P= 0.0002. Lines represent individual birds.

	SOUND			LAME		
	Before injection (sec)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	8	25	15	87	8	6
2	4	20	25	268	272	134
3	34	6	11	145	40	46
4	28	5	7	85	100	67
5	6	8	8	142	300	70
6	27	6	12	48	29	34
7	9	12	9	60	15	5
8	5	7	6	46	150	249
9	4	4	4	53	40	28
10	29	19	22	54	98	10
11	DNF	DNF	DNF	DNF	DNF	DNF
12	DNF	DNF	DNF	DNF	DNF	DNF
MEAN	**15.40**	**11.20**	**11.90**	**98.80**	**105.20**	**64.90**
SEM	**3.91**	**2.36**	**2.18**	**22.10**	**33.29**	**23.91**

Table 21: Study one. Obstacle course observations. Before, 30 minutes and 2 hours after intravenous injection of normal saline in sound and lame broiler chickens. (DNF- did not finish)

	SOUND			LAME		
	Before injection (sec)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	568	225	600	136	170	102
2	600	600	600	115	DNS	DNS
3	600	217	600	35	25	29
4	600	600	600	600	600	600
5	600	600	600	600	308	258
6	600	600	600	172	218	600
7	600	600	600	600	600	600
8	600	600	600	66	150	90
9	600	600	142	600	360	600
10	600	600	132	600	44	130
11	600	600	600	DNS	DNS	DNS
12	600	600	600	DNS	DNS	DNS
MEAN	**597.30**	**536.80**	**522.80**	**352.40**	**247.50**	**300.90**
SEM	**2.66**	**42.59**	**52.03**	**83.34**	**69.39**	**84.16**

Table 22: Study one. Latency to lie test observations. Before, 30 minutes and 2 hours after intravenous injection of normal saline in sound and lame broiler chickens. (DNS- did not stand)

Study Two: Analgesic efficacy of butorphanol, morphine and salicylic acid in lame broiler chickens.

Butorphanol

Obstacle course: The results for obstacle course completion after butorphanol injection for both lame and sound birds are shown in table 23. Three lame birds were not able to finish the course and were not included in the analysis. There was a significant reduction in time taken to finish the course 30 minutes after injection as compared to before injection. This difference was also significant after 2 hours of injection (figure 34) (P = 0.01). There was no significant difference in time taken to finish the course after the butorphanol injection in sound birds (P=0.7). The sound broiler chickens took approximately the same time in all three observations.

Latency to lie test: The results for LTL test for butorphanol injection are shown in table 24. There was no significant difference between sound and lame broiler chickens. These results were similar to the study 1 results. Though the lame chickens after the injection of butorphanol stood 140 seconds longer than before injection this difference was not statistically significant (P=0.1). In the case of sound broiler chickens, there was not much difference in the standing time between the three observations (P=0.8).

Morphine

Obstacle course: The results of the obstacle course for morphine injection are shown in table 25. Only one bird was not able to finish the course and was excluded from the study. The lame broiler chickens took approximately 35 seconds more to finish the course at 30 minutes after the injection of morphine compared to before the injection (84 secs). At 2 hours, lame birds took only 2 seconds less than at 30 minutes. This increase was non-significant for both the observation time points (P=0.9). In sound broiler chickens, the time taken to finish the course after 30 minutes was significantly higher than before the injection (24 secs) (Figure 35). This difference further increased by 11 seconds after 2 hours (P=0.02).

Latency to lie test: The results of the LTL test for the birds injected with morphine injection are shown in Table 26. There was non-significant increase in the standing time after the injec-

	SOUND			LAME		
	1 (SEC)	2 (SEC)	3 (SEC)	1 (SEC)	2 (SEC)	3 (SEC)
1	38	56	31	70	44	53
2	39	30	45	98	33	15
3	14	30	22	93	53	57
4	19	20	9	46	28	59
5	19	13	14	62	35	13
6	15	12	13	69	33	20
7	10	13	13	45	26	15
8	6	8	16	63	34	37
9	22	5	11	75	39	78
10	13	23	28	DNF	DNF	DNF
11	15	11	15	DNF	DNF	DNF
12	DNF	DNF	DNF	DNF	DNF	DNF
MEAN	**19.09**	**20.09**	**19.73**	**69.00[a]**	**36.11[b]**	**38.56[ab]**
SEM	**3.18**	**4.38**	**3.28**	**6.06**	**2.76**	**8.02**

Table 23: Study two. Obstacle Course observations. Before, 30 minutes and 2 hours after intravenous injection of butorphanol at 2mg/kg dose rate in sound and lame broiler chickens. (DNF- did not finish)
Significant difference is shown by the superscript alphabets (P value = 0.0103)

	SOUND			LAME		
	1 (SEC)	2 (SEC)	3 (SEC)	1 (SEC)	2 (SEC)	3 (SEC)
1	600	600	600	150	78	150
2	600	600	600	135	600	306
3	203	264	460	98	70	111
4	600	600	600	80	246	330
5	600	600	600	104	550	373
6	600	432	600	210	100	90
7	600	600	600	190	364	404
8	600	494	482	294	373	247
9	600	600	600	270	600	600
10	600	600	600	DNS	110	213
11	435	600	600	470	600	600
12	600	600	600	600	600	600
MEAN	**553.20**	**549.20**	**578.50**	**216.50**	**357.60**	**335.30**
SEM	**34.65**	**30.32**	**14.56**	**49.56**	**65.90**	**53.99**

Table 24: Study two. Latency to lie test observations. Before, 30 minutes and 2 hours after intravenous injection of butorphanol at 2mg/kg dose rate in sound and lame broiler chickens. (DNS- did not stand)

	SOUND			LAME		
	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)
1	19	33	17	300	160	94
2	14	28	22	29	18	20
3	16	10	16	56	242	106
4	19	44	29	45	29	30
5	17	24	58	40	34	24
6	35	102	132	42	88	140
7	38	59	22	69	178	100
8	5	20	12	121	128	139
9	45	163	104	80	300	300
10	46	168	101	80	70	300
11	23	15	25	68	65	32
12	14	37	300	DNF	DNF	DNF
MEAN	**24.25[a]**	**58.58[b]**	**69.83[ab]**	**84.55**	**119.30**	**116.80**
SEM	**3.86**	**16.04**	**24.06**	**22.88**	**27.79**	**30.42**

Table 25: Study two. Obstacle Course observations. Before, 30 minutes and 2 hours after intravenous injection of morphine at 2mg/kg dose rate in sound and lame broiler chickens. (DNF- did not finish)

Significant difference is shown by the superscript alphabets (P value = 0.0242)

	SOUND			LAME		
	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)
1	600	416	600	583	341	600
2	600	441	513	170	70	145
3	510	78	130	138	72	207
4	600	108	199	43	36	12
5	600	237	120	271	405	141
6	600	314	262	103	79	65
7	600	390	240	192	540	570
8	600	600	600	136	600	600
9	142	99	173	25	80	134
10	108	469	600	212	130	300
11	600	190	600	122	116	236
12	600	39	155	86	140	72
MEAN	**513.30[a]**	**281.80[b]**	**349.3[ab]**	**173.40**	**217.40**	**256.80**
SEM	**52.93**	**52.72**	**60.92**	**42.29**	**57.65**	**62.18**

Table 26: Study two. Latency to lie test observations. Before, 30 minutes and 2 hours after intravenous injection of morphine at 2mg/kg dose rate in sound and lame broiler chickens.

Significant difference is shown by the superscript alphabets (P value = 0.0094)

tion in lame broiler chickens (P=0.59). Lame broiler chickens stood for 217.4 ± 57.6 and 256.8 ± 62.1 after 30 minutes and 2 hours of injection, as against 173.40 ± 42.29 seconds before the injection.

In sound broiler chickens, there was a significant reduction in standing time 30 minutes after the injection (P=0.01) (figure 36).

Salicylic Acid

Obstacle course: The results of the obstacle course test for birds injected with salicylic acid are shown in table 27. Two lame birds were not able to finish the course and one sound bird wandered around and did not finish the course. These birds were omitted from the analysis. The lame birds took a shorter time 30 minutes after the injection compared to before the injection of salicylic acid (64 secs). This reduction in time was statistically significant (P=0.001) (figure 37), but at 2 hours, they took 24 seconds more than the first observation to finish the course. It was significantly different from the 30 minutes observation but non-significant from the before injection observation time point. In the sound birds, there was no difference in the time taken to finish the obstacle course after the injection (P=0.6).

Latency to lie test: The results for LTL test are shown in table 28. There was a significant increase in the standing time by lame birds (figure 38) 30 minutes after the injection of salicylic acid (P=0.1). After 2 hours, the birds were able to stand longer than at 30 minutes, but this difference was non-significant.

Placebo

Obstacle course: The results of OC test for placebo injection in both sound and lame broiler chickens are shown in table 29. All the birds were able to finish the course. There was no significant difference in the time to finish the course for both sound (P=0.6) and lame broiler chickens (P=0.4). In the lame broiler chickens, the time taken was increased by 56 seconds after 30 minutes and after 2 hours the time to finish the course doubled as compared to the initial observation (82 secs). There was no difference in sound broiler chickens and the time difference was only 7 seconds more than the before injection observation.

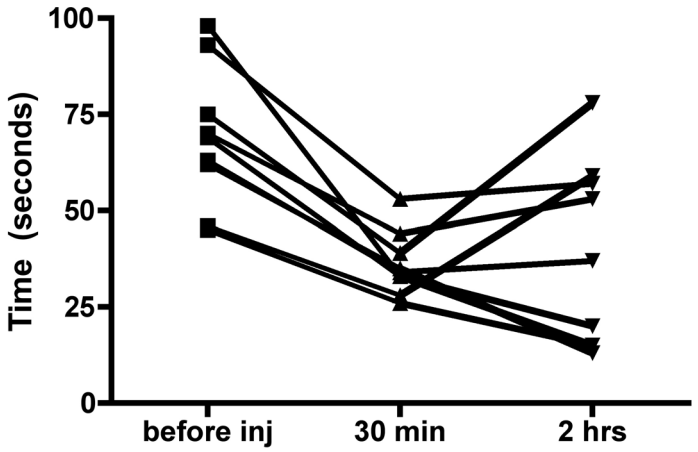


Figure 34: Lame broiler chickens during study two in obstacle course test after injection of Butorphanol at 2 mg/kg. There was significant decrease in time to finish after the injection. P= 0.0103. Lines represent individual birds.

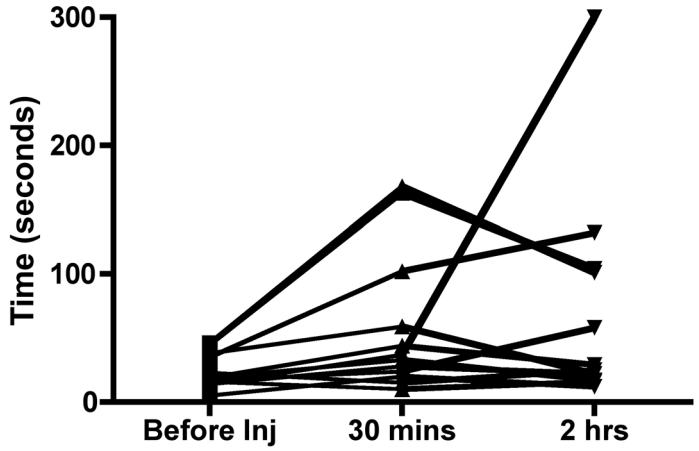


Figure 35: Sound broiler chickens during study two in Obstacle course test after injection of morphine at 2 mg/kg. There was significant increase in time to finish after the injection. P= 0.0242. Lines represent individual birds.

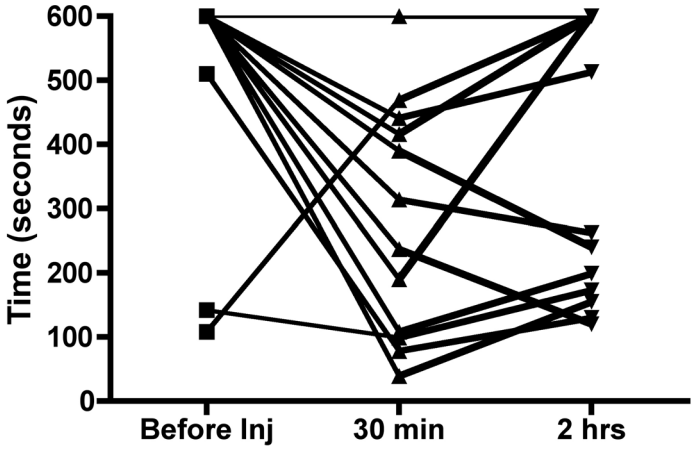


Figure 36: Sound broiler chickens during study two in Latency to lie test after injection of morphine at 2 mg/kg. There was significant decrease in standing time after the injection. P= 0.0094. Lines represent individual birds.

	SOUND			LAME		
	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)
1	19	11	15	67	19	41
2	45	23	14	71	60	300
3	14	15	35	40	44	115
4	23	48	48	65	33	38
5	20	52	54	53	47	56
6	8	36	11	49	32	44
7	25	30	57	101	36	120
8	18	15	27	70	29	63
9	39	41	27	79	37	60
10	26	58	26	47	18	50
11	20	17	12	DNF	DNF	DNF
12	DNF	DNF	DNF	DNF	DNF	DNF
MEAN	**23.36**	**31.45**	**29.64**	**64.20[a]**	**35.50[b]**	**88.70[a]**
SEM	**3.18**	**5.00**	**5.09**	**5.67**	**4.00**	**25.21**

Table 27: Study two. Obstacle Course observations. Before, 30 minutes and 2 hours after intravenous injection of Salicylic acid at 50mg/kg dose rate in sound and lame broiler chickens. (DNF- did not finish)
Significant difference is shown by the superscript alphabets (P value = 0.0013)

	SOUND			LAME		
	1 (SEC)	2 (SEC)	3 (SEC)	1 (SEC)	2 (SEC)	3 (SEC)
1	342	406	345	484	359	129
2	600	304	600	125	220	230
3	590	474	210	210	428	396
4	600	600	600	400	600	600
5	380	372	117	220	600	600
6	43	35	46	78	224	600
7	600	600	600	290	600	600
8	600	600	600	150	156	259
9	600	600	600	45	68	20
10	223	240	180	81	122	75
11	600	600	381	270	600	600
12	600	600	600	194	342	600
MEAN	**481.50**	**452.60**	**404.90**	**212.30[a]**	**359.90[b]**	**390.80[ab]**
SEM	**54.97**	**53.74**	**63.09**	**38.30**	**58.84**	**67.62**

Table 28: Study two. Latency to lie test observations. Before, 30 minutes and 2 hours after intravenous injection of Salicylic acid at 50 mg/kg dose rate in sound and lame broiler chickens.

Significant difference is shown by the superscript alphabets (P value = 0.0115)

Latency to lie test: The results for LTL test are shown in table 30. There was no difference seen in either the lame and sound broiler chickens for LTL test. The sound birds were able to stand for approximately same time for all the three observations.

Handling Control

Obstacle course: The results of obstacle course for the control group are shown in table 31. In this case only one lame bird was not able to finish the course and was excluded from the study. There was a significant increase in the time taken to finish the course at 30 minutes and 2 hours (figure 39) (P=0.01). At 2 hours, the lame birds took three times longer than the first observation (58 secs). In sound birds there was a non-significant increase in time taken to finish the course (P=0.12). The birds took 11 seconds and 12 seconds more during the second and third observation compared with first observation (24 secs).

Latency to lie test: The results for LTL test for the control group are shown in table 32. One lame bird did not finish the obstacle course, was not able to stand and was excluded from the study. There was no significant difference seen in either lame or sound broiler chickens at the three observation time points, but in case of lame birds at 2 hours, the standing time halved compared to the 1st observation (149 sec) (P=0.47). The P value for sound birds in LTL test was 0.9.

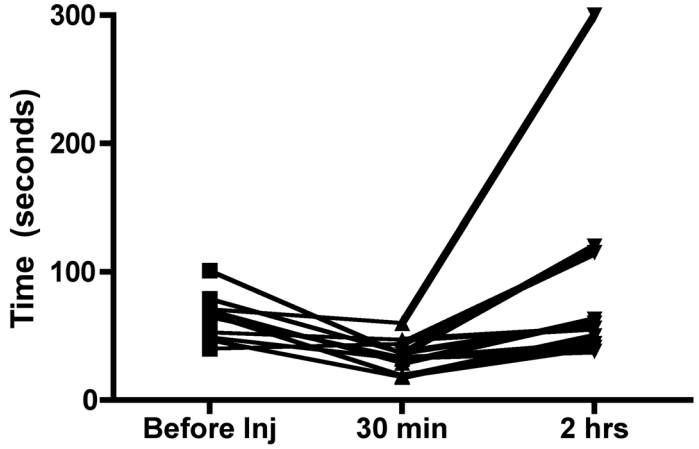


Figure 37: Lame broiler chickens during study two in Obstacle course test after injection of Salicylic acid at 50 mg/kg. There was significant decrease in time to finish after the injection. P= 0.0013. Lines represent individual birds.

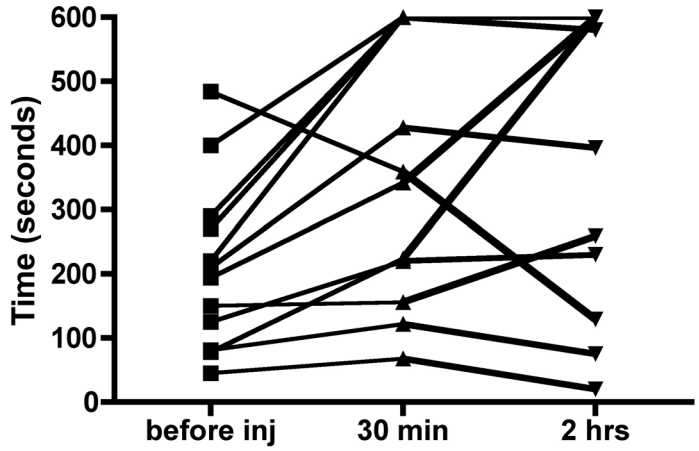


Figure 38: Lame broiler chickens during study two in Latency to Lie test after injection of Salicylic acid at 50 mg/kg. There was significant increase in standing time after the injection. P= 0.0115. Lines represent individual birds.

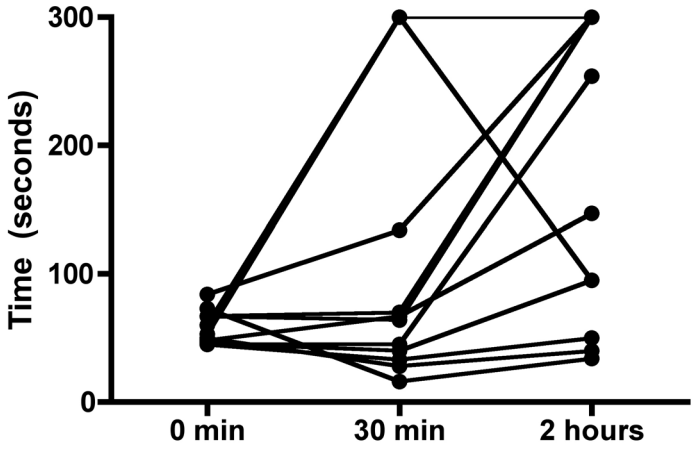


Figure 39: Lame broiler chickens of control group during study two in Obstacle course test. There was significant increase in time to finish after the injection. P= 0.0129. Lines represent individual birds.

	SOUND			LAME		
	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)
1	40	39	28	193	300	300
2	26	26	12	23	185	275
3	16	26	21	160	85	74
4	19	12	18	24	23	37
5	12	20	18	118	300	183
6	10	30	45	59	15	30
7	25	84	70	61	128	180
8	12	10	13	40	300	300
9	12	17	39	146	146	136
10	10	15	11	63	73	53
11	25	26	23	42	76	300
12	36	27	34	62	34	80
MEAN	**20.25**	**27.67**	**27.67**	**82.58**	**138.80**	**162.30**
SEM	**2.95**	**5.65**	**4.96**	**16.45**	**31.52**	**31.47**

Table 29: Study two. Obstacle Course observations. Before, 30 minutes and 2 hours after intravenous injection of normal saline in sound and lame broiler chickens.

	SOUND			LAME		
	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)	Before injection (sec)	30 minutes after injection (sec)	2 hours after injection (sec)
1	600	537	595	337	DNS	DNS
2	600	600	600	285	294	248
3	600	600	600	170	145	166
4	600	600	600	112	118	211
5	600	538	420	92	50	99
6	577	337	98	600	421	395
7	600	159	185	404	475	348
8	600	600	600	300	292	430
9	600	600	600	115	67	460
10	600	570	600	540	362	600
11	557	600	600	155	76	98
12	600	600	600	600	600	600
MEAN	**594.50**	**528.40**	**508.20**	**309.20**	**241.70**	**304.60**
SEM	**3.90**	**39.97**	**51.89**	**55.03**	**55.90**	**57.30**

Table 30: Study two. Latency to lie test observations. Before, 30 minutes and 2 hours after intravenous injection of normal saline in sound and lame broiler chickens. (DNS- did not stand)

	SOUND			LAME		
	Before injection (sec)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	14	28	20	48	67	147
2	30	101	24	60	300	300
3	14	51	50	45	33	50
4	30	12	18	46	40	95
5	10	20	36	553	300	95
6	21	23	42	45	45	254
7	24	35	50	67	70	300
8	37	19	22	50	28	40
9	30	32	38	84	134	300
10	33	55	86	67	64	300
11	25	16	17	73	16	34
12	20	28	33	DNF	DNF	DNF
MEAN	**24.00**	**35.00**	**36.33**	**58.00^{ab}**	**99.73^{b}**	**174.10^{a}**
SEM	**2.43**	**7.08**	**5.65**	**3.97**	**31.30**	**35.17**

Table 31: Study two. Control group. Obstacle Course observations. Before, 30 minutes and 2 hours in sound and lame broiler chickens. (DNF- did not finish)
Significant difference is shown by the superscript alphabets (P value = 0.0129)

	SOUND			LAME		
	Before injection (SEC)	**30 minutes after injection (sec)**	**2 hours after injection (sec)**	**Before injection (sec)**	**30 minutes after injection (sec)**	**2 hours after injection (sec)**
1	74	585	284	DNS	25	35
2	313	134	183	56	83	98
3	600	600	600	10	48	33
4	600	85	58	76	600	21
5	600	600	251	48	21	44
6	463	600	600	25	38	39
7	491	600	600	150	156	176
8	600	600	600	41	59	45
9	560	358	587	376	526	187
10	600	600	600	600	482	90
11	96	90	90	267	178	160
12	600	600	600	DNS	DNS	DNS
MEAN	**466.40**	**454.30**	**421.10**	**149.90**	**201.50**	**84.36**
SEM	**57.15**	**64.35**	**65.45**	**57.33**	**67.00**	**18.88**

Table 32: Study two. Control group. Latency to lie test observations. Before, 30 minutes and 2 hours in sound and lame broiler chickens.

Discussion

In this study, we correlated the pharmacokinetic parameters of healthy broiler chickens with the pharmacodynamics of morphine, butorphanol and salicylic acid in lame broiler chickens. Figures 40 to 42 show the relationship between the plasma concentration of butorphanol, morphine and salicylic acid and time taken to finish the obstacle course and standing time in latency to lie test in case of lame broiler chickens in both the studies.

Commercial broilers were assessed for severity of lameness by previously validated tests. Gait scoring (Kestin et al., 1992) was used to select lame birds from the flock. Gait scoring divides the walking ability of chickens into six categories from GS 0 to 5. We randomly selected the birds from the flock of 500 chickens. Those with gait score 0 to 2 were considered sound and 3 were lame. The birds with gait score 5 were not included in the study as we considered it unethical to use birds in severe pain. The gait score of broilers gets worse over time as they become heavier (Kestin et al., 1992). After the gait scoring, the birds were subjected to Obstacle Course (OC) and Latency to lie (LTL) testing. The OC test was earlier used by (McGeown et al., 1999). They constructed a U shaped course with 2 obstacles, but we simplified this course and made a 2 metre long straight course with only one obstacle. The feeder was kept on one side of the course such that the test birds had to clear the obstacle in order to reach the feeder. All the birds were kept off feed for 4 hours before the start of the test. Another bird was allowed to eat the feed as the test bird started its course to encourage the test bird to complete the course and join it at the feeder. Some severely lame birds were not able to walk while some healthy birds just wandered around and did not finish the course. Such birds were excluded from the analysis. This test is based on the fact that the lame birds will walk slowly and thus will take longer time to finish the course compared to sound and healthy birds. The walking ability and pace of lame broiler chickens should increase after the injection of an analgesic drug as the birds should feel less pain than before injection or the birds in the control groups. In both the pharmacodynamic studies the lame birds took significantly more time to finish the course compared to the sound broiler chickens.

The latency to lie test was first described by Weeks, (2001). This test is based on the fact that

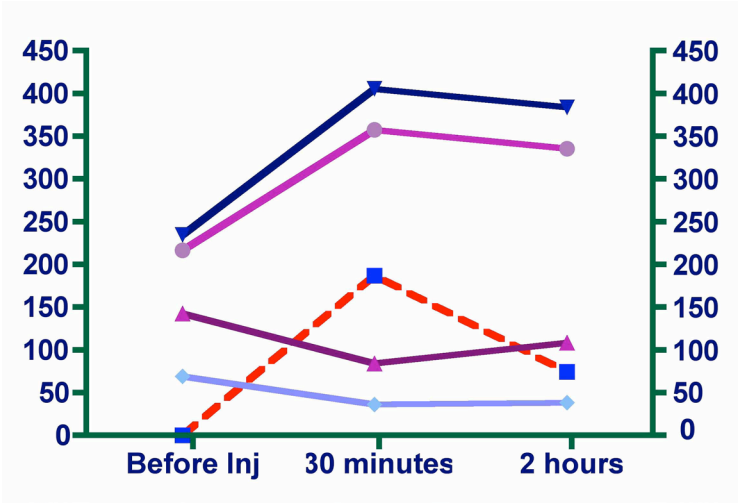


Figure 40: Analgesic effect of Butorphanol in lame broiler chickens

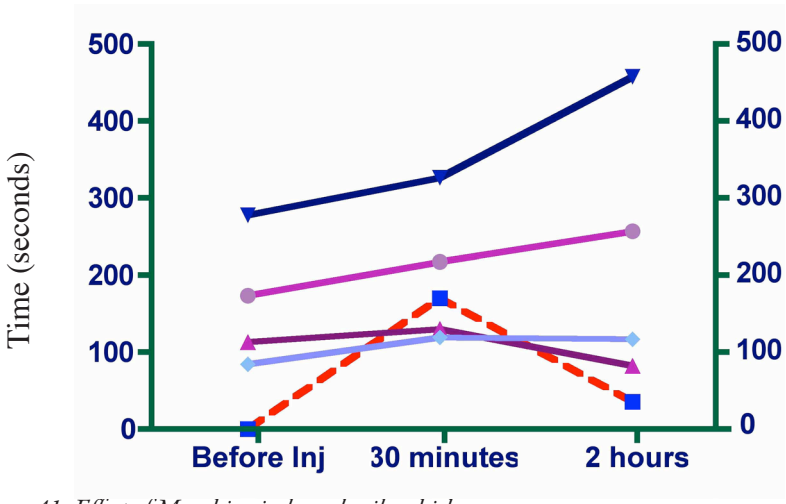


Figure 41: Effect of Morphine in lame broiler chickens

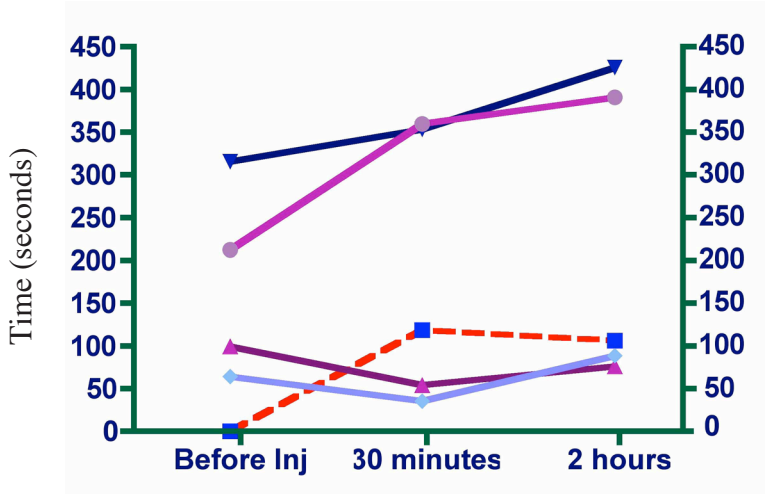
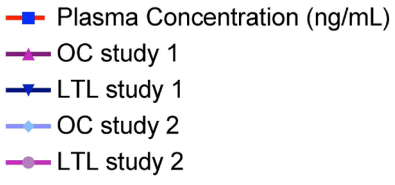


Figure 42: Analgesic effect of Salicylic acid in lame broiler chickens



broiler chickens have strong disinclination to sit in water. The better the leg health of the bird, the longer it will stand. The test involved making the broiler chickens stand in shallow tepid water in a waterproof pen. The broiler chickens to be tested were placed in a waterproof pen and were given 30 minutes to acclimatise in the novel environment. Then, the floor pen was filled with tepid water (32°C) up to 30 mm depth. The cut off time for this test was 15 minutes. Weeks et al., (2002) did a little modification in the test. They did not find any significant difference in LTL scores and different settling time periods (Weeks et al., 2001; 2002)). (Berg and Sanotra, 2003) modified the LTL test. They tested each bird individually, omitted the settling time period and also kept the cut off time at 10 minutes. All the birds they tested at 14 farms sat at or before 10 minutes. Each bird to be tested was placed in a small plastic tub filled with shallow tepid water. They found strong negative correlation between gait scores and LTL standing time. Birds with lower gait scores stood longer in LTL. We used the modified LTL test described by Berg and Sanotra, (2003) because it is less time consuming and inexpensive. The modified LTL tested the birds individually, thus preventing a group effect, in which one bird can influence the result of others.

Butorphanol reduced the time taken by lame broiler chickens to finish the course. This reduction was non-significant in study one, but became significant in study two. In study one, the lame broiler chickens before the injection took more time to finish the course compared to study two. The leg health of the chickens in study one was worse than in study two, so the drug effect was not as profound as in study two. The average gait score of lame birds in study one was 3.41 while in study two, it was 3.15. Time taken to finish the course by sound broiler chickens was similar in both experiments. In study two, the effect of butorphanol was persistent until 2 hours after the injection. This trend was not seen in study one. This may be due to the higher severity of lameness in lame chickens used in study one. The latency to lie test gave the same trend in both the studies. The lame broiler chickens stood for longer time after the injection of butorphanol but the difference was non significant. The increased plasma concentrations of butorphanol decreased the time taken to finish the obstacle course while increasing the standing time. The plasma concentration at 2 hours was 74.37 ng/mL compared to 186.86 ng/mL at 30 minutes. Therefore, there is a relationship between the plasma levels of butorphanol and

the degree of analgesia in lame broiler chickens (figure 40). There was no effect of butorphanol in sound broiler chickens both in OC and LTL. In study one, there was non-significant reduction in the time taken to finish in the course during third observation. This could be due to a "learning effect", but this was not seen in study two. The results of this experiment agreed with the observations of Buchwalder and Huber-Eicher, (2005) in turkeys. An analgesic effect of butorphanol was also demonstrated by Gentle et al., (1999) in domestic fowl, (Curro et al., 1994; Paul-Murphy et al., 1999; Sladky et al., 2006; Paul-Murphy et al., 2009a) in psittacines and (Paul-Murphy et al., 2009b) in conures.

An injection of morphine induced sedation in broiler chickens. This sedation slowed their walking ability and they tend to sit down rather than walk. Thus, there was a significant increase in the time taken to finish the course after the injection of morphine. This trend was seen in both sound and lame broiler chickens in both the studies. In the case of sound birds in study one, the time taken to finish the course after 2 hours, was approximately the same as the observation made before injection. This suggests that with a decrease in plasma concentrations of morphine, the level of drowsiness also diminished (figure 41). According to our pharmacokinetic study, the plasma concentrations at 30 minutes and 2 hours were 170 ng/mL and 35 ng/mL, respectively. In lame broiler chickens, after 2 hours there was a reduction in the time taken compared to the observation before injection, but this difference was non-significant. This suggests that morphine could be analgesic at lower concentration, but at higher concentration, it causes sedation and drowsiness in broiler chickens. In study two, the drowsiness and sedation was seen until 2 hours unlike in study one.

In LTL testing during study one, there was no significant difference seen in sound birds, but in case of lame birds, there was significant difference, but the post test Dunn's multiple column comparison did not show any significant difference among the three observations. Also only 8 out of 12 birds could stand. There was no significant difference in standing time of lame birds in study two. The standing time for sound chickens 30 minutes after the injection was significantly less than before the injection. The birds were sedated and were not able to stand as long as before the injection of morphine. After 2 hours, the difference was non significant. These results with morphine suggest that it causes sedation in broiler chickens. Morphine could be analgesic at

lower doses, but at lower dose, the plasma concentration did not last long enough to produce as persistent effect. Gentle et al., (1999) injected morphine at lower dose rates and did not find any analgesic effect in sodium urate induced arthritis in domestic fowl. Hughes, (1990) and Sufka and Hughes, (1990) found that morphine causes hyperalgesia in domestic fowl, but in our study we did not find any such effect. The chickens in our study looked sedated and sleepy after injection of morphine. The LTL test, does not differentiate between sedation and analgesia. Some birds braced on the walls of the tub and were able to stand for long time. But in OC, these birds did not perform well, because of sedation.

Salicylic acid provided good analgesia in lame broiler chickens, but this effect did not last longer than 2 hours. After 2 hours there was non significant increase in the time taken to finish the course when compared with before the injection time point. This trend was seen in both studies. In case of LTL in study two, lame birds stood for a significantly longer time after 30 minutes compared to before the injection of salicylic acid, but no significant difference was seen in the first study. There was no effect of salicylic acid in sound birds either in OC or in LTL. These results indicate that salicylic acid is analgesic in broiler chickens but this effect diminishes after 2 hours. The relationship between the plasma concentration of salicylic acid and both these test is shown in figure 42. Increase in salicylic acid plasma concentration increased the analgesic effect. Birds were able to walk faster and stood for a longer time. The non-significant difference in sound chickens in both the tests suggests that gait score 0 to 2 birds do not feel much pain and thus the analgesic drugs do not produce any effect. Hocking et al., (2005) demonstrated analgesic effects of salicylic acid in domestic fowl. They induced lameness in domestic fowl by intraarticular injection of sodium urate microcrystals. The behaviour parameters of grooming, standing, feeding and drinking were recorded. Salicylic acid improved these behaviour parameters but they were not fully restored. The dose rate required for analgesia ranged from 100 to 200 mg/kg.

All the results from the placebo group in both the studies were non-significant, but the trend suggested that the lame birds progressively became worse with time. The lame birds from the control group took longer to finish at 30 minutes, but the difference was not significant. At the 2 hours observation time point, this difference further increased and became significant. The

results of the control group are similar to the placebo. This shows that there was no stress or pain caused by injection. In the second study we did not observe any "learning effect" in control and placebo birds. The reduction in time to finish the OC and increase in standing time in case of LTL were due to analgesic effect of butorphanol and salicylic acid. When the lame birds were not given any treatment in case of control or placebo group, the severity of lameness increased with time.

The results from both the behaviour studies suggests that butorphanol and salicylic acid are analgesic in broiler chickens, but their effect did not last long. This correlated with the short half lives as seen in the pharmacokinetic study. The approximate therapeutic range in birds for butorphanol is 50 to 80 and salicylic acid is 50 to 110 ng/mL. At these dose rates these levels lasted for 2 hours after intravenous bolus administration. In order to increase the duration of analgesia, sustained released formulations e.g. liposome encapsulated (Sladky et al., 2006) or slow released drug delivery systems such as mini osmotic pumps could be used.

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CHAPTER FIVE

Plasma concentrations of butorphanol in a Northern Royal Albatross

ABSTRACT

Butorphanol is an opioid analgesic drug that acts predominantly on κ opioid receptors. It is considered an effective analgesic in birds but its use is limited by our lack of knowledge of its pharmacokinetics. In this experiment, we analysed the plasma concentration of butorphanol after 4 mg/kg, I/V, in a Northern Royal Albatross. This sea bird was admitted to the Massey wildlife clinic for treatment of a fracture. The $T_{1/2}$, *Vd*, *Cl* and *MRT* were 85.5 minutes, 896.1 ml/kg, 7.26 mL/kg/min, and 194.5 minutes, respectively. The levels remained above the minimum effective concentration for mammals for about 4 hours. Though we have data from only one albatross for butorphanol kinetics, it confirms the chicken data: butorphanol does not last as long in birds as in mammals.

5.1.1 INTRODUCTION

Avian veterinarians always face problems when deciding the dosing regimen of drugs to be used for treatment of various conditions in birds because of the scarcity of information on the pharmacology of even the most commonly used drugs in birds. Class *Aves* contains more than 9500 species and such a large number makes it impossible to conduct pharmacokinetics in each and every wild avian species. Also, administration of drugs in endangered birds without any prior knowledge of its effects in birds can raise ethical problems. The extrapolation of pharmacokinetic parameters for birds from mammalian studies has been shown to generate a higher percent error compared to extrapolation between bird species (Hunter et al, 2008). There is no established model of drug research for avian species. This is also true for pharmacology of analgesic drugs in birds.

Butorphanol is currently the recommended analgesic drug for birds basaed on anaecdotal evidence. Birds have higher number of kappa receptors and since butorphanol is a kappa agonist, it is assumed to be the analgesic of choice in birds.

Butorphanol improved the welfare and increased the walking ability of lame turkeys (Buchwalder and Huber-Eicher, 2005). The analgesic efficacy of butorphanol has also been demonstrated by its isofurane sparing effect. When injected intramuscularly at 1 mg/kg, butorphanol reduced the minimum alveolar concentration for isoflurane in cockatoos (Curro et al. 1994). At this dose rate, butorphanol also increased the threshold for electrical stimulus in African grey parrots (*Psittacus erithacus*) (Paul-Murphy et al. 1999). Sladky et al. (2006), found that analgesic effects of butorphanol did not last long after intramuscular injection at 5 mg/kg. They used liposome encapsulated butorphanol to increase the duration of analgesia. The analgesic efficacy of liposome encapsulated butorphanol was also demonstrated in Conures (Paul-Murphy et al. 2009a) and Hispaniolan Parrots (Paul-Murphy et al. 2009b). Butorphanol has also been considered safe for perioperative analgesia (Klaphake et al. 2006).

All these studies described the analgesic efficacy of butorphanol in avian species, but none of the studies are supported by pharmacokinetics. There is a single study reporting pharmacokinetics of butorphanol in red tailed hawks and great horned owls (Riggs et al. 2008). The best fit for

butorphanol's pharmacokinetic profile was a two and one compartmental model after 0.5 mg/kg, I/V and I/M administration in both species. The elimination half-life was 0.94 hours in red tailed hawks and 1.79 hours in great horned owls. The clearance (L/h/kg) and volume of distribution (L/kg) at steady state was 3.4 and 2.8, for red tailed hawks and 1.5 and 2.06 for great horned owls, respectively.

In our previous study (chapter two) we described the pharmacokinetics and pharmacodynamics of butorphanol in chickens. The terminal half life was 71.3 minutes, volume of distribution was 6.9 L/kg and clearance was 67.5 mL/min/kg after a 2 mg/kg I/V dose. Butorphanol had higher tissue distribution and faster clearance in chickens than mammals. In our pharmacodynamic study butorphanol increased the walking pace and standing time of lame broiler chickens. The minimum effective concentration in plasma that should be maintained for analgesia in chickens is 50 to 80 ng/mL and these levels were maintained for 2 hours after 2 mg/kg I/V.

A Northern Royal Albatross was admitted in Massey University Teaching Hospital's wildlife ward with a history of spiral femur fracture. The fracture was repaired using a standard Type 1 external fixation method with an intramedullary pin. The surgery was performed under isoflurane anaesthesia. Butorphanol was injected pre-operatively in this injured albatross at 4 mg/kg I/V, which is the standard dose used in Massey Veterinary Teaching Hospital. The objective was this study was to assess the duration of therapeutic plasma concentration of butorphanol in the albatross under surgical conditions.

5.1.2 EXPERIMENT NINE: Plasma concentrations of butorphanol in a Northern Royal Albatross

Case History

An 8 months old, Northern Royal Albatross was admitted to the wildlife ward of Massey University Teaching Hospital with the history of a sore left leg. The affected leg was radiographed under isoflurane anaesthesia. Radiographs showed a long oblique femur fracture with minor displacement. The fracture was repaired by placing a intramedullary pin and external fixator. The

surgery was performed under isoflurane anaesthesia. Butorphanol injection 10 mg/mL (Lloyd Laboratories, New Zealand) was injected intravenously at 4 mg/kg before surgery. An intravenous fluid line was set up in the left wing vein and 0.9% saline was given at 15 mL/kg/hr. Co-amoxiclav was given intravenously during the surgery at 50 mg/kg. Blood samples were taken at 0, 0.25, 0.5, 1. 1.5, 2 and 4 hours throughout surgery and during the post-operative recovery period. The heparinised vials were kept chilled immediately after collection and were centrifuged at 3000 rpm for 10 minutes. Plasma was pipetted out and kept at -70 ºC until day of analysis.

Sample Analysis

The plasma samples were analysed by high performance liquid chromatography described in chapter 2. Briefly, the HPLC system consisted of LC-20AD pumps, SIL-20AC HT auto-injector, diode array detector SPD- M20A, CTO-20A column oven, DGU-20A3 Degasser (Shimadzu, Japan). The chromatographs were analysed in LC solutions. The analytical column used was Phenomenex C18(2) (150 X 4.6 mm i.d, 5 µm particle size). The mobile phase consisted of 0.1 M phosphate buffer pH 4.8: acetonitrile (80:20) with flow rate of 1 mL/min. The separation was achieved under isocratic conditions at 30°C. The injection volume was 50 µL and the detector was set at 202nm wavelength.

Sample Preparation

The plasma samples were prepared by a solid phase extraction procedure using Phenomenex Strata X Reversed Phase SPE cartridges. The standard solution used to spike the plasma samples was prepared in the Milli-q water. 300 µL of plasma was spiked with 300 µL of the standard solution and 300 µL of concentrated HCl was added and vortex mixed for 1 minute. Then this solution was centrifuged at 3000 rpm for 10 minutes. The supernatant was separated and loaded into the SPE cartridge preconditioned with one volume of methanol followed by one volume of water. The first wash used 3 mL of water and dried for 2 minutes followed by a second wash with 2 mL of 40% methanol and again the cartridge was dried for 2 minutes. The elution was made with 100% methanol . The sample was dried under gentle stream of air at 20°C and was reconstituted with 200 µL of mobile phase. The injection volume was 50 µL and each sample was injected three times in the HPLC system.

Data Analysis

All the chromatograms were analysed for peak height, area, width and the concentration for the unknowns using the software LC solutions (Shimadzu). The standard calibration curve was also processed in the same software

Pharmacokinetic Analysis

Pharmacokinetic parameters were calculated using standard equations in a spreadsheet (Excel, Microsoft). These included half life of the terminal phase ($T1/2_{\lambda z}$), area under the curve extrapolated from time zero to infinity ($AUC_{0-\infty}$), area under the moment curve extrapolated from time zero to infinity ($AUMC_{0-\infty}$), volume of distribution (Vd), clearance (Cl) and mean residence time (MRT). The plasma concentration data fitted well in a 2-compartment model with first order rate constant using the equation $Cp=A_1 \cdot e^{-\alpha t} + A_2 \cdot e^{-\beta t}$. The micro rate constants k_{10}; elimination rate constant from the central compartment, k_{12} and k_{21}; distribution rate constants within the central and peripheral compartments were also calculated.

Results

The concentration time curve and semi log plot of concentration time curve for butorphanol after intravenous administration at 4 mg/kg in albatross is shown in figures 43 and 44, respectively. The pharmacokinetic parameters thus calculated are given in table 33. The terminal half life was 94 hours, volume of distribution was 0.8 L/kg and clearance was 0.6 mL/min/kg.

Discussion

Butorphanol is recommended by some people for providing analgesia in avian species. However, little information is available on its pharmacokinetics in different birds. An injured albatross was admitted in wild life ward and provided us a good opportunity to analyse the plasma concentration of butorphanol and to find out how long the minimum effective concentrations in plasma are maintained. In our previous study on the pharmacodynamics of butorphanol in chickens (chapter 4), we found that 50 to 80 ng/mL should be maintained for effective analgesia in birds. In this albatross, after 4 mg/kg I/V, the concentrations of butorphanol were higher than the minimum effective concentration for 4 hours. We did not conduct any behavioural analysis

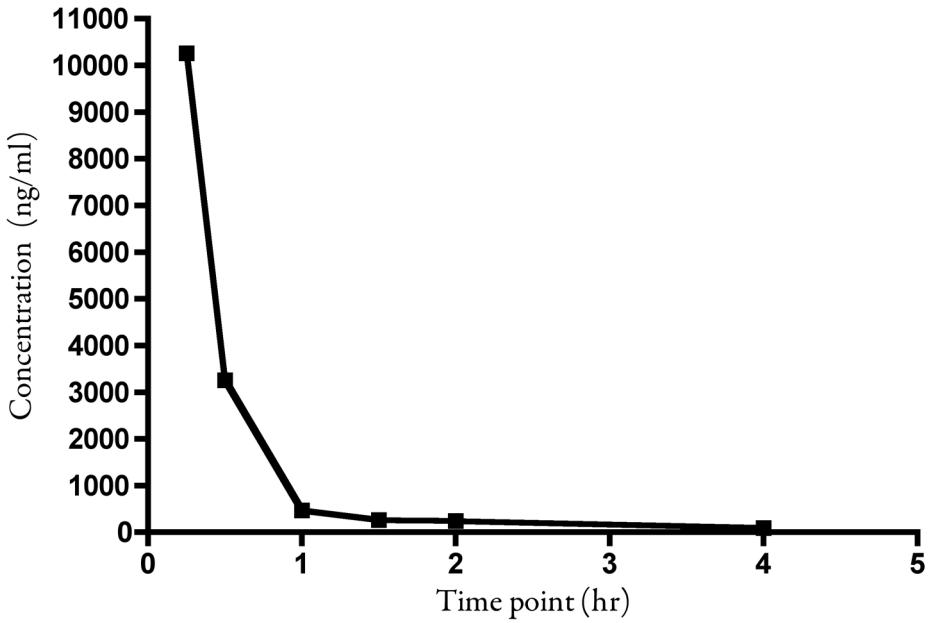

Figure 43: Concentration time curve for Butorphanol after intravenous administration at 4 mg/kg in a Northern Royal Albatross

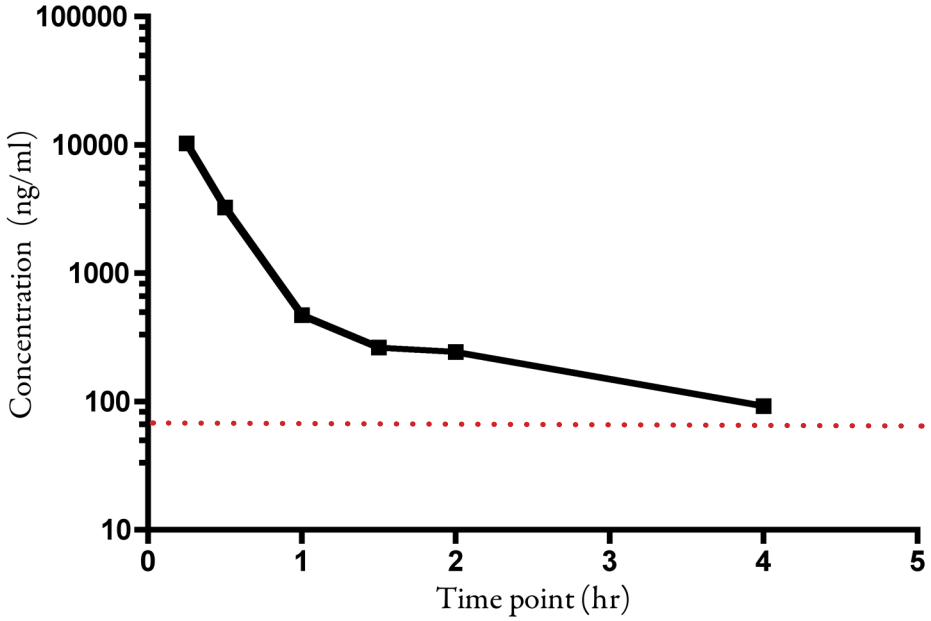

Figure 44: Semi-log plot of concentration time curve for Butorphanol after intravenous administration at 4 mg/kg in a Northern Royal Albatross. Red dotted line represents the minimum effective plasma concentration required to maintain analgesia in broiler chickens.

Parameters	Units	Mean
Noncompartmental		
$AUC_{(0-\infty)}$	ng.min/mL	640716.4
$AUC_{(0-t)}$	ng.min/mL	628153
$AUMC_{(0-\infty)}$	$ng.min^2/mL$	14957395.8
Vd_{area}	mL/Kg	851.2
Cl	mL/min/Kg	6.23
$T_{1/2\lambda z}$	minutes	94.5
$MRT_{\lambda z}$	minutes	146.5
Compartmental		
A_1	ng/mL	46789.43
A_2	ng/mL	543.85
α	1/min	0.098
β	1/min	0.007
k_{21}	1/min	0.008
k_{12}	1/min	0.011
k_{10}	1/min	0.086
$T_{1/2\alpha}$	minutes	7.07
$T_{1/2\beta}$	minutes	99
$Vd_{(central)}$	mL/Kg	84.5
Vd_{ss}	mL/Kg	1246.37
$Vd_{(peripheral)}$	mL/Kg	1161.87
Cl	mL/min/Kg	0.59

A_1 and A_2 are mathematical constants, α distribution rate constant, β elimination rate constant, k_{21}, k_{12}, k_{10} are the microconstants, $T_{1/2\alpha}$ distribution half-life, $T_{1/2\beta}$ elimination half-life, $T_{1/2\lambda z}$ half-life of terminal phase.

Table 33 : Compartmental and noncompartmental pharmacokinetic parameters for butorphanol after 4 mg/kg intravenous dose rate in Northern Royal Albatross

on this albatross as it was under anaesthesia, thus our assumptions on the analgesia are based on the pharmacodynamics in chickens. If these assumptions are correct then these levels will also provide good analgesia in the albatross. Butorphanol provided analgesia in parrots after 1 and 2 mg/kg intramuscular injection, but it did not last longer than 1 and 2 hours respectively (Paul-Murphy et al. 1999; Sladky et al. 2006). Butorphanol was cleared faster in albatross as compare to hawks and owls (Riggs et al. 2008) but much slower as compared to chickens. Faster clearance in chickens could be due to smaller body weight and higher metabolic rate as compared to hawks, owls and albatross. The volume of distribution was much less in this albatross compared to other avian species. The pharmacokinetic parameters in this albatross differed from other stated species. In this study, the number of subjects was only one and that too was injured albatross, suffering from pain. The distribution and metabolism of drugs vary with change in physiological state of an animal. The levels of creatinine kinase were elevated in this albatross, which is associated with rhabdomylosis and also could be onset of acute renal failure (De Meijer et al, 2003). Human patients with partial renal impairment cleared butorphanol much slower as compared to the healthy subjects (Shyu et al, 1996). Kidney function in some salt water animals is also different from terrestrial mammals. Thus the slower clearance and lower volume of distribution seen in this experiment could be due to some degree of renal impairment and traumatic shock. Shock also reduces the liver blood flow and hepatocellular function, as well as circulating blood volume, resulting in low clearance and volume of distribution. The blood samples were collected during the surgery. The pharmacokinetic parameters vary when the subject is under general anaesthesia. In the horse a significant reduction is seen in the clearance of fentanyl metabolites under isoflurane anaesthesia primarily because of reduction in renal blood flow (Thomasy et al., 2007).

This experiment was not intended to study pharmacokinetics. The major limitation of this study is the number of subjects, and the fact that the albatross was not healthy. It presents valuable information about butorphanol dose regimens in injured birds, as the pharmacokinetics were studied under real-life surgical conditions. It is the only pharmacokinetics study of an analgesic drug in a sea bird.

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CHAPTER SIX

General Discussion

Pain has been extensively studied in mammals. A wide range of analgesic drugs are used to alleviate pain in both humans and animals. There is a paucity of information regarding pharmacokinetics and pharmacodynamics of analgesics and pain mechanisms in birds. Avian veterinary surgeons are always forced to extrapolate the avian pharmacokinetic parameters from mammalian studies. Though extrapolations should always be done with caution, parameters for birds extrapolated from mammals had higher percent errors compared to those calculated from other birds (Hunter et al. 2008). The objective of this research project was to conduct PK and PD studies for opioids and NSAIDs in broiler chickens. The data generated from this research could be used as basis of extrapolation for other wild and rare avian species. The pharmcokinetic parameters and dosing regimens can be calculated by allometeric scaling. However, the albatross study shows that direct pharmacokinetics analysis is preferred.

Opioids and NSAIDs are the most widely used and researched analgesic drugs. In this study, we chose I/V route of administration, though oral route is more easier. There is not much information available regarding absorption and bioavailablity of analgesic drugs chosen in this study. The I/V study provide accurate rate of metabolism without any interference from the gut contents. We can calculate the percent drug absorption after other routes after comparison with I/V route. It can be argued that even after I/V route, bioavailability is not 100% (Toutain and Bousquet-Melou, 2004). The drug can undergo first past metabolism in lungs, but it is easier to deliver drugs and withdraw blood from veins as compared to arteries. Intravenous blood sampling is standard protocol for pharmacokinetic studies.The pharmacokinetics of morphine have been reported in various other species including man (Hoskin et al. 1989; Brunk and Margrieta 1974; Stanskl et al., 1979), dogs (Hug et al., 1981), horses (Combie et al., 1983) and cats (Taylor et al., 2001). However, no one has reported the pharmacokinetics of morphine in broiler chickens or any other avian species. In the pharmacokinetic experiments on morphine in broiler chickens we found that clearance and volume of distribution were much higher in chickens compared to other species. The pharmacokinetics studies of other drugs in chickens also show higher clearance, which could be due to chicken's higher metabolic rate and body temperature when compared to mammals of similar body size (Baert et al., 2002; Graham et al., 2005). There is only one study reporting pharmacokinetics of butorphanol in red tailed hawks

and great horned owls (Riggs et al., 2008). They found that butorphanol has shorter half life and higher tissue distribution. The pharmacokinetic results in chickens also agree with the findings by Riggs et al., 2008.

Aspirin has a much shorter half life in chickens than mammals. The plasma concentration of aspirin was also much below the therapeutic levels seen in mammals. The maximum plasma concentration achieved in chickens was 46.72 μg/mL. If the therapeutic effects of aspirin are due to salicylic acid, which is debatable as not many comparative studies between the two salts have been published (Levy, 1979), aspirin could have useful analgesic effect in chickens as the duration for which salicylic acid remained within the mammalian therapeutic levels was from 1 until 4 hours after aspirin injection. However, intravenous administration is not recommended, because aspirin is highly insoluble in water and it rapidly hydrolyses to salicylic acid in aqueous media.

Salicylic acid, in contrast to aspirin, is highly soluble in water, which makes it easier to use. The maximum concentration for salicylic acid (93.75 μg/mL) was achieved 1 hour after aspirin injection and at this time, plasma concentration of aspirin was at its lowest. Salicylic acid remained in the circulation until 16 hours after i.v bolus injection at 50 mg/kg, but only for 4 hours within the therapeutic range for humans. Pharmacokinetic parameters obtained after I/V injection of salicylic acid in chickens were similar to the previous study conducted by Baert and De Backer (2002). Broiler chickens had a lower volume of distribution for salicylic acid compared to morphine and butorphanol (Singh et al., 2010) which is typical for all the NSAIDs (Davis, 1980). NSAIDs have lower tissue distribution as compared to opioids (Lin et al., 1987) because of higher plasma protein binding and lower pKa. However, the site of analgesic action of NSAIDs is unknown. If the site is inflamed tissue in the periphery, as for their protein binding and pKa mean that, they may be concentrated at the site of action. This would mean their analgesic effects may not be directly related to plasma concentrations. Chickens have higher volumes of distribution and faster clearance compared to other species. This trend was seen in the pharmacokinetics of all the analgesic drugs studied in this thesis.

In this study we used a HPLC method for analysis of drugs in plasma. The precision and ac-

curacy was similar to the methods already published. We did not use any internal standard in the HPLC assays followed in our study. Internal standards are useful in liquid chromatography primarily to correct precision problems caused by injection volume variations. This problem does not arise in case of new, highly efficient and advanced HPLC machines. We obtained excellent precision by using external standardization, so the use of internal standard did not provide any advantage. Also, the use of an internal standard method in the case of biological samples can result in faulty data because of the different behaviour of the analyte and internal standard during sample preparation.

We developed a simple and inexpensive HPLC method for analysis of butorphanol. The precision and accuracy of our method is similar to reported LC MS methods, <6.5% (Chang et al., 1999), 6.2 to 9.2% (Sellon et al., 2001) and 2.7 to 6.8% (Boulton et al., 2002). Though the LLQ for these methods are lower than our method, with small volume samples we were able to achieve LLQ well below the MEC using a standard HPLC machine with DA detection. The diode array detector is the most commonly used detector in pharmaceutical laboratories. Lower sample volume and higher recoveries as compared to other methods makes it possible to detect lower levels of butorphanol.

In our pharmacodynamic study we used lame broiler chickens to evaluate the analgesic effects of the drugs used in pharmacokinetic experiments. Naturally occurring lameness in broiler chickens is painful. (Weeks et al., 2000) found that lame broiler chickens spend significantly more time lying than the healthy chickens and also preferred to consume their feed while lying down. There was lack of dust bathing in lame broiler chickens (Vestergaard and Sanotra, 1999). Dust bathing involves forceful movements with legs and wings, which might be difficult for broiler chickens suffering from lameness and pain. Administration of carprofen at 1 mg/kg subcutaneously in lame broiler chickens increased their walking pace and ended to return their behaviour towards normal (McGeown et al., 1999). A study conducted by Danbury et al., (2000) also strengthens this assumption that lame broiler chickens suffer from pain. In her study, both sound and lame broiler chickens were fed with standard and carprofen mixed feed. The lame chickens preferentially opted to eat carprofen mixed feed. The intake of feed was correlated with increase in walking time of lame broiler chickens. Thus the clinically lame broiler

chickens can be used as a model of chronic pain and there are minimal ethical problems associated with their use.

Butorphanol and salicylic acid reduced the time taken by lame broiler chickens to finish the course. They also improved the standing time in case of lame broiler chickens. Analgesic effect of butorphanol was also demonstrated by Buchwalder and Huber-Eicher (2005) in turkeys, Gentle et al., (1999) in domestic fowl, Curro et al., (1994) in cockatoos, Paul-Murphy et al. (1999), Sladky et al., (2006), Paul-Murphy et al., (2009a) in conuress and Paul-Murphy et al., (2009b) in psittacine. Hocking et al., (2005) demonstrated analgesic effects of salicylic acid in domestic fowl induced with acute arthritic pain but the dose rate required for analgesia ranged from 100 to 200 mg/kg. The duration of analgesia was not reported in that study. In our study, salicylic acid provided good analgesia but the duration was only 2 hours.

Unlike butophanol and salicylic acid, morphine seems to have mainly sedative actions in broiler chickens. The chickens were the injected at 2 mg/kg intravenously, which is a very high dose of morphine in mammalian terms. In our pilot pharmaockinetics study, morphine was injected at 0.5 mg/kg - a dose rate commonly used subcutaneously in dogs. At that dose, morphine lasted only for 10 minutes, though the limits of detection for our method was 20 ng/mL. Reduction in walking and increased sitting or lying down was observed both in the sound and lame broiler chickens. Chickens injected with morphine depicted reduced grooming behaviour and were more passive as compared to normal saline group. Decrease in plasma levels of morphine also reduced the level of drowsiness. In the case of lame broiler chickens, after 2 hours there was a reduction in the time taken as compared to the observation before injection, but this difference was non-significant. This suggests that morphine could be analgesic at lower concentration, but at higher concentration, it causes sedation and drowsiness in broiler chickens. The tests we used were not able to differentiate sedation from analgesia. Morphine injected at low dose rates, 0.05 and 0.1 mg/kg S/C, decreased the sleeping time and there was non significant increase in percent time spent walking and standing in lame chickens as compared to the normal saline-injected lame group (Danbury et al., 1999). However, morphine at the higher dose of 1 to 4 mg/kg S/C in the same experiment, increased the time spent lying and sleeping in lame chickens. Hughes (1990a) and Sufka and Hughes (1990) found that morphine causes hyperalgesia

in domestic fowl. There was no evidence of this in our study. The broiler chickens in our study looked sedated and sleepy after injection of morphine.

All the results from the placebo group in both the studies were non-significant, but the trend suggested that the lame birds progressively became worse with time. The results of the control group are similar to the placebo. This shows that there was no stress or pain caused by injection but cannot rule out stress induced analgesia caused by handling. When the lame birds were not given any treatment as in the case of control or placebo group, the severity of lameness increased with time.

In both the pharmacodynamic studies the lame birds took significantly more time to finish the course as compared to the sound broiler chickens. The lame birds also stood for significantly lesser time as compared to sounds birds in LTL test. The lame birds in the second study took significantly less time to finish the course as compared to the lame birds in first study. In the case of LTL, the lame birds in the second study stood for less time than the lame birds of first study, but this difference was not significant. The OC test seems to be more robust as compared to the LTL, as the pace of the bird is directly related to lameness, but there are more distractions in OC as compared to LTL. In case of LTL, there were no distractions as the birds could not wander around, but some lame birds seem to brace the walls of the tub and stood for a longer time period. Both these tests are good to differentiate between the lame and sounds birds, but not very sensitive for evaluating degree of lameness. There are many variables involved in these two tests, e.g. state of hunger, noise and disturbance in their surroundings, stress due to being examined or handled. Increasing the number of subjects might increase the sensitivity of these tests and give more conclusive results. It is difficult to conclude analgesic efficacy only from the behaviour studies, but there correlation with pharmacokinetics that strengthen the evidence of pain relief with the use of analgesic drugs.

Conclusion

Butorphanol is analgesic at 2 mg/kg I/V, while morphine causes sedation at this dosage. The MEC levels for butorphanol that should be maintained for analgesia ranged from 50 to 80 ng/mL. At 2 mg/kg I/V dose, the plasma concentration exceeded this range for about 2 hours.

Morphine at 2 mg/kg I/V dose rate caused sedation and drowsiness. It may produce analgesia at lower dose rates, but owing to its shorter elimination half life, it should be administered more frequently at a lower dose rate, which needs to be evaluated further.

Salicylic acid can effectively be used as an analgesic agent in chickens at 50 mg/kg I/V bolus dose. The therapeutic plasma concentration of salicylic acid for analgesia ranged from 110 to 150 ng/mL. These concentrations lasted for 2 hours after 50 mg/kg I/V. Sustained released formulations such as liposome encapsulation (Sladky et al., 2006) or slow release drug delivery systems such has mini osmotic pumps could be used to increase the duration of analgesia.

Chickens can be used as a model of drug research for other birds. They are cheap and easily available as compared to Albatross, kiwi or any other wild bird. It is impossible to carry out pharmacokinetic studies in critically endangered avian species. In our wild life ward, we get certain species eg kakapos (*Strigopidae habroptila*), for which no animal ethics committee will grant permission to conduct any experiment as there are only 122 kakapos left. Therefore, it very important to use chickens or other easily available avian species as a model of drug research to extrapolate the pharmacokinetic parameters without much error. This extrapolation should be done with caution, as the pharmacokinetic parameters vary significantly within a species. Further pharmacokinetic studies should be conducted to establish the extrapolation of drugs from chickens to other birds.

Lame chickens can be fed with analgesic drugs to reduce the pain and thus increasing their quality of life. The pharmacokinetics of opioids have not been reported in food animals, thus no data is available for determining drug residues. There is a need to conduct drug residue studies to comment further on this statement. Pharmacokinetics of opioids in chickens exhibit rapid elimination from plasma. If tissues containing residues are consumed, the oral absorption of these drugs is poor, which minimises the risk of adverse reactions in people. A conservative approach would be a 48 hours withdrawal period. Aspirin is commonly used in food animals. The FDA considers the use of aspirin to be of low a regulatory concern. The drug does not present a substantial food safety issue, therefore formal approval is not necessary. The withdrawal time is 24 hours in meat animals.

Future work

The results of this study revealed that butorphanol and salicylic acid provide adequate analgesia in birds, but they lasted only for a short duration. Therefore, to maintain adequate levels of the analgesic drugs in birds, either they should be injected at very high dose rates, which could be lethal, or should be administered every 3-4 hours. This repeated administration could be a challenge for avian veterinary surgeons and the repeated handling of the bird could either elicit fear and stressful behaviour, or acclimatise the bird to people to the extent that its survival after release could be compromised. Therefore, there is a need to develop a sustained release drug delivery system that can control and slow down the release of these drugs in birds. There are various sustained release devices being used in humans and animals, including oral, dermal, ocular and implants for continuous intravenous, intramuscular and sub-cutaneous drug delivery. Some of these drug delivery systems have already been successfully used in birds such as mini-osmotic pumps for the continuous administration of fadrizole (a non-steroidal aromatase inhibitor) to study the courtship behavior in doves (Fusani et al., 2001). However, these have not been optimized so far for other drugs. Therefore in future studies, mini-osmotic pumps for the subcutaneous delivery of analgesic drugs may be useful as they are easy to implant because of their small size. They can also maintain a constant flow of drug. Mini-osmotic pumps may enhance the efficacy of these analgesic drugs by maintaining adequate plasma concentration required for analgesia. They may also reduce side effects by reducing the total required dosage of drug.

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