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Insights into Pain Management Strategies in Veterinary Medicine: Unraveling the Pharmacokinetics of Some Coxibs in Geese, Sheep, and Goats

Director: Prof. Enrico Pietro Luigi De Santis

Coordinator: Prof.ssa Fiammetta Berlinguer

Tutor: Prof. Mario Giorgi

PhD candidate: Dr. Charbel Fadel

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To be cherished with memories of friends and family.

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CHAPTER I: Comprehending Pain Management Strategies: Insights into Non-Steroidal Anti-Inflammatory Drugs and COXIBs

1. Insights on pain management in veterinary medicine

In recent years, the treatment and alleviation of pain in animals have gained unprecedented attention due to the heightened awareness and concern for the well-being of animals within society (Fraser, 2006). The recognition of animals as sentient beings capable of experiencing pain and suffering has sparked a paradigm shift in veterinary medicine and animal care. Scientific advancements in understanding animal physiology, behavior, and cognitive abilities have underscored the ethical and moral responsibility to provide adequate pain management. This growing awareness is evident in both public sentiment and regulatory frameworks, which are increasingly demanding the implementation of effective pain management strategies for animals across various contexts, including veterinary care, livestock production, research, and wildlife conservation (Mellor and Stafford, 2003). Moreover, research has demonstrated that unaddressed pain not only compromises the quality of life for animals but can also lead to adverse physiological, psychological, and behavioral consequences. Consequently, the integration of comprehensive pain assessment and tailored pain management protocols has become imperative, reflecting a more compassionate and ethical approach to animal care (McMillan, 2003).

In farm animals specifically, pain has often not received as much attention as it has in pets. Managing pain in farm animals hasn't improved as much as it has for pets. There are several reasons for this. Finding good pain medicines is challenging, and some farmers and veterinarians have differing opinions. Also, concerns exist about how farm animals are used in studies. However, things are changing now. More and more people are concerned about how animals are treated on farms. Additionally, in recent years, consumers have demanded that agricultural industries provide products that are kind to animals and also meet strict standards for food quality and safety. Despite evidence showing that using pain-relief medicines is beneficial for the well-being of animals, in many countries, it's still common and allowed to perform procedures like removing horns and castrating

young calves without using pain relief. The reasons given for not using pain relief in farm animals include practical and financial factors, challenges in giving the medicines, perceiving the animals as having low value, the cost of treatment, a shortage of approved pain-relief medicines for animals intended for human consumption, and concerns about medicine residues in food (Vinuela-Fernandez et al., 2007).

2. What is pain?

Pain constitutes a neural sensation and sentiment, brought forth by real or potential harm to tissue. The interpretation of pain varies among diverse individuals, frequently retaining a subjective nature, and is intertwined with emotions and past encounters (Fong and Schug, 2014). The International Association for the Study of Pain (IASP) defines pain as: “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage”. (ISAP, 1979). However, this definition has not been accepted as being relevant to non-verbal animals as it relies on self-report. Therefore, Molony and Kent (1997) defined pain as “an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal’s physiology and behaviour to reduce or avoid damage, to reduce the likelihood of recurrence and to promote recovery”. Pain is favorable from an evolutionary standpoint and is viewed as essential for survival. Although there is some dispute about what constitutes pain, researchers generally agree that assessing pain in animals is a challenging undertaking.

3. Classification of pain

3.1. In terms of time frame

Pain lasting less than 3 months is typically categorized as acute pain, often arising from surgery or injury. In response to acute pain, animals swiftly adapt their physiological mechanisms to mitigate harm and initiate the healing course. The intensity of acute pain varies, ranging from mild to severe

based on the underlying cause. On the other hand, when pain persists for over 3 months without resolution, it falls under the label of chronic pain (Epstein et al., 2015; Mathurkar, 2016).

3.2. In terms of sensory perception

Nociceptive pain (musculoskeletal/somatic or visceral) involves a localized sense of pain originating from peripheral sensory neurons called nociceptors, triggered by mechanical, chemical, or thermal injuries. Nociception primarily serves as a protective mechanism, setting in motion the repair of harmed tissues (Loeser and Treede, 2008). Pain induced by nociceptor activation stems from various sources. Somatic pain emerges from harm to local muscles, skin, and joints, while visceral pain arises from trauma to visceral organs. Inflammatory pain results from tissue damage, prompting the release of diverse inflammatory agents like cytokines, kinins, eicosanoids, and neuropeptides at the injury site, culminating in peripheral sensitization (Mathews, 2008). Inflammation assumes a crucial role in pain physiology (Xu and Yaksh, 2011), significantly influencing the transduction of pain signals to the central nervous system (CNS), potentially mitigated through non-steroidal anti-inflammatory drugs (NSAIDs). Conversely, opioids operate on central sensory responses (Stein, 2016), interacting with opioid receptors to inhibit neuronal excitation, thereby preventing neurochemical release from primary afferent nerve fibers in the spinal cord. This interruption hinders depolarization, reducing pain perception and facilitating central pain modulation (Koneti and Jones, 2016).

Neuropathic pain entails a feeling of pain where the peripheral pain signal is conveyed to the CNS through neurons (Meintjes, 2012; Mathews, 2008). Various pathological conditions, including diabetes, endocrine disorders, viral and bacterial infections, as well as other neurodegenerative ailments, have the potential to harm nerve cells, giving rise to neuropathic pain (Jay and Barkin, 2014). This type of pain can originate from sensory and/or motor origins, encompassing descriptions such as mild, severe, burning, and shooting pain (Steeds, 2009), contingent upon the underlying cause of the damage.

3.3. Whether physiological or pathological

Livingston and Chambers (2000) in accordance with Woolf and Chong (1993) considered simply two types of pain for more convenient and easier understanding as physiological and pathological pain.

Physiological pain serves as a vital defense mechanism, alerting the body to potential harm and prompting immediate protective responses. This type of pain is crucial for survival as it helps prevent further damage and facilitates the healing process. This type of pain typically falls under the category of acute pain. The experience of physiological pain correlates with the intensity of the noxious stimulus (Livingston and Chambers, 2000). Examples of physiological pain are abundant in everyday life: touching a hot/burning surface, stepping on a sharp object, undergoing menstruation, experiencing bone and teeth growth, being bitten by an insect, sustaining a cut on a finger, paw, or limb, and more.

Pathological pain, on the other hand, pertains to the perception of pain that surpasses the expected response to a noxious stimulus. This heightened perception involves the presence of inflammatory changes resulting from peripheral stimuli that cause tissue damage. This type of pain can manifest as either acute or chronic. Moreover, pathological pain extends beyond scenarios involving inflammation or physical lesions. It encompasses instances where the nervous system is damaged or functioning improperly (Loeser and Treede, 2008; Livingston and Chambers, 2000). For instance, acute or inflammatory pain arises due to tissue damage, initiating an inflammatory process. In contrast, visceral pain does not necessarily involve inflammation; it can stem from the distension of organs, such as the colon. Phantom pain presents itself in a limb or body part that has been amputated or removed. Tooth pain can also result from nerve damage. Ischemic pain has its own distinct characteristics (Julius and Basbaum, 2001; Gebhart, 2004). Numerous theories, including the neuromatrix theory and body schema theory, attempt to explain such pain (Giummarra et al., 2007; Katz and Melzack, 1990). Consequently, an array of explanatory frameworks exists for these pain phenomena. These are a few examples of pathological pain can arise from a variety of sources,

including nerve damage, altered pain processing, and complex interactions within the nervous system: neuropathic pain, fibromyalgia, Complex Regional Pain Syndrome (CRPS), post-herpetic neuralgia, central sensitization syndrome, phantom limb pain, chronic back pain, and more.

Therefore, the comprehensive coverage of all pain aspects exceeds this thesis's scope. As a result, this thesis will focus primarily on highlighting inflammatory pain and inflammation itself.

Table 1: Characteristics of the different types of chronic pain (Source: https://www.openanesthesia.org/keywords/types-of-pain/)

	Neuropathic pain		**Nociceptive pain**		**Nociplastic pain**
Etiology	Nerve injury		Tissue Injury		Sensitization of the nervous system
Further classification	Central	Peripheral	Somatic	Visceral	-
Qualities	Burning, stinging, electric		Throbbing, aching, pressure-like		Similar to neuropathic pain Diffuse, gnawing, aching, sharp pain. Hypersensitivity, hyperalgesia, and sensitivity
Location	Nerve distribution can be central (CNS) or peripheral neurons		Bones Muscles Joints Skin	Mucosal injury, obstruction, ischemia, tissue Injury	Nondermatomal, diffuse
Examples	Postherpetic neuralgia, diabetic peripheral neuropathy, complex regional pain syndrome, sciatica/radicular pain		Bone fracture, metastases, dystonia, muscle spasm, osteoarthritis, postoperative pain, burns	Peptic ulcer, angina, gallstones, kidney stones, mesenteric ischemia, cancer, cirrhosis	Fibromyalgia, irritable bowel syndromes, interstitial cystitis, complex regional pain syndrome
Non-opioid treatments	Tricyclic antidepressants (TCAs), serotonin-norepinephrine reuptake inhibitors (SNRIs), gabapentinoids, capsaicin or lidocaine patch, tramadol		Nonsteroidal anti-inflammatory drugs (NSAIDs), muscle relaxants, SNRIs, TCAs, disease modifying anti-rheumatic drugs, nerve growth factor inhibitors, tramadol		TCAs, SNRIs, gabapentinoids, ketamine infusions

4. Inflammatory pain mechanism and pathways

Before addressing pain, it's crucial to comprehend its underlying mechanisms. The process of pain encompasses four distinct phases: Transduction; Transmission; Perception; and Modulation. Transduction signifies the reception of pain via nociceptors, which are activated by injury or trauma

(Meintjes, 2012). Inflammatory pain, induced by the release of inflammatory mediators like histamine, bradykinins, and prostaglandins, also plays a role in transduction (Meintjes, 2012). Transmission pertains to the conveyance of action potentials from peripheral nociceptors to the thalamus cortex through the spinal cord and brain stem, employing excitatory neurotransmitters like glutamate and aspartate (Fong and Schug, 2014). Perception involves the transfer of pain signals from the spinal cord's dorsal horn to the brain via the spinothalamic and spinoreticular tracts, prompting autonomic and behavioral responses to injury (Meintjes, 2012). Modulation signifies the release of inhibitory neurochemicals, such as γ-aminobutyric acid (GABA), that inhibit depolarization. Additionally, enkephalins and endorphins, opioid peptides, bind to opioid receptors (μ, κ, and δ), contributing to pain modulation (Li et al., 2003).

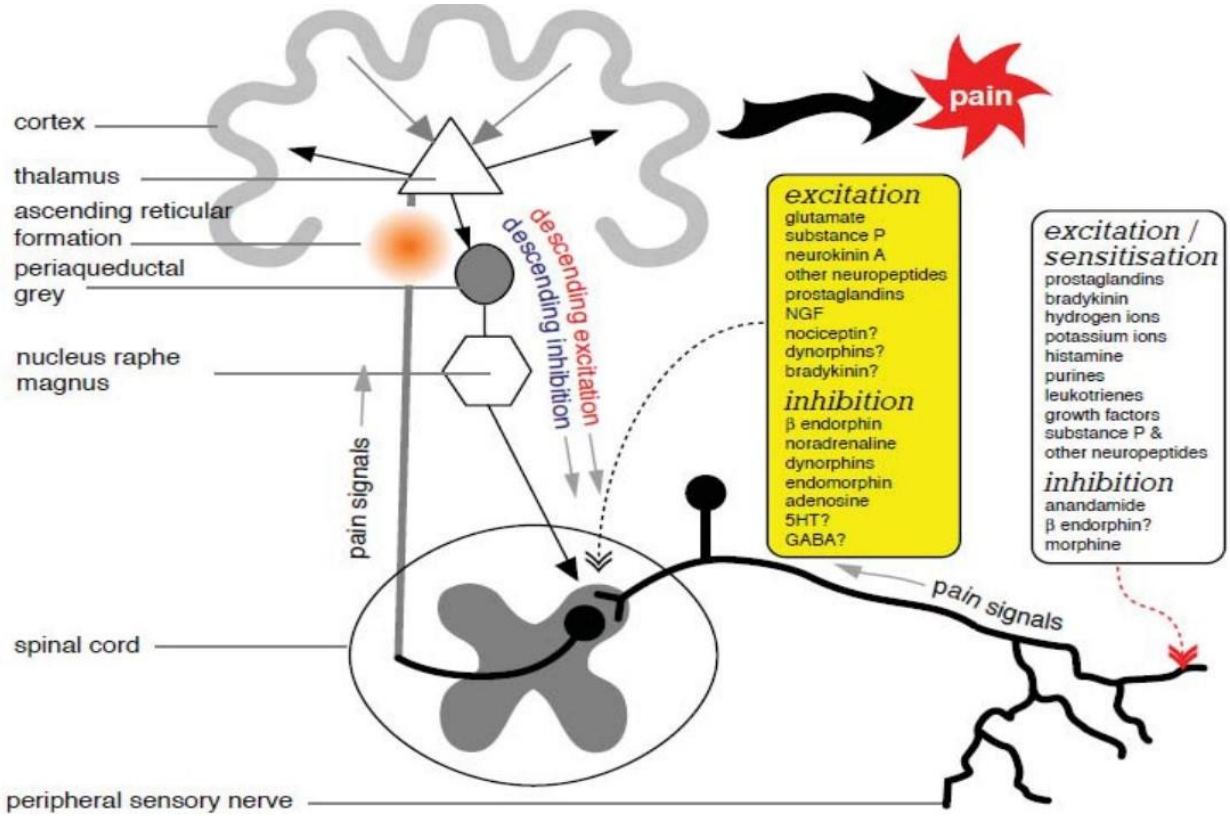


Figure 1: Mechanism of pain (Courtesy: Associate Professor Paul Chambers)

4.1. Peripheral/Afferent Pathway

The initiation of a pain signal occurs when intense noxious stimuli (mechanical, thermal, or chemical) are detected by nociceptive receptors, which are widespread in the skin, mucosa, membranes, deep fascias, connective tissues of visceral organs, ligaments and articular capsules, periosteum, muscles, tendons, and arterial vessels (Almeida et al., 2004). The nociceptive receptors correspond to peripheral free nerve endings/fibers known as nociceptors (Basbaum et al., 2009; Basbaum and Jessell, 2000). Nociceptors transmit the impulses from noxious stimuli to the dorsal horn of the spinal cord (Kidd and Urban, 2001). These nociceptors/fibers originate from nerve cell bodies, and their diameter is classified as large, medium, or small, depending on the size and type of nerve cells they belong to. Aδ-fibers are thinly myelinated, of medium diameter, and fast-conducting. Their activation results in acute sharp pain along with a withdrawal reflex. This pain type is often termed "first pain" and usually acts as a protective or defensive mechanism to prevent tissue damage (Diesch, 2010; Livingston and Chambers, 2000). C-fibers are non-myelinated, of small diameter, and slow-conducting, leading to dull, burning, or prolonged pain sensations (Julius and Basbaum, 2001; Livingston and Chambers, 2000). Another type of fibers, Aβ-fibers, possess large diameters and conduct rapidly, typically responding to innocuous stimuli like light touch or proprioception (Basbaum et al., 2009).

In addition to responding to noxious stimuli, peripheral sensitization occurs primarily through post-translational reorganization of crucial receptors and ion channels (Costigan and Woolf, 2000). For example, phosphorylation of TTX-r (tetrodotoxin-resistant) sodium channels by protein kinase A (PKA) and protein kinase C (PKC-C) increases sodium currents, generating a depolarizing stimulus that leads to additional excitation and lowers the activation threshold of neurons (Tate et al., 1998). Moreover, alterations in voltage-gated sodium channels play a significant role in the pathogenesis of chronic inflammatory and neuropathic pain (Amir et al., 2006).

Tissue injury that damages cells triggers the secretion of numerous compounds, leading to inflammation around peripheral fibers. This inflammatory response comprises various components functioning as inflammatory mediators (Besson, 1999; Dray, 1997a), including substances like prostaglandins, bradykinin, hydrogen ions, potassium ions, histamine, purines, leukotrienes, growth factors, substance P, and neuropeptides. These elements collectively constitute what's known as the "inflammatory soup" (Dickenson, 2008; Julius and Basbaum, 2001; Livingston and Chambers, 2000). Inflammatory mediators contribute to nociception by either exciting or sensitizing afferent nerve fibers, influencing the conduction of nociceptive impulses. Among afferent fibers, there exists a subset called "silent" or "sleeping" nociceptors in the skin, joints, and visceral organs. Normally unresponsive to intense stimuli, these nociceptors become sensitized and responsive to sensory stimuli when influenced by inflammatory mediators (Dray, 1997b).

Prolonged exposure to noxious stimuli results in heightened nociceptive responses from the tissue, a condition termed hyperalgesia (Short, 1998). Conversely, nociceptive responses stemming from the surrounding tissues are referred to as secondary hyperalgesia (Simone, 1992). Inflammation in peripheral tissues leads to spontaneous pain and hyperalgesia (Ikeda et al., 2006). At times, even normal non-noxious stimuli can evoke nociceptive responses, a state known as allodynia (Short, 1998). The nociceptive impulses carried by afferent nerve fibers journey to the spinal cord, where they undergo further processing involving various chemicals, including neurotransmitters, ion channels, amino acids, and more. These signals are then relayed to higher brain centers.

Table 2: Inflammatory mediators at periphery/site of injury (Source: Kongara, 2008).

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

4.2. Spinal cord

The dorsal horn's gray matter neurons in the spinal cord gather sensory information from primary afferents of sensory receptive neurons that innervate the skin and deeper body tissues. These neurons respond to specific types of noxious and non-noxious stimuli (Todd, 2010; Caspary and Anderson, 2003; Costigan and Woolf, 2000). These highly receptive neurons transduce noxious stimuli into electrical activity (Farquhar-Smith, 2008; Costigan and Woolf, 2000). Impulses from nociceptive afferent fibers, including Aδ mechanoreceptive and C polymodal fibers, initially synapse in the gray matter of the dorsal horn within the spinal cord. Additionally, these noxious signals reach the ventral horn to form a spinally mediated reflex arc, contributing to motor neuron-controlled withdrawal responses, as the ventral portion of the spinal cord governs motor output (Caspary and Anderson, 2003; Livingston and Chambers, 2000).

These nociceptive afferents terminate in a distinct distribution pattern within the dorsal horn. This pattern is determined by their sensory modality and the specific body part they innervate. This region also holds significance as a site of drug action (Todd, 2010; Pappagallo, 2005; Livingston and Chambers, 2000). The dorsal horn is anatomically and electro-physiologically divided into distinct laminae (I to X) (Basbaum et al., 2009; Basbaum and Jessell, 2000; Rexed, 1952). Aδ nociceptors project to lamina I and the deeper dorsal horn (lamina V). Conversely, low-threshold, rapidly conducting Aβ afferents—responsive to light touch—project into deeper laminae (III to VI) (Colvin and Power, 2005). On the other hand, C nociceptors project more superficially to laminae I and II (Dickenson, 2008; Pappagallo, 2005).

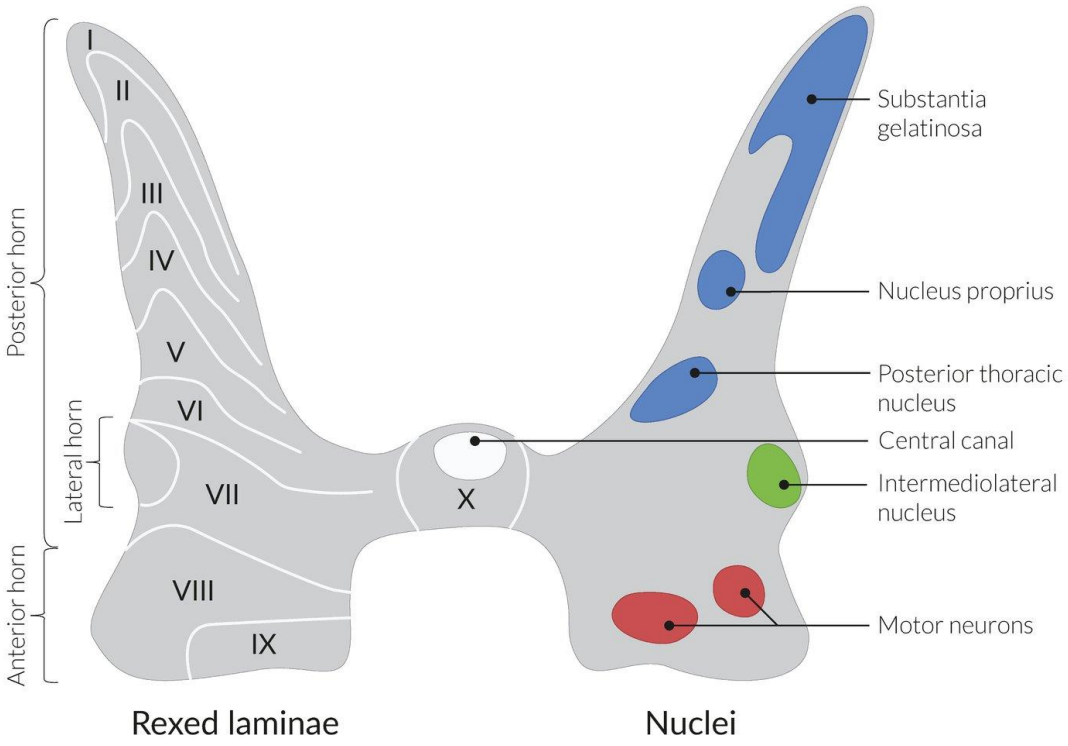


Figure 2: Laminar distribution of spinal dorsal horn. All mammals are thought to have a similar distribution (Source: https://www.amboss.com/us/knowledge/spinal-cord-tracts-and-reflexes).

All these nociceptors utilize glutamate as their primary neurotransmitter, a substance distributed throughout the CNS. However, the effects of glutamate are influenced by distinct neuropeptides within the dorsal root ganglia (DRG), which serve as entry points for nociceptors into the spinal cord

dorsal horn. Additional neuromodulators, like calcitonin gene-related peptide (CGRP), galanin, vasoactive intestinal polypeptide, and somatostatin, play pivotal roles at the initial synapse in the dorsal spinal cord. These neuromodulators adjust the impulses within the spinal cord, determining whether they are directed to brain centers or motor neurons in the ventral horn (as a reflex) (Wilcox et al., 2005).

The functioning of these neurons responsible for transmitting pain signals is also impacted by local inhibitory interneurons located in the spinal cord, as well as mechanisms that originate from higher brain centers and the brainstem and extend down to the spinal cord.

The dorsal horn laminae consist of an array of neurons, including interneurons and projection neurons, which play pivotal roles in transmitting sensory input both within and beyond the spinal cord, reaching even higher brain centers (McMahon et al., 2013). These neurons are categorized based on the specific type of sensory information they receive. For instance, neurons that respond to sensory input from Aδ and C fibers by generating action potentials are termed nociceptive-specific (NS) neurons. Meanwhile, neurons that react to input from Aβ fibers are known as proprioceptive/low-threshold mechano-receptive (LTMR) neurons. A third category, wide dynamic range neurons (WDR), receive input from all three fiber types and are responsible for a phenomenon called 'wind-up,' where repeated stimulation of WDR neurons accumulatively triggers their response (D'Mello and Dickenson, 2008; Herrero and Max Headley, 1995).

In some animals, like sheep, for example, somatosensory neurons within the spinal dorsal horn exhibit wide dynamic range properties. Over 60% of these neurons showcase such properties. This phenomenon is observed in both the superficial and deeper laminae of the spinal dorsal horn (Herrero and Max Headley, 1995).

The received information undergoes intricate processing through circuits involving both excitatory and inhibitory interneurons. Subsequently, this processed information is transmitted to projection neurons, which in turn relay it to various brain regions, including the brainstem and specific thalamic

nuclei, such as the ventral posterior nucleus, intralaminar nucleus, and para-fascicular nucleus (Todd, 2010; Milligan and Watkins, 2009).

Projection neurons are mainly concentrated in lamina I and are scattered across Lamina III to VI, with only a small presence in lamina II (Hylden et al., 1989; Lima and Coimbra, 1988). The majority of neurons contributing to pathways like spino-thalamic, spino-reticular, and spino-mesencephalic tracts are primarily located in lamina I, the outer layer of lamina II, and laminae IV, V, and VI of the dorsal horn (Fein, 2012; Farquhar-Smith, 2008). However, lamina I to III are particularly active in processing nociceptive information, as a significant portion of afferents terminate in these layers, especially in lamina I (Todd, 2010; Yu and Chan, 2003). As a result, the spinal cord functions as the initial site where sensory and nociceptive signals undergo modulation. Depending on the nature of the signal, the accumulated output is subsequently transmitted beyond the spinal cord, as depicted by the gate control theory proposed by Melzack and Wall in 1965 (Livingston and Chambers, 2000). The amplification of pain-related information in lamina I of the spinal dorsal horn contributes to inflammatory pain (Ikeda et al., 2006). Inflammation triggers the release of neuromodulators, including substances like substance P and glutamate in the spinal dorsal horn, which significantly contribute to the modulation of pain impulses (Milligan and Watkins, 2009; Ikeda et al., 2006).

Calcium ions (Ca^{2+}) also hold a significant role in various biological processes, including the broader mechanism of pain. A brief elevation in cytoplasmic Ca^{2+} concentration can trigger the release of neurotransmitters and influence the modulation of cell membrane excitability. The alteration in cytoplasmic concentration stems from the movement of Ca^{2+} ions through membrane channels, their transportation by ion pumps, or their release from internal stores (Prado, 2001). The entry of Ca^{2+} ions is regulated through three main pathways: firstly, via voltage-operated calcium channels (VOCC), secondly, through receptor-activated calcium channels, and lastly, by means of ligand-gated nonspecific calcium channels (Barritt, 1999). Intracellular influx of Ca^{2+} ions escalates upon acute activation of primary afferent terminals, subsequently leading to the release of glutamate. Continued

and sustained stimulation of these afferents intensifies intracellular Ca^{2+} levels, triggering the release of substance P and an increased secretion of glutamate. Moreover, these afferent neurons employ both glutamate and substance P as their neurotransmitters to convey nociceptive information (Bear et al., 2007; Kangrga and Randic, 1990; De Biasi and Rustioni, 1988). Among these mechanisms, N-type calcium channels predominantly facilitate the release of neurotransmitters like calcitonin gene-related peptide (CGRP), glutamate, and SP, both at the peripheral and dorsal horn synaptic levels (Bourinet et al., 2014).

Glutamate interacts with various receptor subtypes, each with distinct affinities. These receptors encompass NMDA (N-Methyl-D-Aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid), and kainate (KA) receptors, facilitating rapid excitatory transmission (Pin and Duvoisin, 1995). In addition, there are metabotropic glutamate receptors connected to G-proteins, enabling slower synaptic transmission (Costigan and Woolf, 2000; Ferraguti and Shigemoto, 2006). The NMDA receptor comprises seven subunits: Glu1, which binds to glycine, Glu2 (Glu2A, Glu2B, Glu2C, Glu2D), and Glu3 (Glu3A, Glu3B), which bind to glutamate (Bourinet et al., 2014; Paoletti et al., 2013; Lizarraga and Chambers, 2006; Dingledine et al., 1999). Notably, NMDA receptors hold significance in central sensitivity and hyperalgesia (Besson, 1999). NMDA receptors amplify excitatory synaptic transmission in nociceptive pathways (Vanegas and Schaible, 2007). These receptors play vital roles in dorsal horn neurons, encompassing the wind-up of dorsal horn neurons and modulation of the flexion reflex (Daw et al., 1993).

At resting membrane potential, NMDA receptors are obstructed by magnesium ions (Mg^{2+}), which are displaced upon depolarization of sufficient amplitude and concurrent glutamate release triggered by Ca^{2+} ion channels (N-type), thereby activating NMDA receptors (Dickenson, 2008; Besson, 1999). All fibers, including C-fibers, convey pain transmission primarily through AMPA receptor activation due to glutamate release at the primary afferent synapse; NMDA receptors are engaged by persistent and sufficiently intense stimuli (Dickenson, 2011). NMDA receptors possess a non-specific cation

channel that permits entry of both calcium ions and sodium ions upon activation (Dickenson, 2011, Dickenson, 2008). Meanwhile, AMPA and KA receptors open Na+ and K+ ion channels; the principal mechanism by which NMDA receptors elicit effects is the substantial influx of Ca^{2+} ions (D'Mello and Dickenson, 2008, Budai, 2000). This results in the heightened response of spinal dorsal horn neurons (WDRs) to C-fiber stimulation due to the persistence of the stimulus, a phenomenon termed "wind-up," which is implicated in central hypersensitivity.

Advancements in molecular cloning of metabotropic glutamate receptors have revealed the existence of eight subunits (mGlu1 to mGlu8). These receptors also participate in numerous brain functions, including synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD), associated with memory and learning (Pin and Duvoisin, 1995).

4.3. Transmission routes of pain signals to higher brain centers:

Upon the processing and modulation of noxious stimuli in the dorsal horn of the spinal cord, these signals are conveyed to the higher brain centers for subsequent pain perception and modulation.

The primary pathways for this transmission include the spinothalamic (STT), spinoreticular (SRT), and spinomesencephalic (SMT) pathways, which transmit noxious stimuli to the brain (Schaible, 2006).

The axons of second-order neurons in lamina IV to VI, collectively known as the nucleus proprius, cross the midline and come together to form the anterolateral pathway. This pathway combines with axons from second-order dorsal horn neurons in lamina I to create the spinothalamic tract, which serves as the primary ascending route from the spinal cord's dorsal horn. This tract projects to various regions in the thalamus, including the lateral complex, nuclei of the posterior medial and intra-laminar complex, and the medial central nucleus.

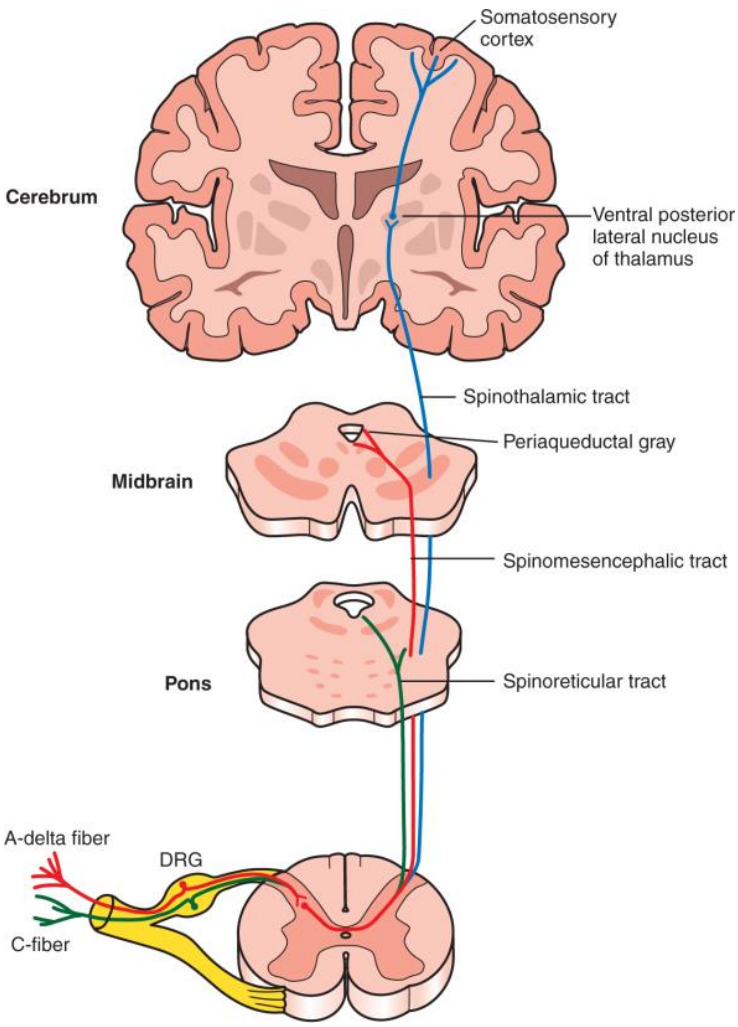


Figure 3: The three primary pain pathways of the anterolateral system (Source: https://loonylabs.org/2020/03/01/spinotectal-tract/).

The SRT tract originates mainly from lamina V, VII, VIII, as well as lamina I and X. This tract ascends towards the brainstem, connecting with the medial rhombencephalic reticular formation, dorsal and lateral reticular nuclei, and the nucleus reticularis gigantocellularis, among others. Some of these connections extend to the intra-laminar thalamic nuclei, ventral thalamus, and hypothalamus. The significance of the SRT lies in its role in establishing connections with the brainstem.

Similarly, the spino-mesencephalic tract involves many STT neurons in the dorsal horn, particularly in laminae VII (ventral horn) and X (mid-region). This tract primarily projects to regions like the lateral and ventrolateral periaqueductal gray matter (PAG), as well as the dorsal PAG, with few projections extending to the medial thalamic region.

Beyond these pathways, additional spinal projection paths exist. The spino-hypothalamic tract, originating from deeper laminae in the dorsal horn, directly projects to the medial and lateral hypothalamus. This pathway contributes to the processing of emotional, somato-sensory, and painful stimuli. The spino-parabrachio-amygdalar tract, originating from neurons in the superficial laminae (I and II) and to some extent from deeper lamina X near the central canal, projects to the parabrachial area or the amygdala. This pathway is associated with the emotional aspects of pain. Furthermore, the spino-cervical tract is observed in certain species (like cats, rats, and monkeys with identified lateral cervical nuclei). This tract ascends from the dorsolateral funiculus and processes both mechano-sensory and nociceptive inputs in its neurons.

4.4. Processing and Perception of Pain in Higher Brain Centers: The Pain Matrix

Transmission of pain signals to the higher brain centers occurs through the ascending pathways discussed earlier. Within this context, the cortex holds a significant role as the primary center for pain perception. This cortical region is subdivided into various segments (Brooks and Tracey, 2005). However, some scholars emphasize the importance of the thalamus (Albe-Fessar et al., 1985). The process of pain perception and processing is intricate, involving numerous elements, leading to the current term "pain matrix" (Tracey and Mantyh, 2007). This pain matrix is further divided into medial and lateral systems based on the pathways responsible for processing, inhibiting, and enhancing pain signals across distinct brain areas (Brooks and Tracey, 2005). Several imaging studies corroborate the involvement of different brain regions, including somatosensory (primary and secondary), insular, anterior cingulate, prefrontal cortices, and the thalamus in acute pain situations (Apkarian et al., 2005). Moreover, in cases of chronic pain, specific activation occurs in the prefrontal, frontal, and anterior insular cortex (Tracey and Mantyh, 2007). Nevertheless, Tracey and Mantyh (2007) suggest exploring innovative investigative methods such as structural imaging, spinal cord imaging, microglial activation imaging, and genetics to precisely delineate the roles of brain centers in distinct

types of pain perception. The neurotransmitters aspartate and glutamate are implicated in the activation of supra-spinal centers (Kelly et al., 2001).

4.5. Regulation of Pain through Descending Pathways

Axons connecting the brainstem to the spinal cord can affect pain sensation in the spinal cord by modulating its activity. McMahon et al. (2013) and Todd (2010) have shown that these descending pathways can inhibit (slow down) and facilitate (speed up) pain-related signals. Initially, the concept of endogenous analgesia was proposed, which posited that brainstem-spinal cord modulation was primarily an inhibitory mechanism, but subsequent research has established the presence of both descending inhibition (DI) and descending facilitation (DF) as means of descending control of pain (Gebhart, 2004).

Heinricher et al. (2009) have identified the periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) as the primary regions responsible for descending control of pain with brainstem centres receiving afferent input from the PAG, nucleus tractus solitaris (NTS), and parabrachial nucleus (PN) forming spinobulbospinal loops (Moffat and Rae, 2011) during chronic pain states. The pathways for DI or DF pass through the RVM, which also receive input from higher brain centres including the thalamus and cortex.

Pain is modulated at the RVM and spinal cord levels by various transmitters, receptors, and groups of neurons (in RVM, on and off cells) that either facilitate or inhibit pain (Palazzo et al., 2008). To date, various pain modulation descending pathways have been studied, and the involvement of each neurotransmitter, receptor, and neuronal circuitry is known (Todd, 2010, Bee and Dickenson, 2009).

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

499 terminals, intrinsic interneurons, ascending tract neurons and terminals of the further descending tract
500 neurons (Bee and Dickenson, 2009).

501 Histamine, acetylcholine, GABA, neuropeptides, neurotensins, galanin, SP and glutamate, 5-HT,
502 noradrenaline (depending on serotonergic and counteracting noradrenergic pathways) are the primary
503 transmitters involved in the various descending modulations (Benarroch, 2008). Pain is modulated
504 by endogenous opioids (endorphins), and opioid receptors in various brain regions (particularly in
505 RVM) contribute to overall nociception processing (Basbaum and Fields, 1984).

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

508 **5. Pain in animals and small-ruminants**

509 Despite the fact that the mechanisms of pain in animals and humans are similar, pain in animals is
510 difficult to understand and accurately detect. Over the years, there have been numerous debates about
511 animal pain. Because animals lack speech, the debate over "can animals feel pain" has raged on for
512 years (Musk et al., 2013, Paul-Murphy et al., 2004). However, it is now almost universally
513 acknowledged that animals feel pain, though the expression of pain differs between species
514 (Rutherford, 2002). As a result, pain detection and alleviation are critical components of animal care
515 and welfare (Anil et al., 2005;). The physiological, pathological, and emotional components of animal
516 and human pain have been reported to be similar (Panksepp, 2005, Yaksh et al., 1999). The majority
517 of human pain management strategies are based on animal models (Morton and Griffiths, 1985). This
518 is possible because animals and humans have similar neuronal pathways and neurotransmitter
519 receptors (Livingston, 2010).

520 A painful procedure is defined by the Animal Welfare Act (1999) as any procedure that could
521 reasonably be expected to cause more than minor and temporary pain or distress in a human being

(AWIC, 2000). Animals, it has been argued, should be given the benefit of the doubt (Anil et al., 2005).

Many animal husbandry procedures, such as castration, tail docking, disbudding or destruction of the horn bud, dehorning, branding, debeaking, and even management practices like shackling, transport, milking, housing, and so on, can cause acute pain, compromising animal welfare (Grant, 2004). In addition to routine surgical and other procedures, farmed animals frequently sustain injuries from fighting and other activities. Pneumonia, enteritis, arthritis, mastitis, foot rot, and other systemic conditions are also painful, resulting in acute or chronic pain (Molony and Kent, 1997). Acute pain is typically associated with the development of protective mechanisms to prevent further pain processing (Greisen et al., 1999). However, ongoing acute pain, which eventually leads to chronic pain, is not beneficial. Chronic pain causes poor appetite, growth, and production in farm animals (Molony et al., 1995; Dantzer and Mormède, 1983). As a result, the animals' welfare and production are jeopardized, and in such cases, analgesic treatment and proper animal care are required (Stafford and Mellor, 2005; Anil et al., 2005). The assessment of pain in animals, both qualitatively and quantitatively, is critical for the management of painful conditions and the improvement of welfare (Fitzpatrick et al., 2006).

As for small-ruminants, they are susceptible to a variety of diseases, and either infectious or non-infectious diseases can impair sheep welfare by causing pain (Fitzpatrick et al., 2006). They are subjected to various husbandry operations such as castration, vasectomy, and tail docking, and are prone to developing painful pathologies such as lameness, foot rot, mastitis, vaginal prolapse, and penis deviation. Moreover, sheep are also widely employed as an experimental animal model for particularly invasive surgeries, for educational purposes, and biological research (Lizarraga and Chambers, 2012).

5.1. Recognition and assessment of pain in small ruminant species

Animal pain assessment is a critical aspect of veterinary medicine and animal welfare. However, pain is an individual experience, and measuring it is extremely difficult (O Callaghan et al., 2003), because there are intra-species and inter-species differences in responses to painful stimuli. Even the same animal's responses may not be the same in all cases (Anil et al., 2002). Individual variation may be related to developmental stage (age), gender, genetic variation, environment, emotional status, and prior pain experience, among other things (Nielsen et al., 2008, Johnson et al., 2005). It is indeed more difficult to assess pain in small ruminants, that tend to be stoic and do not readily show overt signs of discomfort. As prey species, small ruminants often do not exhibit pronounced painful behavior, especially in the early stages of experiencing pain (Smith et al., 2021).

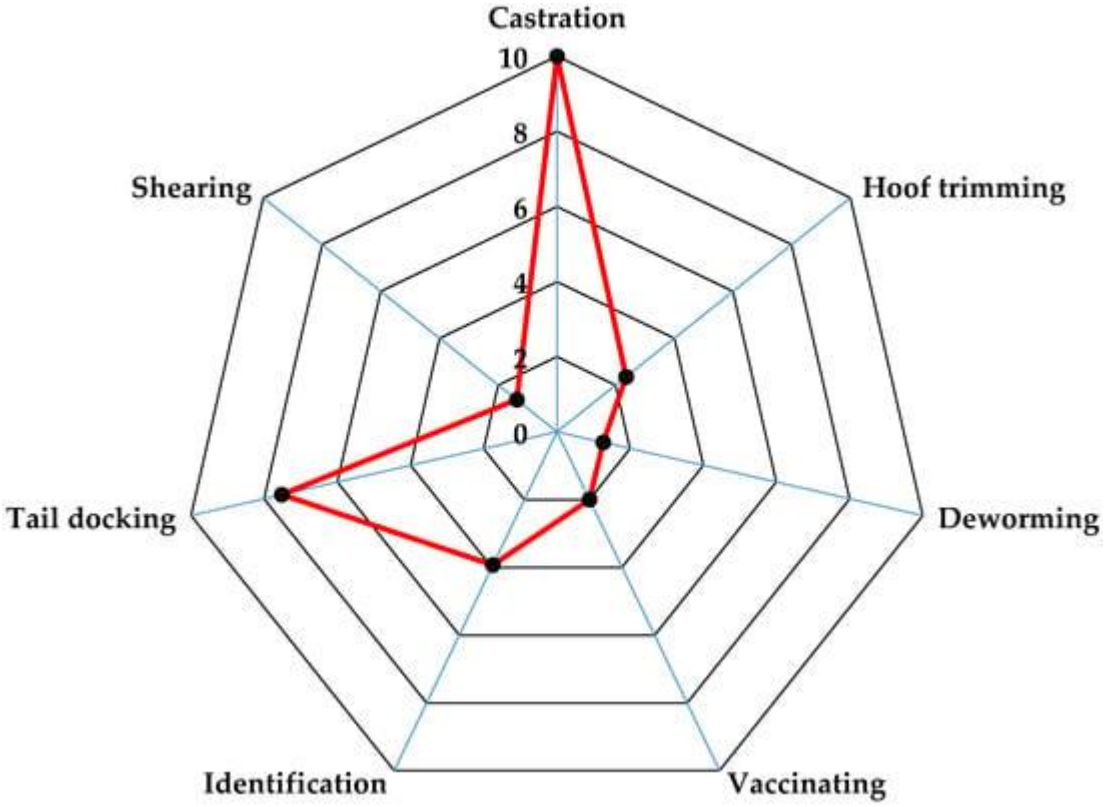
As a result, in the absence of verbal communication, the researcher must rely on other methods to confirm or quantify the nature and intensity of the painful or nociceptive experience in animals (Livingston, 2010). Bufalari (2007) proposed that the neurological, cardiovascular, respiratory, skeletal, endocrine, digestive, and urinary systems be included in the evaluation of pain (Bufalari et al., 2007). According to Landa (2012), direct and indirect indicators such as behavioral, physiological, and/or clinical responses can be used to assess pain in animals.

5.1.1. Behavioral indicators

Behavioral responses of animals due to pain involve changes in postures or gait, vocalization, temperament and others such as alteration in urination and defecation frequency (Morton and Griffiths, 1985), reduced sociability, decreased food consumption, tremors, abnormal vocalization, changes in responses to nociceptive thresholds (Ley et al., 1989) changes in locomotion such as licking, lying down, shaking head, flicking ears, lameness etc. (Duncan, 2006, Molony and Kent, 1997), changes in facial expressions (Love et al., 2011). Additionally, during pain, there may be a change in eating habits (appetite loss) (González et al., 2008). However, caution should be taken into account since that sheep and goats usually tend to mask the effect of pain by expressing normal

behaviour in spite of being in painful conditions, presumably because animals showing signs of injury are more likely to be picked out by predators. Presence of people is also a contributing factor for a sheep's pain hiding and they tend to behave normally when people are around. This does not mean that sheep do not experience pain.

Many strategies for pain evaluation have been proposed based on behavioral changes in animals during pain. To assess pain, researchers created distinct pain scales for different animals. The criteria used to evaluate pain vary and are dependent on the study and the animals (Bufalari et al., 2007). Subjective approaches such as pain scores are considered (Rutherford, 2002). In animals, the simple descriptive scale (SDS), numerical rating scale (NRS), and visual analogue scale (VAS) are the most regularly used pain scales (Holton et al., 1998). There are also several composite pain measures that have been published, such as the Glasgow composite measure pain scale (GCMPS) and Glasgow composite measure pain short form (CMPS-SF), which have been designed to quantify acute pain in dogs and their application in cats (Brondani et al., 2011, Reid et al., 2007, Holton et al., 2001).



583 Figure 4: Median pain scores associated to husbandry practices in lambs using a numerical rating
584 scale, from 0 (no pain) to 10 (maximum pain) (Source: Larrondo et al., 2018).

585 **5.1.2. Nociceptive threshold testing**

586 Nociceptive threshold measurements after mechanical limb stimulation or thermal stimulation of the
587 ear pinna are well-established procedures for researching pain and analgesics in sheep (Nolan et al.
588 1987; Chambers et al., 1994). These techniques have been used to detect pain hypersensitivity in a
589 variety of conditions, including inflammatory pain models (Colditz et al., 2011), ventral midline
590 laparotomy (Welsh and Nolan, 1995), foot rot (Ley et al., 1989; Chambers et al., 1994), and chronic
591 mastitis (Dolan et al., 2000).

592 Mechanical nociceptive testing (MNT) usually involves external application of pressure to produce a
593 noxious stimulus in an animal. During MNT, after application of stimulus, selective nociceptors are
594 activated in response, which includes two types of Aδ and polymodal C fibre nociceptors. This

stimulus is usually quantifiable and the animal responds by lifting its leg (if the stimulus is applied to leg) or flicking of skin, vocalizing, flicking of ear or tail, changing gait or posture and sometimes standing still, without any movement etc. depending upon the species of animal or location of the stimulus. The most commonly used device for MNT, especially in farm animals is a pneumatically driven blunt pin in a specific region on the animal's body to create a noxious stimulus which is terminated as soon as the animal responds. In animals, MNT is mostly used to test the efficacy of analgesic drugs as it is reliable, reproducible and does not damage the tissue (Dixon et al., 2010). Dixon et al. has used the MNT in cats to test the analgesic efficacy of different NSAIDs as well as opioids. MNT testing offers advantages such as direct proportionality of responses to stimulus intensity and precision in evaluating drug analgesic efficacy. However, drawbacks include technical challenges in freely moving animals and the simultaneous activation of both high and low mechanoreceptors, hindering the differentiation of their contributions to behavioral responses (Grant et al., 1996).

Other methods of inducing nociceptive mechanical stimulation include pinching or pin-pricking a specific anatomical area (Aminkov and Hubenov, 1995), but these techniques are incapable of measuring nociceptive thresholds and so quantifying changes in them following drug delivery. Aversive, nociceptive responses have also been elicited by electrical stimulation of a leg (Ludbrook et al., 1995). This approach is beneficial for determining changes in nociceptive thresholds in humans (Grant et al., 1996; Haerdi-Landerer et al., 2005), however it is not unique to any type of pain receptor.

Another method as well would be the thermal nociceptive threshold testing. It consists of applying thermal stimulation (usually heat) using different sources such as a thermode, infrared radiation (usually a laser) and hot water (Dixon et al., 2002, Veissier et al., 2000). This nociception activates the cutaneous thermoceptors including Aδ and polymodal C fibre nociceptors. A thermode based device is usually mounted on either on animal's leg or ear and then the temperature gradually

619 increased until a response is evoked through either lifting of leg, flicking of ear, tail, etc. (Dixon et
620 al., 2002).

621 ### 5.1.3. Physiological responses and plasma constituents

622 Physiological factors measured in animals include plasma cortisol (glucocorticoid hormone) levels
623 after stressful or painful operations. They are frequently utilized as pain markers (Stafford et al., 2002;
624 Mathews, 2000). Blood levels of β-endorphin, lactate, tumour necrosis factor alpha, interleukin-1 β,
625 C-reactive protein, serum amyloid A and haptoglobin have also been evaluated by certain researchers
626 (Moya et al., 2008). However, plasma cortisol continues to be the preferred and most reliable
627 physiological indicator (Landa, 2012). Physiological responses which can alter due to pain include
628 pulse, temperature, respiration, blood pressure, etc.

629 However, the great individual variability, the involvement of the stress response, and the effect of
630 drugs call for caution when interpreting results for these plasma constituents as indicators of pain. All
631 of these physiological parameters are responses to stress, rather than pain, and stress can also be
632 induced by non-painful stimuli such as handling. Thus, it may be useful during normal and painful
633 conditions to relate and compare the parameters before and during pain (Bussières et al., 2008).
634 Further research to identify reliable biomarkers of pain in sheep and goats is necessary.

635 ### 5.1.4. Other

636 Electroencephalography (EEG), which offers an overall measure of cortical activity, can also be used
637 to evaluate neurophysiological reactions during painful circumstances. Because the cerebral cortex is
638 involved in pain perception and Jongman et al., 2000), this can indicate pain. Other processing
639 neurophysiological approaches, such as bispectral index (BIS, a number generated from the EEG)
640 and somatosensory evoked potentials (SEPs), offer advantages and limitations (Murrell and Johnson,
641 2006). Indeed, EEG recording has been investigated as a method to study pain in sheep. EEG
642 recordings of changes in the brain activity of sheep that were subjected to a painful stimulus
643 demonstrated that the response in the brain to pain was similar to that of humans (Ong et al., 1997).

However, the recording of responses to the noxious stimulation is only practical under general anesthesia.

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

5.2. Pain Perception in Avian Species, with a Focus on Geese

It is often assumed that birds sense pain in the same way as mammals do. Birds have similar neurologic components that respond to painful stimuli and endogenous anti-nociceptive mechanisms that modify pain, and several pharmacologic drugs used to treat pain in mammals also modulate pain pathways and behavioral responses in birds (Machin, 2005). Because species that may be preyed on are less likely to display overt pain-associated behavior that may attract predator attention, birds frequently do not indicate pain in an obvious manner. Furthermore, there is significant variation in behavioral responses to pain among avian species, breeds, strains, or individuals, and there is no reliable or universal pain indicator (Gentle, 1992; Holloway et al., 1980; Danbury et al., 1997). Indeed, most practitioners can identify acute severe pain, but chronic pain may go undetected, especially if practitioners are unfamiliar with the species' normal behavior. As a result, when dealing with any condition that is expected to cause pain, it is best to treat for pain.

Geese and, generally birds, tend to respond to noxious stimuli with: altered movement (limping, reduced activity, or reluctance to move altogether), vocalizations, aggression or withdrawal (some animals become more aggressive due to their discomfort, while others may withdraw from social interactions), appetite changes (may eat less or stop eating altogether), change in posture, self-grooming (biting the painful area), restlessness or pacing (or repetitive movements), avoidance behavior, and so on. The responses however can be classified into a fight-or-flight response (i.e., escape reactions, vocalization, excessive movement) and/or conservation–withdrawal responses (immobility, avoidance behavior) (Gentle, 1992). In birds, immobility represents a multifaceted behavioral response triggered by painful or fear-inducing stimuli. Research suggests that the duration

669 of this immobility response is influenced by the level of fear experienced. When fear is heightened,
670 the immobility reaction tends to be prolonged, while strategies that mitigate fear tend to diminish this
671 response. This phenomenon points to the potential role of immobility as an evolved anti-predator
672 tactic. By minimizing movement, the bird aims to avoid exacerbating injuries that could result from
673 struggling and to potentially create an opportunity for escape from danger. Another perspective
674 relates the shift from active escape behaviors to the crouching immobility stance to a concept known
675 as "learned helplessness." This behavioral pattern develops when animals undergo distressing events
676 that are unpleasant and persist despite the animals' efforts to mitigate them (Machin, 2005).

677 When birds were subjected to ‘acute’ pain through methods like electric shock or comb pinch, they
678 displayed active avoidance behaviors characterized by forceful attempts to escape, including actions
679 such as jumping and wing flapping, often accompanied by vocalizations. In contrast, when birds
680 experienced prolonged ‘chronic’ pain, such as through continuous feather removal, they typically
681 exhibited signs of discomfort such as reduced appetite, decreased activity, and an appearance of being
682 fluffed up. In this scenario of prolonged pain, the usual heightened escape response seemed to be
683 absent; instead, the birds assumed a crouched and motionless posture (Paul-Murphy et al., 1999). In
684 a separate experiment, observations made immediately after feather removal revealed changes in
685 blood pressure and EEG readings, suggesting the presence of a painful sensation (Gentle et al., 1989).

686 To summarize, avian pain management is characterized by multiple challenges. Recognizing pain
687 and assessing its intensity are both essential for effective management. Thus, the farmer's appreciation
688 of the intensity of pain, as well as his familiarity with the normal behavior of both animal species and
689 individual birds in order to recognize signs of pain, is critical for the selection of an analgesic drug
690 and its dosage regimen (Hawkins, 2006). Through dedicated research, analgesic drugs have displayed
691 promising potential in avian species, by exhibiting effective outcomes in mitigating pain, as
692 elaborated further in this thesis.

In light of the need to ensure the welfare and well-being of animals, the utilization of analgesic drugs becomes imperative for effective pain management. Delving into the specifics, a comprehensive understanding of these analgesics is essential, given that the assessment of pain serves as a crucial compass in guiding their appropriate and compassionate application.

5.3. Analgesics and pain management

Analgesics are medications designed to alleviate pain. Yet, a majority of these pharmaceuticals target both the sensory and emotional dimensions of pain in order to regulate it, all the while keeping consciousness unaffected. Several of these agents modify the pain threshold by functioning as anti-hyperalgesic agents, generally leading to pain reduction rather than absolute eradication, although this outcome may be contingent on the dosage employed (Hewitt, 2000).

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

- Opioids
- Nonsteroidal anti-inflammatory drugs (NSAIDs)
- Alpha-2 receptor agonists
- N-methyl-D-aspartate (NMDA) receptor antagonists
- Others (Local Anesthetics, Corticosteroids, Myorelaxants, Tricyclic Antidepressants, Anticonvulsants, Biphosphonates, Cannabinoids, Alternative Therapies)

Veterinarians employ analgesics to administer pain relief across various distressing conditions, including post-operative or post-traumatic pain, musculoskeletal discomfort, and soft tissue inflammation, particularly within companion animals such as dogs and cats. Nonetheless, among larger animals, analgesics are primarily administered to horses and cattle as a routine practice (Flecknell, 2008). As reported by (Riviere and Papich, 2013), there has been an upsurge in the use of analgesics in the veterinary market since 1998.

It goes without saying the pain management in small ruminants is still until nowadays inadequate and there are several reasons for this. For instance, in the United States and Europe, there are no drugs approved for the use in managing pain in sheep or goats (Lizarraga and Chambers, 2012; Smith et al., 2021). As a result, these medications are being utilized off-label. Moreover, this off-label utilization often faces constraints due to the limited understanding of the drug's pharmacokinetics (PK), effectiveness, and residual effects within these particular animal species. The challenge of administering injectable drugs without established dosing guidelines, coupled with the absence of precise pain-assessment methods for guiding dosing protocols, insufficient education or awareness among many farmers regarding pain-related concerns, alongside considerations of cost and time, compound this situation (Huxley and Whay, 2007; Lizarraga and Chambers, 2012). As a result, these challenges persist due to the scarcity of PK data specific to these species, which not only discourages efforts in drug development by the pharmaceutical companies for these species, but also hinders regulatory approvals for species lacking of approved medications.

In the management of pain, animals such as dogs and cats commonly rely on opioids as a class of analgesics (Robertson and Taylor, 2004). However, this approach can introduce CNS associated side effects such as sedation, euphoria, dysphoria, and excitement in small animals (Papich, 2000). Similarly, farm animals like cattle and sheep experience side effects from opioids, including mild sedation, vocalization, and restlessness (Bassert and Thomas, 2014). Furthermore, inexpensive opioids like morphine prove ineffective in ruminants and can lead to residues in food animals (Chambers et al., 2002). Alpha-2 adrenoceptor agonists, while used with large animals, commonly cause sedation and ataxia as side effects (Bassert and Thomas, 2014; Chambers et al., 2002). Local anesthetics, while cost-effective and short-acting, pose concerns due to potential carcinogenic metabolites in Europe (Chambers et al., 2002). On the other hand, nonsteroidal anti-inflammatory drugs (NSAIDs) act primarily on peripheral pain sites, minimizing CNS-related side effects and boasting little to no withholding time for milk (Chambers et al., 2002; Papich, 2000). This profile

741 makes NSAIDs particularly advantageous, especially for ruminants, by circumventing the drawbacks
742 of higher costs, CNS-related and systemic side effects, and long-acting nature (and thus residues)
743 commonly associated with non-NSAID analgesics (Chambers et al., 2002).

744 **5.3.1. NSAIDs**

745 NSAIDs have a historical legacy in treating various inflammatory conditions, fever, musculoskeletal
746 pain, and arthritis, dating back to ancient times. Documentation of their use dates as far back as the
747 Assyrian era (4000 B.C.) and the Sumerian era (3000 to 1900 B.C.), involving remedies derived from
748 willow tree bark and leaves (Mahdi et al., 2006; Mackowiak, 2000). The Egyptian Ebers Papyrus
749 (1534 B.C.) describes willow's role as an antipyretic and anti-inflammatory agent (Fuster and
750 Sweeny, 2011), while the fourth-century Greek physician Hippocrates also advocated willow leaves
751 and bark for pain, fever, inflammation, and pain relief during childbirth (Seaman, 2011). Discorides
752 employed willow bark for treating rheumatism (Calixto et al., 2000), and other notable physicians
753 such as Celsus, Gallen, and Pliny the Elder recognized its potential in rheumatism treatment (Vane,
754 2000). The first modern use of willow occurred around 1763 when Sir Edmond Stone shared his
755 findings with the Royal Society of London on using willow to address fever and pain (Vane, 2000).

756 Central to willow's efficacy is the glycoside salicin (Vlachojannis et al., 2011), which metabolizes
757 into salicylic acid and other salicylates, producing anti-inflammatory, antipyretic, and analgesic
758 effects. The initial synthetic NSAID, sodium salicylate, emerged in the early 19th century, followed
759 by the acetyl ester of salicylic acid (aspirin) in 1898, developed by Felix Hoffman of Bayer
760 Pharmaceutical company (Riviere and Papich, 2013). Aspirin gradually gained prominence in
761 rheumatism treatment. Yet, the true mechanism of action behind aspirin and other NSAIDs remained
762 elusive until Sir John Vane elucidated it in 1971 (Vane, 1971), leading to the discovery of numerous
763 other NSAIDs.

5.3.1.1. Mechanism of action of NSAIDs

NSAIDs exert their effects by impeding the activity of the cyclooxygenase (COX) enzyme, an integral component for synthesizing prostanoids, namely prostaglandins and thromboxanes (TXA2) (Vane, 2000b). These prostanoids play dual roles, both in facilitating pain signaling pathways during inflammatory states and in orchestrating vital physiological processes (Zarghi and Arfaei, 2011). The initiation of prostanoid synthesis involves the release of fatty acids from cell membrane phospholipids due to tissue damage. These fatty acids, upon conversion by phospholipase A2, yield arachidonic acid (AA). COX, characterized by two main isoforms, COX-1 and COX-2, as well as other enzymes like lipoxygenase (LOX) and its isoforms, work on AA to create a collection of oxygenated C20 fatty acid-derived lipid mediators collectively known as eicosanoids (Riviere and Papich, 2013).

COX enzymes trigger the formation of prostaglandin G2 (PGG2), which undergoes conversion into prostaglandin H2 (PGH2) via peroxidase. Additional prostaglandins (PGD2, PGE2, PGI2, PGF2, and TXA2) are synthesized through catalytic synthase enzymes (Rao and Knaus, 2008). Notably, prostaglandins are found in inflammatory exudates, their synthesis heightened in response to tissue damage (Davies et al., 1984). Thus, NSAIDs intervene at the COX enzymes' level to hinder prostanoid production, consequently yielding analgesic effects. However, comprehending the intricacies of prostaglandin functions, COX enzymes' roles, and the selective impacts of NSAIDs on COX is essential for a detailed grasp of NSAID mechanisms (Mathurkar, 2016).

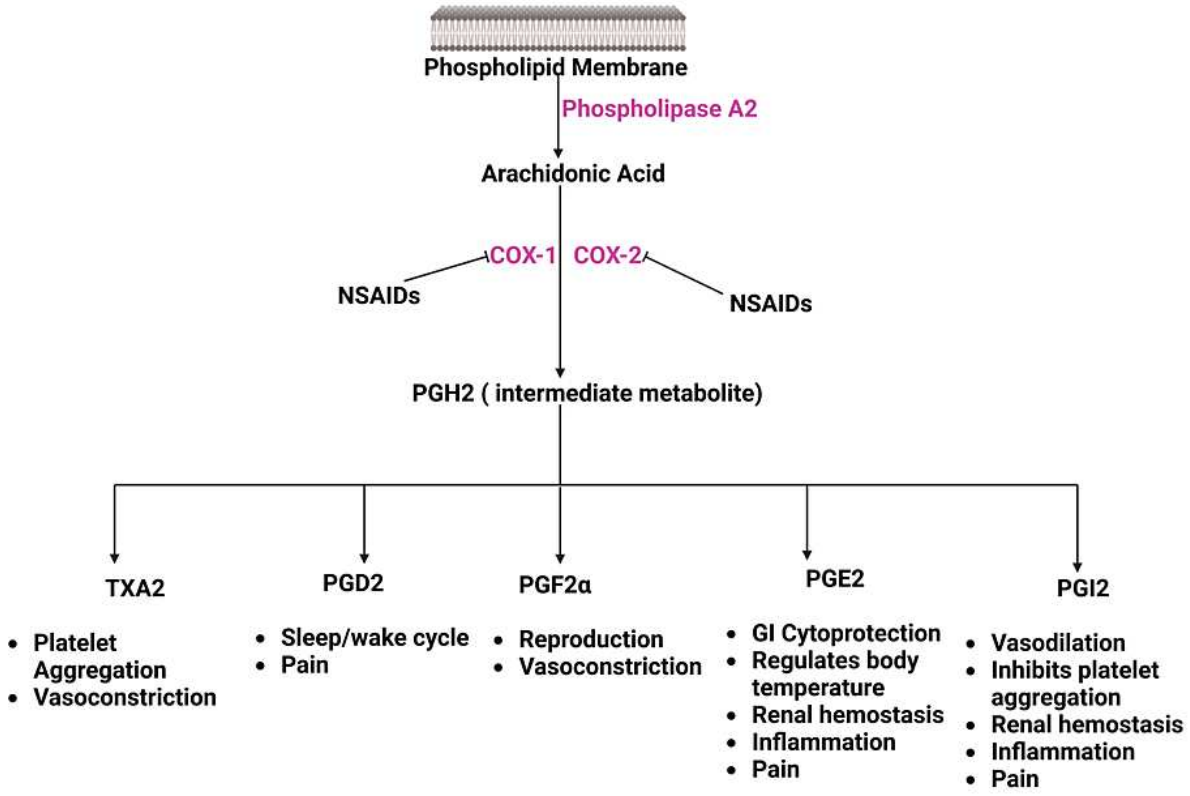


Figure 5: Mechanism of Action of NSAIDs (Source: Sohail et al., 2023)

a. Prostaglandins

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

PGH2 is the precursor for the main bioactive prostaglandins, PGD2, PGE2, PGI2 and PGF2 which are present in most cells; however, their biosynthesis is remarkably increased in response to inflammation, especially in acutely inflamed tissues (Ricciotti and FitzGerald, 2011). Each prostanoid

has specific tissues where preferential synthesis takes place e.g. PGF2α in uterus, PGI2 in endothelium etc (Ricciotti and FitzGerald, 2011, Breyer et al., 2001).

The synthesis of prostaglandins depends upon COX enzymes which have two main isoforms, COX-1 and COX-2. COX-1 is considered to produce the PGs which have constitutive function, while COX-2 is induced by inflammatory processes (Brzozowski et al., 2001). COX-1 preferentially links with TXA2 synthase, PGF synthase, and PGE (cytosol) synthase, while COX-2 prefers PGI and the PGE (microsomal) synthase (Smyth et al., 2009).

PGs bind to specific rhodopsin-like-7-transmembrane-spanning G protein-coupled receptors (Ricciotti and FitzGerald, 2011). There are eight prostanoid receptors, E prostanoid receptor (EP) 1, EP2, EP3 and EP4 which bind PGE; D prostanoid receptor (DP1); F prostanoid receptor (FP); I prostanoid receptor (IP); and TXA2 receptor (TP) (Breyer et al., 2001).

PGE2

PGE2 is a COX-1 and 2 derived PG exhibited in many animal species and is widely involved in biological processes such as immunity, gastrointestinal integrity, fertility and blood pressure; however, impairment in its synthesis is followed by series of pathological conditions such as chronic inflammation, Alzheimer's disease, or tumorigenesis (Legler et al., 2010). PGE2 is involved in all classical processes of inflammation such as redness, swelling and pain which makes the role of PGE2 prominent in inflammation (Ricciotti and FitzGerald, 2011). EP1 receptors are involved in the typical sign of inflammation, hyperalgesia, which occurs through peripheral as well as central activation (Moriyama et al., 2005). EP2 and EP4 are involved in collagen induced arthritis where development of swelling is due to these receptors (Honda et al., 2006). Similarly, EP2 and EP3 are observed in carrageenan induced oedema and pleurisy (Yuhki et al., 2004).

PGD2

PGD2 is widely involved in the various systems of the body such as CNS where it plays role in induction of sleep, regulation of body temperature and hormonal release (Nagata and Hirai, 2003, Kobayashi and Narumiya, 2002) and other systems such as the vascular and immune systems where it has specific roles. In the vascular system it inhibits the aggregation of platelets and in the immune system it is secreted by mast cells after activation with antigen in allergic conditions such as asthma (Nagata and Hirai, 2003, Kobayashi and Narumiya, 2002).

PGI2

PGI2 or prostacyclin, is a prostaglandin that affects many body systems. It has two main functions as inhibition of platelet aggregation and it acts as vasodialator (Kelton and Blajchman, 1980). This eicosanoid has an important role in the cardiovascular system through its receptor IP and along with vasodilation it is an inhibitor of platelet aggregation, leukocyte adhesion, and vascular smooth muscle cells proliferation (Kawabe et al., 2010). Apart from its protective role, this PG is present in inflammatory exudates in arthritis (Ricciotti and FitzGerald, 2011).

PGF2α

This PG has a role in various activities of the reproductive system such as in luteolysis (in ruminants including sheep is most important), uterine smooth muscle contraction, and instigation of parturition; apart from this, it is also involved in the renal function, myocardial function and pain (Ricciotti and FitzGerald, 2011, Kunori et al., 2009, Eguchi et al., 1992, Silvia et al., 1991). PGF2α is present in acute and chronic inflammatory exudates in conditions such as arthritis, obesity, diabetes etc. (Higdon and Frei, 2003). In humans PGF2α has been reported to cause bronchoconstriction, especially asthamatic people are more prone to this action; however, response differs on individual basis (Pasargiklian et al., 1976)

TXA2

TXA2 is largely a COX-1 derivative (Ricciotti and FitzGerald, 2011). It has mixed role as pro- and anti-inflammatory mediator as it is evident in the asthma (Tilley et al., 2001). It is involved in platelet aggregation (Gryglewski et al., 1978). It is also a potent vasoconstrictor and therefore has potential risk in induction of cardiovascular disorders (Cheng et al., 2002).

b. Cyclooxygenase enzymes

COX is the enzyme required to catalyse the process of prostaglandins synthesis. It has two main isoforms COX-1 and COX-2 and these enzymes are also known as prostaglandin endoperoxide H synthases (PGHS) (Smith et al., 1996). The enzyme was first discovered from sheep seminal vesicles when Sir John Vane described the mechanism of aspirin inhibiting the enzyme COX and ultimately preventing the synthesis of prostanoids (Vane, 1971). After about 20 years, COX-2 was discovered in early 90's with about 60% similar amino acid sequencing as that of COX-1 with a different expression pattern and biology (Smith et al., 1996). Recently, third isoform called COX-3 has also been discovered which is considered as the variant of COX-1 (Chandrasekharan et al., 2002) and may only be present in dogs.

COX-1

COX-1 is a constitutive enzyme which is present in almost every tissue and responsible for the synthesis of prostaglandins that are important in many vital physiological functions (Talley et al., 2000). PGE2, PGF2α and TXA2 are predominantly COX-1 derived and they play important physiological functions (Ricciotti and FitzGerald, 2011). Classic NSAIDs such as aspirin and indomethacin inhibit COX-1 and prevent synthesis of prostaglandins required for protective functions such as maintenance of integrity of gastrointestinal mucosa, reproductive functions related to PGF2α (Willoughby et al., 2000, Mitchell et al., 1993).

COX-2

The discovery of COX-2 enzyme led to the development of COX selective NSAIDs. COX- 2 is an isomer of COX-1 with a slight difference in its amino acid sequencing (COX-1 has 576 amino acids as opposed to COX-2 with 581 amino acids) (Rouzer and Marnett, 2009). COX-2 is not present in all tissues normally but has dramatically increased levels after exposure to cytokines (IL-1, TNFα), growth factors, bacterial toxins etc. (Riviere and Papich, 2013, Dubois et al., 1998). COX-2 has wide range of functions which includes both constitutive physiological and pathological processes. In the reproductive system of mice COX-2 is involved in the ovulation, fertilization, and implantation as well as during the completion of pregnancy (Lim et al., 1997). It is also constitutively present in monocytes, macrophages, endothelial cells, spinal cord, brain and ciliary body of the eye etc. (Riviere and Papich, 2013). However, in the brain, it is involved in the neurodegenerative disorders (Alzheimer's disease) and also synthesises PGs which induce fever. It has also a significant role in certain cancers (Riviere, 2009). The presence of COX-2 in the cartilage and synovial fluid in osteoarthritis and rheumatoid arthritis shows its role in inflammatory and painful conditions which can be considered due to its action at peripheral as well as central sites (Dubois et al., 1998). Due to the participation of COX-2 in these non- constitutive functions, COX-2 selective NSAIDs have been developed so that they can specifically inhibit COX-2 without disrupting the COX-1 functions (DeWitt, 1999). Coxibs are a new class of COX-2 selective NSAIDs which includes deracoxib, mavacoxib, robenacoxib, firocoxib for veterinary use (Riviere and Papich, 2013). There are several *in vitro* test systems available for testing the selectivity of COX-2 inhibitors. These tests are classed into three main groups as purified/recombinant enzymes, cultures of intact cells and human whole blood assay (Giuliano and Warner, 1999).

COX-3

Simmons et al. (1999) and Willoughby et al. (2000) proposed a third isoform of this enzyme family, COX-3, which might represent a new therapeutic target. However, Chandrasekharan et al. (2002)

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

5.3.1.2. Additional possible mechanisms of action of NSAIDs

It has been established that some NSAIDs act not only on COX-1 and COX-2, but also inhibit the nuclear transcription factor κB that is essential for cytokine gene expression during inflammation (Vaish and Sanyal, 2011; Lawrence, 2009). Inhibition of NFκB, related transcription factors, or cytokines themselves, could be considered a potential treatment for acute and chronic inflammatory pain (Carr and Goudas, 1999). Another possible mechanism of action could be the inhibition of 5-lipoxygenase (5-LO) which ultimately inhibits leukotrienes synthesis; tepoxalin is an example of a dual inhibitor i.e. COX and 5-LO. Inhibition of NFκB which controls the expression of COX-2 and cyclin-1; also inhibition of TNF (tumour necrosis factor) by most of the NSAIDs such as aspirin, ibuprofen, sulindac, phenylbutazone, naproxen, indomethacin, diclofenac, celecoxib has been demonstrated and should be considered as an additional mechanism of action of NSAIDs (Takada et al., 2004). Similarly, various other mechanisms of action of NSAIDs described are inhibition of action of eicosanoids on their receptors (Funk, 2001), stimulation of nuclear receptor peroxisome proliferator-activated receptor-gamma (PPAR-γ), inhibition of bradykinin (Fahmi et al., 2002),

913 modulation of release of pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF-α), increased intracellular
914 breakdown of ATP to adenosine, inhibition of neutrophil activation and ultimately preventing release
915 of oxygen radicals (superoxide, hydroxyl) as well as lysosomal and non-lysosomal enzymes (Riviere
916 and Papich, 2013), modulation of synthesis of nitric oxide (Bergh and Budsberg, 2005) etc. Further,
917 a spinal mechanism of action of NSAIDs has been reported in different studies where intra-thecal
918 administration of NSAIDs inhibits behavioral hyperalgesia produced by the action SP and NMDA
919 (by formation of PGs) in the spinal cord (McCormack, 1994, Malmberg and Yaksh, 1994).

920 ***5.3.1.3. Therapeutic uses of NSAIDs***

921 NSAIDs are generally used as anti-inflammatory, anti-pyretic and analgesic drugs. NSAIDs have
922 demonstrated efficacy in addressing lameness and various musculoskeletal conditions in a diverse
923 range of animals, extending beyond equines and dogs to include species such as sheep and goats.
924 NSAIDs are indeed used in sheep and goats for indications such as lameness, musculoskeletal
925 injuries, postoperative pain management, arthritis, soft tissue injuries, pain associated with infectious
926 diseases, foot rot, mastitis, respiratory infections, and discomfort related to reproductive issues.
927 Furthermore, the versatility of NSAIDs extends to avian species, where they have shown promise in
928 alleviating pain associated with conditions unique to birds. Those conditions include pododermatitis,
929 egg-laying difficulties, beak and feather disease, gastrointestinal issues, respiratory infections,
930 traumatic injuries, ophthalmic conditions, as well as pain and inflammation associated with
931 orthopedic problems, soft tissue injuries, postoperative recovery, and complications from infectious
932 diseases.

933 NSAIDs also act as antithrombotic agents as they inhibit blood clotting by blocking the formation of
934 TXA2 by COX-1 (Riviere and Papich, 2013) especially aspirin which irreversibly inhibits COX-1 in
935 platelets and therefore is used in cats to treat aortic embolism (Smith et al., 2003).

936 Similarly, the use of NSAIDs in oncology has also been revealed as some promising results were
937 observed in controlling the growth of neoplastic cells in rats and dogs (Bergh and Budsberg, 2005)

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

5.3.1.4. Importance of use in farm animals:

In our experimental endeavors with farm animals, the testing of NSAIDs holds paramount significance for a myriad of reasons. These crucial investigations not only contribute to the overall understanding of the efficacy and safety of NSAIDs in diverse agricultural settings but also play a pivotal role in advocating for the approval of these medications for widespread use in farm animals. Such research initiatives provide invaluable insights that can influence legislation, fostering a more informed and evidence-based approach to the integration of NSAIDs into farm animal management practices. By systematically assessing the benefits and potential challenges associated with NSAID use, our experiments contribute to shaping policies that prioritize the well-being, health, and productivity of farm animals while adhering to regulatory standards. Several key aspects highlight the importance of NSAID use in these animals:

1. Pain Management: NSAIDs play a crucial role in alleviating pain associated with various conditions, including injuries, surgeries, and chronic musculoskeletal disorders. By providing pain relief, NSAIDs contribute to the overall well-being and comfort of farm animals.

2. Improved Welfare: Pain and inflammation negatively impact the welfare of farm animals, affecting their behavior, productivity, and overall health. NSAIDs help improve animal welfare by addressing pain and discomfort, allowing for a better quality of life.

3. Enhanced Recovery: In the case of surgical procedures or injuries, NSAIDs aid in the recovery process by reducing postoperative pain and inflammation. This, in turn, promotes faster healing and a smoother return to normal activities.

4. Management of Lameness: Lameness is a common issue in farm animals, affecting their mobility and, consequently, their ability to access food and water. NSAIDs are valuable in

managing lameness by addressing the pain and inflammation associated with joint and hoof problems.

5. Increased Productivity: Healthy and pain-free animals are more likely to exhibit normal behaviors, consume adequate nutrition, and produce optimally. NSAIDs contribute to maintaining the productivity of farm animals by ensuring they can move, eat, and perform essential activities without hindrance.

6. Prevention of Secondary Complications: NSAIDs can help prevent secondary complications arising from conditions such as inflammation, which, if left unmanaged, could lead to further health issues.

7. Facilitation of Veterinary Interventions: NSAIDs are often used in conjunction with veterinary treatments, enabling veterinarians to perform necessary procedures or administer medications more effectively. The reduction of pain and inflammation facilitates veterinary interventions and improves the overall success of treatments.

8. Cost-Effective Approach: Managing pain and inflammation with NSAIDs can be a cost-effective approach in farm animal husbandry. By addressing issues promptly, farmers may prevent the development of more severe conditions that could lead to higher veterinary costs or loss of productivity.

4.1.1.4.Side effects of NSAIDs

The most common side effects of NSAIDs are gastrointestinal irritation and ulcer in monogastric animals and humans (Beck et al., 2000). Rarely renal failure (especially with COX-inhibitors) has also been observed in animals such as dogs and in humans (Lomas and Grauer, 2015). Apart from these, some other very rare adverse effects are also reported in humans which are similar for all NSAIDs. CNS associated symptoms such as headaches, tinnitus and dizziness. Cardiovascular symptoms include fluid retention, hypertension, oedema and rarely, congestive heart failure.

Gastrointestinal symptoms involve abdominal pain, dysplasia, nausea, vomiting, ulcers and bleeding. Other side effects such as thrombocytopenia, neutropenia, abnormal liver enzymes, asthma, skin rashes (pruritis) and renal insufficiency (Katzung et al., 2004). In ruminants such as cattle only reduction in fertility i.e. irregular oestrous cycles, reduction in pregnancy rates and reduced formation of corpus leutium have been reported (Stahringer et al., 1999). Only one report of gastrointestinal impairment (abomasal ulceration) due to NSAID (ibuprofen) in ruminants (calves) is evident as far as our knowledge till date (Walsh et al., 2016), though it is commonly listed as a potential cause of abomasal ulceration in ruminants.

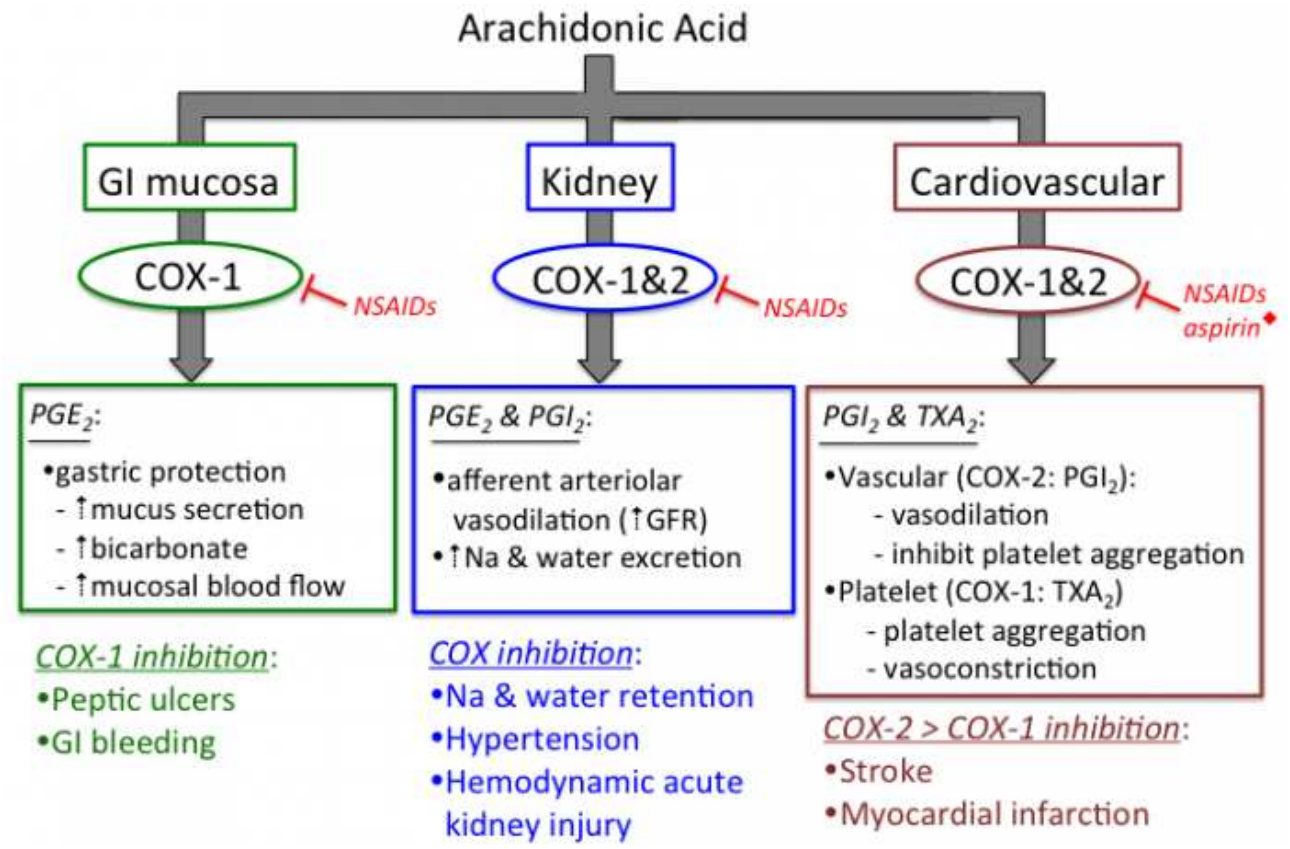


Figure 6: Side effects of NSAIDs (Source: https://tmedweb.tulane.edu/pharmwiki/doku.php/nsaid_side_effects).

4.1.1.5.Classification of NSAIDs

All NSAIDs have potentially similar properties. Chemically, all are weak acids and have similar pharmacological actions i.e. anti-inflammatory, anti-pyretic and analgesic properties, and also clinical

uses (Riviere and Papich, 2013). However, they can be classified considering different criteria such as chemical properties, clinical uses, COX enzyme selectivity etc (Conaghan, 2012). Therefore, researchers are attempting to improve NSAIDs classification. Frölich (1997), have classified NSAIDs on their COX selectivity and other criterion nonetheless, Griswold et al. (1997) disagreed with this classification to some extent, though he agrees the necessity of re-classification of NSAIDs.

According to chemical properties, classically NSAIDs are described as two weak acid groups namely, carboxylic acids (R-COOH) and enolic acids (R-COH) (Nolan, 2000). Further classification of these acid groups' compounds, based on the chemical structure, is shown in the figure below. In addition to these, another group of NSAIDs is COXIBs on the basis of their COX selectivity i.e. the ability/preference of the NSAID to inhibit COX-1 or COX-2 or both and is expressed as the ratio of the COX-2 IC_{50} to the COX-1 IC_{50}, so that the more COX-2-selective an agent the smaller is the ratio expressed (Hawkey, 1999). IC_{50} is the half maximal inhibitory concentration. It is a measure of the effectiveness of a substance/drug in inhibiting a specific biological or biochemical function; here, inhibition of enzymes COX-1 and/or COX-2. Some NSAIDs inhibit COX-1 enzyme, some inhibit specifically COX-2 enzyme while some are non-selective. The existing drugs which selectively inhibit COX-2 enzyme are the coxibs; These drugs are efficient in reducing gastric ulceration and irritation due to their selectivity to COX-2 in animals such as rats and in humans (Silverstein et al., 2000; Hawkey, 1999). Other specifically designed drugs preferentially select COX-2 (Hawkey, 1999), such as meloxicam, nimesulide and etodolac. It is worth noting however that despite the classification, the COX-2 selectivity of these drugs can exhibit significant variation among different animal species. A drug that is COX-2 selective in one species may demonstrate non-selectivity in another, emphasizing the importance of considering chemical structure for a more accurate classification. For example, carprofen is COX-2 selective in dogs, but not in cats or horses; meloxicam is COX-2 selective in humans and dogs, but not in cats; and piroxicam shows COX-2

selectivity in dogs, but not humans. Thus, COX-2 selectivity of NSAIDs is a species-dependent phenomenon that thus fur is not predictable based on drug class or structure.

Considering the focus of this thesis, attention will be solely directed towards coxibs in the next part, while the latter portion of the research will be dedicated to the examination of robenacoxib and deracoxib exclusively.

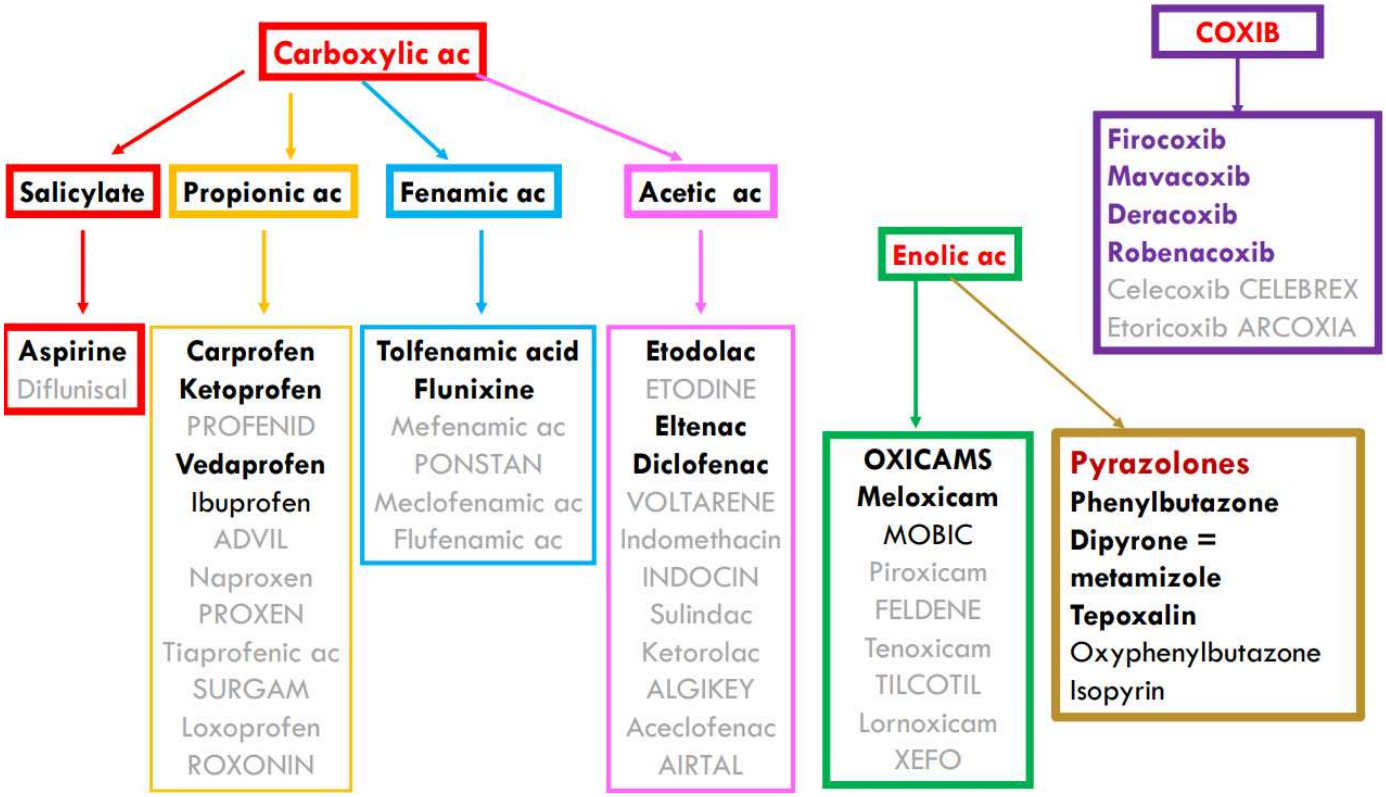


Figure 7: Classification of NSAIDs on the basis chemical properties. Bolded medications are employed in veterinary medicine, including the purple-bolded coxibs + enflicoxib (Courtesy: Associate Professor Racha Karaky).

a. COXIBS

Coxibs represent a subset of NSAIDs with selective effects on COX-2, sparing COX-1 activity. Steric hindrance results in the smaller size of the COX-1 active site compared to COX-2. The greater bulk of coxibs obstructs their inhibition of COX-1, while simultaneously facilitating thorough inhibition

of the COX-2 pathway. Furthermore, the lack of a carboxyl group (-COOH), which otherwise prevents the interaction with arginine 120 and diminishes the capacity to bind to COX-1, alongside the presence of functional groups that engage with the amino acids situated in the lateral pocket of the cyclooxygenase channel, collectively contribute to the specific targeting of COX-2. Coxibs, considered the third NSAID generation (Sternon, 2001), have been introduced in the human field. Celecoxib and rofecoxib were the forerunners of this family of drugs (first generation), with the latter being removed from the market in 2004 due to substantial adverse effects on the cardiovascular system. More recent compounds (valdecoxib, parecoxib, etoricoxib, and lumiracoxib), termed second generation, exhibit higher COX-2 enzyme selectivity (Stichtenoth, 2004; Andersohn et al., 2006). In the realm of veterinary medicine, deracoxib (2002), firocoxib (2007), mavacoxib (2008), and robenacoxib (2009) have been introduced for animal use (Bergh and Budsberg, 2005). Cimicoxib (2011), initially developed for human use (Emmerich, 2012), later found its way into the veterinary market. The most recent addition to the veterinary coxib landscape is enflicoxib, also known as E-6087, designed for treating pain and inflammation associated with osteoarthritis in dogs (VMD, 2021). Indeed, the notably prolonged half-lives and high efficacy observed in both enflicoxib and mavacoxib may prompt further exploration of their pharmacological behaviors in diverse animal species in future research projects.

CHAPTER II: Robenacoxib and Deracoxib Features, Examination of Previous Data, and Significance of Clinical Pharmacokinetic Parameters

1. **Robenacoxib**

Robenacoxib (RX), marketed under the brand name Onsior®, is an innovative veterinary COXIB medication employed for managing pain and inflammation in both dogs and cats. It received approval for distribution in Europe in the year 2008. This drug is accessible in tablet form, with five distinct dosages (6 mg for cats and 5 mg, 10 mg, 20 mg, and 40 mg for dogs), as well as in an injectable solution (20 mg/mL for both dogs and cats). The tablets are administered once daily at a consistent time, with the specific dosage adjusted based on the animal's body weight and the intended usage. In feline patients, treatment duration is limited to six days for acute musculoskeletal issues, while for chronic musculoskeletal problems, it can extend over a longer period, all under the careful monitoring of a veterinarian. In dogs, the treatment of osteoarthritis should continue for the necessary duration (Anonymous, 2008). It is used for: pain management, osteoarthritis, post-operative pain, Chronic MusculoSkeletal Disorders (CMSD), acute musculoskeletal disorders, feline stomatitis, fever reduction and so on.

- **Description**

RX falls within the category of organic compounds known as aniline and substituted anilines. This class is a subset of benzenoids, which are organic compounds featuring a benzene ring. Specifically, aniline and substituted anilines are characterized by the presence of an aminobenzene moiety in their molecular structure.

- Empirical formula: C16-H13-F4-NO2.
- IUPAC name: [5-Ethyl-2-(2,3,5,6-tetrafluoro-phenylamino)-phenyl]acetic acid
- Synonyms: Onsior; 220991-32-2; robenacoxibum; CHEBI:76269; Z588009C7C; UNII-Z588009C7C, ect.

- Structural formula is:

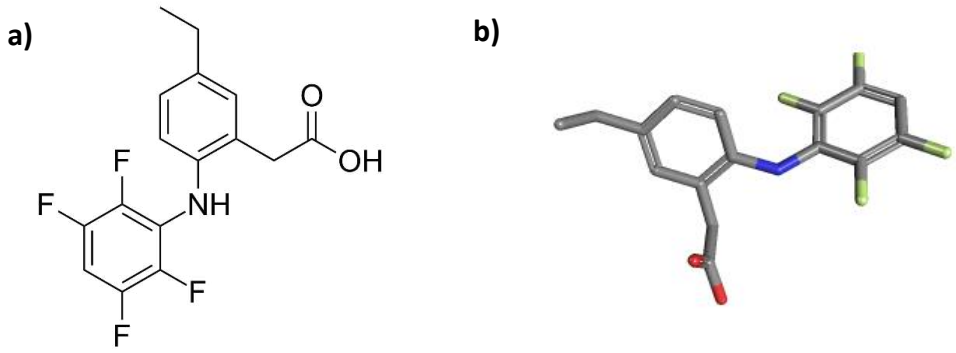


Figure 8: a) Robenacoxib chemical structure b) Robenacoxib-3D chemical structure

In terms of structure, RX is closely related to diclofenac, a COX-2 preferential inhibitor, as well as to lumiracoxib, a COX-2 selective inhibitor developed for human use (Esser et al., 2005). Both RX and lumiracoxib exhibit distinct structural characteristics when compared to other selective COX-2 inhibitors. They lack a sulfur-containing group but do possess a carboxylic acid component, the latter being a common trait among most conventional NSAIDs (Esser et al., 2005).

- **Physicochemical proprieties**

- Water solubility at pH 3 is 0.01 g/l
- Water solubility at pH 6.8 is 0.17 g/l
- Molecular mass: 237.27 g/mol

1.1. Previously reported pharmacokinetics of robenacoxib

1.1.a. Absorption and bioavailability

After being administered orally to rats, RX was rapidly absorbed, with peak plasma concentrations observed at 1.3 hours (T_{max}), and the bioavailability or F % measured at 80% (King et al., 2009). This can be attributed to its relatively high aqueous solubility at the absorption site. The pKa value of 4.7 ensures that the majority of molecules remain non-ionized in the acidic environment (compared to plasma) of the stomach and small intestine, promoting diffusion into the plasma (Mangold et al., 2004). Moreover, the non-ionized form's considerable lipid solubility contributes to this process.

In a canine study, rapid peaks in blood concentrations were achieved after oral administration (T_{max}= 0.5 hours fasting; T_{max}= 0.25 hours fed), with C_{max} reaching 0.947 µg/ml without food and 0.832 µg/ml with food (Jung et al., 2009). RX's absorption rate from the gastrointestinal tract remained unaffected by a fed state, yet its F % decreased. Fasted administration in dogs showed high bioavailability (84%), contrasting with significantly lower bioavailability (62%) under fed conditions. Several aspects of gastrointestinal physiology are influenced by food, including gastric emptying time, acid secretion, blood flow, intestinal motility, bile secretion, and enzyme production. Inter-animal variability was relatively low in this context (Jung et al., 2009). The rapid onset of action applies as well following subcutaneous (SC) administration in dogs, in which RX exhibited a T_{max} of 0.5 hours and a slightly lower C_{max} compared to oral administration (C_{max}= 657 ng/ml), with a bioavailability of 88%.

In fasted cats, RX achieved absolute bioavailability of 69% via SC injection, with a T_{max} of 1 hour, and 49% via oral administration without food, with a T_{max} of 0.5 hours. Administering a third of the daily diet led to a 104% bioavailability compared to the full daily ration (80%) (King et al., 2013). Hence, data analysis suggests that RX administration with the full daily ration could decrease absorption rate and amount. A separate study involving rabbits by Jeffrey et al. (2022) found RX to reach peak concentration at 1.2 hours (oral) and 0.31 hours (SC). Oral delivery resulted in notably lower C_{max} (0.23 µg/ml) than SC administration (5.82 µg/ml). Given rabbits' prolonged gastric emptying times and the challenge of achieving a fully empty stomach even under fasting, they have not been considered optimal for oral F % studies due to their extended gastrointestinal residence time, potentially leading to higher C_{max} values compared to feline patients (Paulson et al., 2001).

In the case of ruminants and small-ruminants, RX's oral F % might be impacted by its binding to hay or digesta, similar to other NSAIDs such as phenylbutazone and flunixin meglumine (Lees et al., 1998).

1.1.b Distribution

The typically low volume of distribution (V_d) observed in NSAIDs is often associated with their notably high plasma protein binding. Indeed, upon IV administration in cats and dogs, the Vd values were considered low with values of 190 ml/kg and 240 ml/kg, respectively (King et al., 2009; Borer et al., 2017). Similar results were reported in other feline studies as well (volume of distribution at steady state around 0.20 ml/kg) (Giraudel et al., 2009; Pelligand et al., 2016).

RX's in vitro binding to rat, dog, monkey, and human plasma proteins was assessed using a plasma concentration of 50 ng/ml, revealing over 99% binding across all animal species (King et al., 2009). Notably, RX displayed robust plasma protein binding, with dogs and cats exhibiting over 98% protein binding at a 2 mg/kg dosage (Jung et al., 2009). The competition between NSAIDs and RX for protein binding sites is unlikely to significantly impact the likelihood of medication interactions, potentially leading to temporary increases in free concentrations at most (Toutain and Bousquet-Mélou, 2002).

As for the partition in the blood department, when researchers conducted the study (Jung et al., 2009), they measured the levels of RX in both blood and plasma of dogs and cats. In their findings, they reported the ratios of RX concentrations in blood compared to plasma. For dogs, the ratio was 0.44:1. For cats, the ratio was 0.65:1. These ratios, given the estimated red cell volume of approximately 45% in dogs and around 35% in cats, suggest that RX is predominantly located within the plasma component and that it doesn't strongly bind to red blood cells.

In mammals, RX tends to accumulate and remain in tissues for longer periods than in plasma. Contrary to what one might expect based on its short blood terminal half-life or t1/2, RX's tendency to amass in inflammatory sites results in prolonged activity duration in conditions related to peripheral inflammation. RX displayed a preference for distribution into inflammatory exudate within tissue cages compared to blood, attributed to its physicochemical property as a weak acid (pKa= 4.7), as well as its high plasma protein binding capacity (King et al., 2009). The carboxylic acid group seems to contribute to the significant protein binding (Brune et al., 2004). Comparable to RX, diclofenac

and lumiracoxib also exhibit selective distribution to inflamed areas and human synovial fluid (Esser et al., 2005; Scott et al., 2004). In another study, after oral administration, low levels of RX were found in aqueous humour, demonstrating that the drug crossed the intact blood-aqueous barrier, and thus signifying a high penetration rate (Sharpe et al., 2018).

In another study involving eight Beagle dogs with urate-induced stifle inflammation and osteoarthritis diagnosis, RX's population PK profile was evaluated in both blood and stifle joint synovial fluid (Silber et al., 2010). While initially assuming the parameters for healthy and osteoarthritic dogs to be the same, differences were subsequently examined for their significance, particularly related to the absorption model and disposition parameters such as joint distribution. RX was estimated to enter the joint of osteoarthritic dogs 1.8 times faster than in healthy dogs. RX's residence time in inflamed stifle joint synovial fluid was extended compared to non-inflamed synovial fluid or blood (Silber et al., 2010).

1.1.c. Metabolism

RX undergoes significant liver metabolism in cats and dogs. Radiolabeled studies involving RX metabolism included the regular collection and analysis of blood, feces, and urine samples. Radioactivity (total residues) was assessed using High Performance Liquid Chromatography (HPLC) mass spectrometry for the parent compound and TLC for the metabolites. In both dogs and cats, a metabolite was notably persistent, alongside the lactam metabolite which is a synthetic precursor of RX and might also be a by-product or degradation product. The specific composition of other metabolites in cats or dogs remains undisclosed, and no pharmacological effects resulting from these metabolites were demonstrated (Anonymous, 2008).

Indeed, the hepatic metabolism was swift. The metabolite(s) exhibited prolonged presence in the bloodstream compared to the parent molecule. Even at the 24-hour mark after oral administration, the parent compound was undetectable, but significant levels of radioactivity, represented by unidentified hydrophilic breakdown products, persisted in the blood. The parent compound wasn't identified in

urine samples. The feces revealed a rather intricate array of metabolites, with a fraction displaying higher lipophilicity, and some instances of the parent compound detection as well (Anonymous, 2008).

1.1.d. Clearance

RX is primarily eliminated through the biliary route, accounting for 70% of the excretion, with the remaining 30% being excreted through the kidney (Anonymous, 2008). Similar trends of predominantly biliary excretion, as opposed to renal excretion, have been observed for other NSAIDs in dogs (carprofen, meloxicam, and mavacoxib) and cats (meloxicam) (Grudé et al., 2010). Indeed, the excretion of IV administered 14C-radiolabelled RX was primarily in feces, 64.6% in dogs and 72.5% in cats, consistent with elimination in bile following hepatic metabolism (King and Jung, 2021). The clearance (Cl) values were 0.81 and 0.29 L/h/kg for dogs and cats, respectively. Variations in cardiac output can explain the variability in Cl of RX between animals. The lower hepatic extraction ratio found in cats, which would explain the differences in the species isoform composition, expression, and activity of biotransformation enzymes, could justify the lesser ability to eliminate RX when compared to dogs (King et al., 2009). Similarily, in the study conducted by Giraudel et al. (2009), body Cl in cats was relatively low at 0.44 l/kg/h. Findings from Pelligand et al. (2012) also indicated comparable outcomes, with a body Cl of 0.502 l/kg/h for cats. In contrast, dogs exhibited a moderate body Cl of 0.81 l/kg/h. Notably, neither age, body weight, nor sex exerted any discernible influence on the Cl of RX in either species. Silber et al. (2010) observed a distinct variation, reporting a 75% higher Cl rate in healthy Beagle dogs compared to dogs with osteoarthritis. Plausible explanations for this divergence encompass the marginally older age and slightly lower average weight of OA-afflicted dogs, alongside the potential inhibition of cytochrome P450 (CYP) due to chronic inflammation observed in osteoarthritis cases (Renton, 2001).

The renal elimination of unchanged RX is anticipated to be minimal. In cases of renal dysfunction in animals, adjusting the RX dose is usually unnecessary or only marginally needed, owing to the minor

contribution of urinary excretion (King et al., 2009). Given its strong binding to plasma proteins, glomerular ultrafiltration is expected to be restricted, leading to a decreased renal Cl of RX in its original form. Moreover, due to the typically lower pH of urine in cats and dogs compared to human blood (pH 7.4), there's a propensity for elevated passive absorption of RX within the tubules.

1.2. Previously reported pharmacodynamics of robenacoxib

1.2.a. Inhibition of cyclooxygenase

Although other modes of action cannot be excluded, all important pharmacodynamic (PD) properties of RX have been at- tributed to COX-2 inhibition. Increased molecular bulk and altered shape account for RX's COX-2 selectivity. In all species tested, RX is a potent and selective COX-2 inhibitor, producing no significant COX-1 inhibition at clinically recommended dosages.

In early studies, RX was evaluated in purified enzyme assays. Binding to ovine COX-1 was weak and rapidly reversible (dissociation t1/2 <1 min). Binding affinities were 0.8 µM (COX-1) and 0.03 µM (COX-2), indicating both selectivity and high potency for COX-2 inhibition. Compared with naproxen (non- selective) and diclofenac (moderately COX-2 selective), RX was also highly COX-2 selective in cell-based assays (King et al., 2009).

Additional information was gathered through experiments conducted on rats using inflammatory exudate and whole-blood assessments. In a model involving lipopolysaccharide (LPS)-induced air pouch inflammation, the ID50 values for inhibiting COX-2-derived prostaglandin (PGE2) were found to be 0.3 mg/kg when administered orally as RX and 0.1 mg/kg when given orally as diclofenac (King et al., 2009). Moreover, in a zymosan-induced tissue chamber inflammation model, the oral administration of 2 mg/kg of RX inhibited COX-2 by 83% after 12 hours, while having no inhibitory effect on COX-1 (King et al., 2009). In a study assessing gastric tolerability in rats, diclofenac, administered orally at a high dose of 30 mg/kg, inhibited serum TXA2, PGE2, and 6-keto-PGF1α in gastric and ileal biopsies, indicating COX-1 inhibition. In contrast, the same high dose of RX (30 mg/kg, orally) did not induce significant changes compared to the vehicle (King et al., 2009). When

it comes to clinical relevance, whole-blood COX-1 and COX-2 assays hold particular importance (Pairet and Engelhardt, 1996). In in vitro whole-blood assays comparing different NSAIDs in dogs, the IC50 values for COX-1 and COX-2 indicated a lack of selectivity for ketoprofen, moderate COX-2 selectivity for R-carprofen, meloxicam, diclofenac, and S-carprofen, and a high degree of selectivity for RX (King et al., 2010). However, it's worth noting that COX-2 mean 80% inhibitory concentration (IC_{80}) is a more relevant predictor of efficacy than IC50, as most NSAIDs inhibit COX-2 by approximately 80% at clinically effective concentrations (Lees et al., 2004; Warner et al., 1999). Furthermore, to minimize side effects related to gastrointestinal and homeostatic functions, it's important to maintain a concentration of COX-1 inhibition not exceeding IC20 (Giraudel et al., 2009). This principle likely applies to RX as well.

In dogs, there was a close similarity between the ED80 for COX-2 inhibition (1.21 mg/kg) and the ED50 for improvement in weight-bearing in the urate synovitis model (1.23 mg/kg; Schmid et al., 2010b), indicating consistency in the dosages required for these effects. In studies involving Beagle dogs, COX inhibition in the blood was assessed after administering therapeutic and higher dosages of RX (1–8 mg/kg orally, 0.5–4 mg/kg subcutaneously) (Borer et al., 2017; King et al., 2011; Schmid et al., 2010b). While all doses inhibited COX-2, the clinically recommended dosages (1–4 mg/kg orally, 2 mg/kg subcutaneously) did not induce COX-1 inhibition, except for a transient effect at C_{max} with the 8 mg/kg oral dosage.

Similarly, in the case of cats, during in vitro whole-blood assays, RX demonstrated a remarkable selectivity for COX-2, with an IC50 ratio of COX-1 to COX-2 at 502:1 (Giraudel et al., 2009). In fact, the expected COX-1 inhibition with RX was quite low in two separate studies, measuring at 5.2% and 7.6% when aiming for 90% COX-2 inhibition. Additionally, the IC_{80} for COX-2 inhibition by RX In cats showed a correlation with its effectiveness in addressing pain, inflammation, and fever in the kaolin model (Giraudel et al., 2009). Furthermore, the in vivo COX-2 selectivity of RX in cats was confirmed at clinically recommended dosages, ranging from 1 to 2 mg/kg orally and 2 mg/kg

subcutaneously. This COX-2 inhibition came with the preservation of COX-1 function (Schmid et al., 2010a).

1.2.b. Inhibition of pain, inflammation, and fever

The molecular mechanism behind RX's effects involves inhibiting COX, which forms the basis for its ability to alleviate pain (anti-hyperalgesia), reduce inflammation, and lower fever. These actions have been verified in studies involving mice, rats, dogs, and cats.

In a rat paw swelling experiment induced by carrageenan, RX displayed a dose-dependent reduction in swelling. Additionally, in a rat Randall–Selitto assay, RX exhibited anti-nociceptive effects, as demonstrated in a study by King et al. in 2009. Moreover, in a rat model of fever induced by LPS, both RX and diclofenac effectively and dose-dependently inhibited fever. The ID50 value for RX was found to be 1.12 mg/kg.

For Beagle dogs experiencing acute synovitis in a stifle joint due to urate crystals, the dose-response relationships for improved weight-bearing and analgesic and anti-inflammatory effects were established. The ED50 values for enhanced weight-bearing were in the range of 0.6–0.8 mg/kg orally and 0.90–1.23 mg/kg subcutaneously. Based on criteria that included superior efficacy compared to a placebo and at least equivalent efficacy to meloxicam, dosages of 2 mg/kg (both subcutaneous and oral) were chosen for surgery, while 1 mg/kg (oral) was selected for osteoarthritis.

In feline subjects, using a paw inflammation model induced by kaolin, various parameters such as lameness score, locomotion, body and skin temperatures, and thermal pain threshold positively responded to RX at a dose of 2 mg/kg subcutaneously. PK/PD modeling indicated a duration of action lasting 5 to 7 hours (Giraudel et al., 2009).

1.2.c. Renal pharmacodynamics

NSAIDs possess the potential to alleviate inflammation in chronic kidney disease (CKD), but they also carry a risk of nephrotoxicity. For instance, they can hinder the dilation of afferent arterioles and induce apoptosis through hyperosmolality.

In a study involving rats, RX at a dosage of 30 mg/kg orally did not produce any significant impacts on renal function (King et al., 2009). While serum creatinine levels showed a slight increase with RX (0.50 mg/dL) compared to the control group (0.47 mg/dL), this effect was numerically minor and had no influence on parameters like urine creatinine and PGE2 concentrations, urine volume, and glomerular filtration rate (GFR). Conversely, diclofenac significantly reduced urine volume and PGE2 concentration.

Another investigation in healthy cat kidneys examined the effects of ketoprofen (COX-1 selective) and RX (COX-2 selective) on renal responses induced by furosemide and the immunolocalization of COX isoforms (Pelligand et al., 2015). Neither drug altered the diuresis and natriuresis induced by furosemide. The study concluded that both COX-1 and COX-2 contribute to the production of prostaglandins that signal macula densa renin secretion and the aldosterone response to furosemide. Additionally, COX-2 may play a role in regulating pathways beyond angiotensin II-stimulated aldosterone secretion. Concurrent use of ACE inhibitors and NSAIDs may harm human kidneys, but could be suitable for animals with pain, inflammation, cardiovascular issues, or CKD. Studies in cats and dogs found that RX and benazepril together were well-tolerated. In cats, benazepril boosted GFR (females only), while RX lowered it (males only). In dogs, GFR remained unaffected, and urine aldosterone levels decreased. RX and benazepril also counteracted furosemide-induced aldosterone increases. This suggests potential benefits in conditions like proteinuric CKD (King et al., 2016; Panteri et al., 2017; Whelton, 1999).

1.2.d. Additional pharmacodynamics properties

Non-selective NSAIDs work by inhibiting COX-1 to prevent blood clotting. This did not happen with RX, which is consistent with its COX-1 sparing activity. Over a dosage range of 3.2-100 mg/kg SC, RX did not decrease clotting or impact hematology characteristics in mice (Beninson et al., 2018). Both clinical and higher RX dosages had no effect on activated partial thromboplastin, prothrombin, or buccal mucosal bleeding time (BMBT) in healthy cats and dogs (Heit et al., 2020; King et al., 2011; King et al., 2012; Toutain et al., 2017).

RX had no effect on buccal mucosal bleeding time (BMBT) in dogs following orthopaedic or soft tissue surgery (2 mg/kg, SC) (Gruet et al., 2011, 2013) or cats undergoing ovariectomy (1 mg/kg, PO) (Sattasathuchana et al., 2018). Indeed, wound healing inhibition (including pre-existing gastrointestinal ulcers) and an increased risk of myocardial ischemia or stroke are potential safety issues with coxib NSAIDs. In RX safety and clinical tests in cats and dogs, no signs of these effects were found.

In canine cruciate ligament cells, RX, like carprofen and meloxicam, inhibited sodium nitroprusside-induced apoptosis (Waldherr et al., 2012), indicating a putative cytoprotective activity.

Oh et al. (2014) studied the compensatory effects of four NSAIDs (carprofen, meloxicam, indomethacin, and RX) on osteogenic differentiation in canine bone marrow-derived mesenchymal stem cells. PGE2-related receptor and enzyme gene expression was elevated, while osteocalcin synthesis was not decreased. These findings could explain the disparity between NSAIDs' suppressive effect on osteogenesis in vitro and the rarely documented worsening of bone repair caused by NSAID clinical usage.

Carprofen, meloxicam, and RX all reduced the viability of cultured canine vascular endothelial cells in a dose-dependent manner. As a result, these NSAIDs could be used as adjuvant anti-angiogenic medicines in dogs with cancer (Horikirizono et al., 2019).

RX (2 mg/kg SC in dogs) reduced the minimum alveolar concentration of sevoflurane necessary to blunt the adrenergic response (MAC- BAR). MAC- BAR measures anaesthetic potency quantitatively. Tamura et al. (2014) found that RX had a minor (17%) effect on sevoflurane need. RX and meloxicam had no effect on insulin secretion in either conscious or anaesthetized dogs, nor on the attenuation of lowered body temperature and heart rate in anaesthetized animals (Takashima et al., 2019). In vitro, ketoprofen and RX showed very poor activation-induced CD25 expression on murine CD4+ and CD8+ T cells (Gregorczyk and Malanka, 2019).

1.3. Safety in pre-clinical studies

In rats, the gastric and intestinal tolerability of RX was greater than that of diclofenac. The data correlated with COX- 1 inhibition by diclofenac but not by RX. The mean ± standard deviation (SD) number of gastric ulcers was 0 (vehicle control), 1.3 ± 1.8 (RX at 100 mg/kg/day) and 18.7 ± 6.6 (diclofenac 100 mg/kg/day). RX (10, 30 and 100 mg/kg over 4 days) increased intestinal permeability to a lesser degree than 10 mg/kg diclofenac. Furthermore, RX had no toxicologically relevant renal effects at a dosage of 30 mg/kg (King et al., 2009).

In Beagle dogs, RX administered orally once daily, at dos- ages of 10, 20 and 40 mg/kg for one month and 0, 2, 4, 6 and 10 mg/ kg for 6 months, produced no significant adverse effects, based on clinical observations, hematological and clinical chemistry variables, and the absence of macroscopic and microscopic lesions at necropsy (King et al., 2011). In the 6-month study, there were no ad- verse effects on BMBT and stifle joint tissues, electrocardiographic and ophthalmoscopic examinations, and urinalysis. The highest dosages administered correspond to 20– 40 (one month) and 5–10 (6 months) multiples of the clinical RX PO dosage for long-term use (OA). In another trial, single RX doses (2 and 4 mg/kg IV and 2 mg/kg SC) exerted no significant effects on arterial blood pressure, heart rate, electrocardiogram (ECG), body temperature, BMBT, blood hae- matology, coagulation and clinical chemistry variables (Desevaux et al., 2017). To support interchangeable use of injectable and tablet formulations, a safety study was conducted in cross- bred hound dogs administered 2, 4

and 6 mg/kg RX, with three 20-day treatment cycles, separated by 14-day washout periods (Toutain et al., 2017). There were no RX formulation- related changes in body weight, food consumption, ophthalmic and neurological examinations, ECG, BMBT, clinical pathology and organ weights. Treatment-related differences, of low incidence at all dosages, comprised macroscopic and microscopic changes at injection sites and microscopic gastrointestinal tract findings.

In cats, RX administration PO (5 and 10 mg/kg once daily for 28 days and 2, 6 and 10 mg/kg twice daily for 42 days) produced no toxicological effects based on general health, haematological and clinical chemistry variables; urinalyses; and organ weight, gross pathology and histopathology (King et al., 2012). Single-dose RX administration, IV (2.0 and 4.0 mg/kg) and SC (2 mg/kg), was well tolerated in healthy cats (Panteri et al., 2017). To support interchangeable use of injectable and tablet formulations, cats were administered RX at 2, 4 and 6 mg/kg (SC) and 2.4, 4.8 and 7.2 mg/kg (PO) (Heit et al., 2020). Ten- day treatment cycles comprised seven days of oral followed by three days of SC administration, once daily and, after the third cycle, an additional seven- day oral dose (total of 37 days). All cats remained in good health. There were no changes in body weight and food consumption and no ophthalmic, physical or neurological adverse effects. Treatment- related abnormalities were of low occurrence, comprising transient edema with mild, subacute/chronic inflammation at injection sites and QT prolongation on ECG. No adverse effects were attributable to interchanging administration route.

These pre-clinical safety studies indicated that RX produces minimal adverse effects, even at high dosages, in healthy rats, dogs and cats.

Before commencing the research, a brief overview will be provided on PK, clinically significant PK parameters, compartmental and non-compartmental PK analyses, as well as the definition and validation of analytical methods in accordance with international guidelines.

2. Deracoxib

Deracoxib (DX), marketed under the brand name Deramaxx® by Novartis, stands as a pioneering coxib in the realm of veterinary medicine, obtaining approval as the inaugural drug of its kind (Papich, 2008). Comprising a sulfonamide moiety, its chemical composition is characterized by a 4-[3-(difluoromethyl)-5-(3-fluoro-4-methoxyphenyl)-1H-pyrazole-1-yl] benzene sulfonamide, with a molecular weight of 397.38 g/moL. Classified as a diarylheterocycle drug, DX operates through a time-dependent pseudo-irreversible inhibition of COX-2, as elucidated by Walker et al. in 2001. Initially sanctioned for addressing postoperative orthopedic pain in dogs, it was administered orally at a daily dose of 3-4 mg/kg for a maximum duration of 7 days. Subsequently, in 2003, regulatory approval extended to chronic usage at a dosage of 1-2 mg/kg orally once daily (Smith, 2003).

- **Description**

DX is classified within the organic compound category known as phenylpyrazoles. This specific class falls under the broader classification of organic compounds and belongs to the super class of organoheterocyclic compounds. Further categorization places it in the class of azoles, with a sub-classification as pyrazoles. The compound's structural composition features a phenylpyrazole skeleton, characterized by the linkage of a pyrazole to a phenyl group, exemplifying its placement as a direct parent in the hierarchy of phenylpyrazoles.

- Empirical formula: $C_{17}H_{14}F_3N_3O_3S$
- IUPAC name: 4-[3-(difluoromethyl)-5-(3-fluoro-4-methoxyphenyl)pyrazol-1-yl] benzenesulfonamide
- Synonyms: deramaxx; NSC 758935
- Structural formula is:

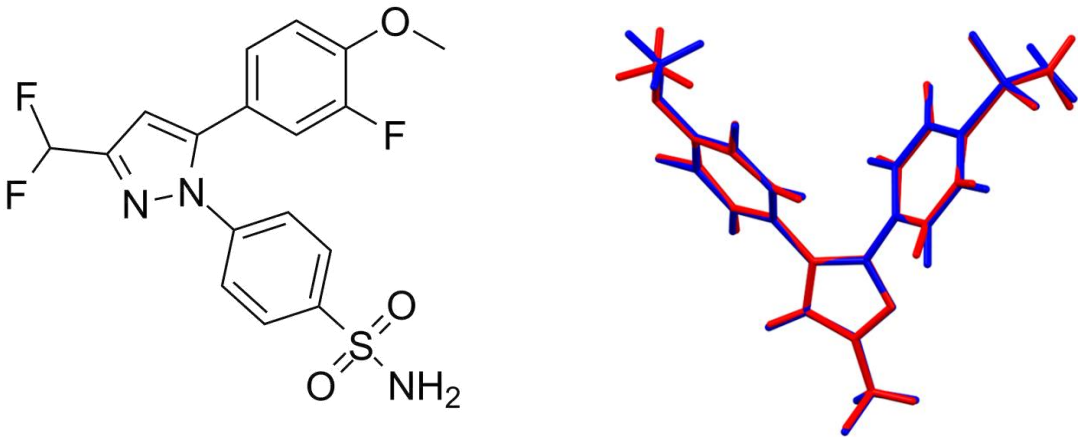


Figure 9: a) Chemical structure of deracoxib b) Crystal structure of deracoxib

- **Physicochemical proprieties**

- Molecular Weight: 397.4 g/mol
- Physical state: Brown speckled powder
- Melting point: 157 °C
- Water Solubility: 0.0104 mg/mL
- Log P: 3.39
- pKa (Strongest Acidic): 10.7
- pKa (Strongest Basic): 0.68

2.1. Previously reported pharmacokinetics of deracoxib

The Deramaxx® leaflet outlines the PK profile of DX, shedding light on its behavior post a single dose of 2.35 mg/kg. Information from the leaflet details estimates derived from intravenous administration of DX as an aqueous solution at a dose of 2 mg/kg, considering in vitro plasma concentrations ranging from 0.1 to 10.0 µg/ml. Key parameters and their corresponding values are highlighted:

-T_{max}: 2 hours

- Oral Bioavailability: Exceeds 90% at 2 mg/kg.

- Terminal Elimination Half-life: 3 hours at 2-3 mg/kg, extending to 19 hours at 20 mg/kg.

- Systemic Clearance: Approximately 5 ml/kg/min at 2 mg/kg, decreasing to about 1.7 mL/kg/min at 20 mg/kg.

- Volume of Distribution: Around 1.5 L/kg.

- Protein Binding: Over 90 %.

The Deramaxx® leaflet underscores non-linear elimination kinetics at doses exceeding 8 mg/kg/day, potentially leading to competitive inhibition of constitutive COX-1. It emphasizes hepatic biotransformation, yielding four major metabolites, with two being products of oxidation and o-demethylation. Notably, DX is not excreted as the parent drug in urine; instead, the primary mode of elimination is through feces, with the majority exiting the body as either the parent drug or its metabolite. Data also acknowledges notable inter-subject variability in drug metabolite profiles of urine and feces, with no statistically significant differences observed between genders.

Furthermore, insights into the PK of DX extend to diverse animal species, revealing distinctive patterns in cats and horses. In cats, administered at a dose of 1 mg/kg, and horses, at 1~2 mg/kg, a notably prolonged t1/2 was observed, standing at 7.9 hours and 12 hours, respectively, surpassing the duration recorded in dogs (Davis et al., 2011; Gassel et al., 2006). This variance is attributed to potential lower concentrations of hepatic enzymes involved in DX's biotransformation in cats and horses compared to dogs. The possibility of enzyme saturation at lower concentrations in these species contributes to the observed longer t1/2 (Davis et al., 2011). Additionally, the time to reach maximum concentration exhibited differences in cats, with a value of 3.6 hours (PO), and horses, showing a mean of 6.33 ± 3.44 hours, further emphasizing the species-specific variations in the PK profile of DX.

2.2. Previously reported pharmacodynamics of deracoxib

During lab assessments, DX was identified as a potent COX-2 inhibitor, displaying a ratio of 1275 in isolated enzyme tests (Gierse et al., 2002). However, when tested in whole blood from dogs, this ratio dropped significantly to just 12 (McCann et al., 2004). The variation in these results stems from the use of different types of cells in each test, adding complexity to the interpretation (Vane and Botting, 1995).

In a separate study involving dogs, DX demonstrated similar levels of COX-1 and COX-2 inhibition when compared to carprofen, a drug with a preference for blocking COX-2. Despite significant differences in COX-1/COX-2 ratios observed in lab tests for both drugs, their actual effects in dogs were found to be quite similar (Sessions et al., 2005). The disparity between lab and real-world findings underscores the limitations of relying solely on lab results to gauge the effectiveness or safety of a drug, as highlighted by Papich (2008). This emphasizes the importance of considering both types of data when evaluating a drug's performance.

Furthermore, DX exhibited potent inhibition of prostaglandin biosynthesis during tests (Deramaxx® leaflet), specifically impeding the production of PGE1 and 6-keto PGF1. Notably, its inhibitory effects extended to COX-2 mediated PGE2 production in LPS-stimulated human whole blood. Despite a plasma t1/2 of approximately 3 hours for Deramaxx® tablets, there is a noteworthy extension in the duration of clinical effectiveness. These findings underscore the complex relationship between DX's PD actions and its clinical impact, suggesting that the drug's effects go beyond what the plasma half-life alone might indicate.

2.3. Efficacy studies:

In the course of efficacy investigations, Deramaxx® tablets underwent scrutiny in blinded, placebo-controlled multi-site field studies involving client-owned animals to assess their effectiveness. The ‘osteoarthritis pain and inflammation field study’ enrolled 209 client-owned dogs presenting clinical and radiographic signs of osteoarthritis in at least one appendicular joint. Of these, 194 dogs were subjected to safety evaluation, and 181 dogs were included in the effectiveness evaluation. In a

masked, placebo-controlled study, Deramaxx® tablets were administered by owners at approximately 1-2 mg/kg/day for 43 consecutive days. Statistically significant differences ($p \leq 0.05$) favoring Deramaxx® were observed for force plate parameters (vertical impulse area, peak vertical force) and owner assessments (quality of life, lameness, and overall activity level). This field study establishes that Deramaxx® tablets, when administered at 1-2 mg/kg/day for 43 days, effectively control pain and inflammation associated with osteoarthritis.

Moving to the 'postoperative orthopedic pain and inflammation field study', 207 dogs undergoing veterinary hospital admission for cranial cruciate injury repair were randomly assigned Deramaxx® tablets or a placebo. Commencing the evening before surgery and continuing for 6 days postoperatively, tablets were administered once daily. Of the evaluated dogs (119 for effectiveness and 207 for safety), statistically significant differences in favor of Deramaxx® tablets were evident for lameness during walk and trot, as well as pain on palpation values across all postsurgical time points. This field study demonstrates the effectiveness of Deramaxx® tablets when administered daily for 7 days in controlling postoperative pain and inflammation associated with orthopedic surgery.

In the context of the 'postoperative dental pain and inflammation field study', 62 dogs admitted for dental extractions were randomly assigned Deramaxx® tablets or a placebo. Administration began approximately 1 hour before surgery and continued once daily for 2 days postoperatively. Effectiveness was assessed in 57 dogs, with safety evaluated in all 62. The Deramaxx® treated group exhibited a statistically significant reduction ($p=0.0338$) in the proportion of dogs requiring rescue therapy for post-surgical pain compared to the placebo control group. Pain assessment, utilizing a modified version of the Glasgow Composite Pain Scale (mGCPS), led to rescue intervention if a dog scored ≥ 4 on the combined mGCPS variables or if the investigator deemed pain intervention necessary at any point. This field study affirms the efficacy of Deramaxx® when administered once daily for 3 days in controlling postoperative pain and inflammation associated with dental surgery.

In further studies as well, clinical trials in dogs revealed that DX (1~2 mg/kg PO for 3 days) effectively reduced postoperative pain and inflammation following dental extraction surgery (Bienhoff et al., 2012). Additionally, Millis et al. (2002) reported that DX administration (1, 3, or 10 mg/kg PO) proved more effective in alleviating pain associated with urate crystal-induced synovitis compared to carprofen (2.2 mg/kg PO). Notably, DX treatment showed no significant adverse effects (Millis et al., 2002).

2.4. Safety studies

According to the Deramaxx® leaflet, in a 6-month investigation, dogs received tablets at doses ranging from 0 to 10 mg/kg with food once daily for six consecutive months. No abnormalities were observed in feces, clinical assessments, food and water intake, body weights, physical examinations, ophthalmoscopic evaluations, macroscopic pathological examinations, hematology, or buccal bleeding time. Urinalysis revealed hyposthenuria (specific gravity <1.005) and polyuria in one male and one female in the 6 mg/kg group after 6 months. After this duration, mean blood urea nitrogen (BUN) values for dogs treated with 6, 8, or 10 mg/kg/day were 30.0, 35.3, and 48.2 mg/dL, respectively. Dose-dependent focal renal tubular degeneration/regeneration was observed in some dogs treated at 6, 8, and 10 mg/kg/day, with renal papillary necrosis seen in 3 dogs dosed at 10 mg/kg/day and one dog dosed at 8 mg/kg/day. No renal lesions were observed at label doses of 2 and 4 mg/kg/day, and no evidence of gastrointestinal, hepatic, or hematopoietic pathology was noted.

In a laboratory study, healthy young dogs received DX tablets once daily within 30 minutes of feeding, at doses of 0, 4, 6, 8, and 10 mg/kg body weight for 21 consecutive days. No adverse events were reported, and no abnormalities were noted in clinical observations, food and water consumption, body weights, physical examinations, ophthalmic evaluations, organ weights, macroscopic pathologic evaluation, hematology, urinalyses, or buccal mucosal bleeding time. Statistically significant (p< 0.0009) dose-dependent trends were observed in BUN levels, but mean BUN values remained within historical normal limits at label doses. No effects on other clinical chemistry values

associated with renal function were reported, and there was no evidence of renal, gastrointestinal, hepatic, or biliary lesions during gross necropsy.

In another study, healthy young dogs received micronized DX in gelatin capsules once daily at doses of 10, 25, 50, and 100 mg/kg body weight for up to 14 consecutive days. Food was withheld before dosing. Non-linear elimination kinetics were observed at all doses, with reduced body weight, vomiting, and melena noted at doses of 25, 50, and 100 mg/kg. Necropsy revealed gross gastrointestinal lesions in all dose groups, with frequency and severity increasing with escalating doses. At 10 mg/kg, moderate diffuse congestion of gut-associated lymphoid tissues (GALT) and erosions/ulcers in the jejunum occurred. At 100 mg/kg, all dogs exhibited gastric ulcers and erosions/ulcerations of the small intestines. No hepatic or renal lesions were reported at any dose in this study.

In a 13-week study, DX in gelatin capsules was administered to healthy dogs at doses of 0, 2, 4, and 8 mg/kg/day. No test-article related changes were identified in clinical observations, physical exams, or other measured parameters. However, one dog in the 8 mg/kg dose group died from bacterial septicemia secondary to a renal abscess, and the relationship between DX administration and the renal abscess is not entirely clear.

In subsequent academic investigations, the safety profile of DX was assessed. Following a 28-day regimen of once-daily DX administration at 1.6 mg/kg orally, it demonstrated a superior safety profile compared to aspirin concerning the risk of gastric ulceration in healthy dogs (Sennello and Leib, 2006). Furthermore, prolonged DX therapy for up to 6 months at the labeled dose was determined to be safe and well-tolerated in dogs, showing no significant nephrotoxicity (Roberts et al., 2009). Conversely, at doses higher than recommended or in conjunction with other NSAIDs or corticosteroids, DX has been associated with causing gastrointestinal perforations in dogs (Lascelles et al., 2005).

While there have been no notable instances of hypersensitivity reported thus far, the use of sulfonamide coxibs in animals with a known allergy to sulfonamides should be approached with caution. There exists a potential for cross-reaction with other sulfonamides, including antimicrobials, or the triggering of hypersensitivity reactions (Shapiro et al., 2003; Sanchez-Borges et al., 2004; Bergh and Budsberg, 2005; Ayuso et al., 2013). It is important to note that the hypersensitivity of sulfonamide coxibs such as DX has yet to be definitively confirmed.

3. Pharmacokinetics definitions

PK encompasses the examination of the processes involving absorption, distribution, metabolism, and excretion (ADME) of a drug within the body once the drug's dosage form is administered (Smith et al., 2012). The collective actions of metabolism and excretion are commonly referred to as elimination, while the entire journey from distribution to elimination is generally referred to as drug disposition (Rosenbaum, 2012). This intricate drug disposition process exhibits variability among individuals due to factors such as age, gender, genetic makeup, and the species or breed of animals (Riviere, 2009).

a. Absorption

Drug absorption refers to its movement from the administration site into the bloodstream or systemic circulation, as described by Riviere (2009). The extent of drug absorption is contingent upon both the method of administration and the drug's formulation. Intravenously administered drugs directly enter the circulatory system, whereas extravascular routes entail a longer absorption process. Additionally, liquid drug formulations exhibit rapid absorption due to their inherent solubility, while solid forms like tablets or capsules necessitate dissolution before absorption can occur. Dissolution, in part, relies on the drug's dissociation constant. Orally administered drugs are predominantly absorbed by the gastrointestinal tract epithelium, with the potential limitation of extensive hepatic metabolism preventing sufficient drug concentrations from reaching systemic circulation for therapeutic efficacy. In essence, the absorption of a drug is influenced by various factors, including formulation, particle

size, physicochemical properties (e.g., pH, lipophilicity), route of administration, drug solubility, animal species, systemic conditions, and both pathological and physiological states (Riviere and Papich, 2013; Brunton et al., 2011).

It's important to note that drug absorption plays a pivotal role in determining the bioavailability of a drug, which represents the proportion of the drug that successfully enters the systemic circulation—a matter of significant clinical concern (Brunton et al., 2011). Consequently, bioavailability is also subject to the same factors that influence drug absorption.

b. Distribution

Following absorption or administration into the circulatory system, drugs undergo distribution among various bodily fluids, including plasma, interstitial fluid, and intracellular fluid, with the ultimate goal of reaching different organ tissues. This distribution process is contingent upon a multitude of physiological factors within the body and the physicochemical characteristics of the drug itself. Physiological factors encompass variables like cardiac output, regional blood flow, capillary permeability, and tissue volume, while the drug's physicochemical properties involve parameters such as molecular weight, pKa, and lipid solubility (Brunton et al., 2011; Riviere, 2009).

In most cases, drugs initially gravitate towards highly perfused organs like the heart, liver, kidneys, and brain, before gradually diffusing into less vascularized tissues such as the skin, adipose tissue, and various viscera (Riviere, 2009). Indeed, lipophilic drugs exhibit a more extensive distribution (Fahr et al., 2005).

c. **Metabolism**

Drug metabolism, also known as biotransformation, comprises a series of enzymatic or chemical reactions that alter a drug to either generate its therapeutic effects or terminate its biological activity. Typically, metabolites resulting from these processes exhibit increased polarity (hydrophilicity) (Brunton et al., 2011). These transformations are primarily categorized into phase I and phase II reactions, predominantly occurring in hepatocytes. Phase I reactions involve straightforward

biotransformation mechanisms, including hydrolysis, oxidation, and reduction, where the parent drug is generally converted into a more polar metabolite through the introduction or exposure of functional groups like -OH or -NH. The resultant metabolite may either be more active than the original compound or, if adequately polar, can be readily excreted by the kidneys. Phase I reactions are facilitated by isoforms of the cytochrome P450 (CYP450) enzyme family. On the other hand, phase II reactions typically encompass conjugation reactions, and most phase I metabolites undergo these transformations to increase their polarity (Gibson and Skett, 2001).

To illustrate phase I reactions, the case of nabumetone is taken into a consideration, a NSAID that is converted into its active form, 6-methoxy-2-naphthylacetic acid, through CYP450 enzyme activity. This transformation enables nabumetone to exert its analgesic effects by inhibiting COX-2 while minimizing gastrointestinal irritation. Another example is losartan, an exceptionally selective and competitive antagonist of angiotensin II receptor type 1, which undergoes oxidation by cytochrome P450 to produce its 5-carboxylic acid derivative, known as EXP3174. Remarkably, EXP3174 exhibits 10–40 times greater potency than losartan itself (Montellano, 2013).

In phase II conjugation reactions, covalent bonds are formed between the functional groups of the parent compound or phase I metabolite and molecules like glucuronic acid, amino acids, acetate, glutathione, or sulfate. This results in the creation of highly polar, inactive compounds that are rapidly eliminated via urine and feces. Morphine provides a notable exception, where its active conjugate, 6-glucuronide metabolite, possesses greater analgesic potency than the parent drug (Brunton et al., 2011).

While the liver is the primary site housing enzymes responsible for drug metabolism, it's worth noting that other organs, including the gastrointestinal tract, kidneys, and lungs, possess substantial metabolic capabilities. This extended metabolic involvement can significantly impact drug processing. For instance, a considerable portion of an orally administered drug may undergo metabolic inactivation either within the gastrointestinal tract or in the liver before it can enter systemic

circulation. This metabolic phenomenon is commonly referred to as first-pass metabolism, and it notably diminishes the oral bioavailability of drugs that are highly susceptible to metabolic alterations, such as morphine (Brunton et al., 2011). Consequently, drug metabolism, or biotransformation, assumes a pivotal role in modulating a drug's activity, either to curtail or enhance its effects.

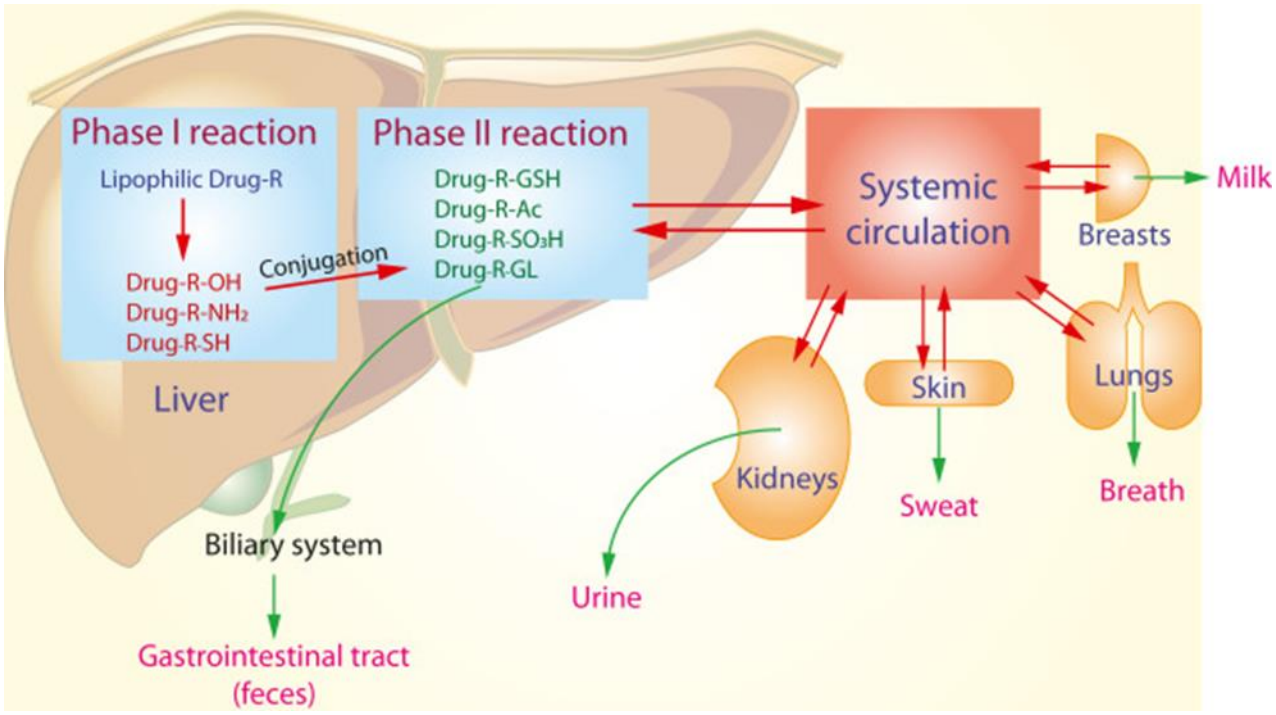


Figure 10: An overview of the drug metabolism in the liver (Source: Handbook of Dialysis Therapy (Fifth Edition), 2017).

d. **Excretion**

The process of drug excretion involves the elimination of the substance from the body, either in its original unchanged state or following conversion into metabolites. Among the organs responsible for drug excretion, the kidney holds paramount importance in expelling both drugs and their metabolites. Three distinct mechanisms participate in drug excretion: glomerular filtration, active tubular secretion, and passive tubular reabsorption. Any alterations in renal function can have a profound impact on all three of these processes. Excretion is contingent upon the GFR and the degree of plasma binding, with only unbound drug molecules being filterable by the kidneys. Several other factors

come into play in renal excretion, including the ionization state of the metabolite, active carrier-mediated tubular secretion in the proximal renal tubule, the presence of transporters like multi-drug-resistance-associated protein type 2 (MRP2) localized in the apical brush-border membrane, which facilitates the secretion of conjugated metabolites, and blood pressure, among others.

In the proximal and distal tubules, passive reabsorption of uncharged weak acids and bases occurs. Tubular cells exhibit lower permeability to ionized forms of weak electrolytes, so the passive absorption of these electrolytes is influenced by the pH of the urine. When the urinary pH is adjusted to alkaline conditions, weak acids become ionized and are rapidly excreted. For instance, the excretion of salicylic acid is enhanced following urine alkalization (Brunton et al., 2011).

In addition to the kidneys, certain organs like the lungs play a vital role in eliminating specific drugs, such as anesthetic gases. Furthermore, some compounds find their route of elimination through fecal excretion. This can occur because they are primarily unabsorbed following oral administration, or they may represent metabolites, especially glucuronides, that are excreted through bile or secreted into the intestinal lumen without subsequent reabsorption (Brunton et al., 2011).

It's noteworthy that certain metabolites undergo reabsorption within the intestinal lumen, a process known as enterohepatic recycling. This phenomenon prolongs the presence of the drug in circulation, consequently extending its t1/2 (Roberts et al., 2002). Factors such as the drug's lipophilicity, ionization, polarity, and molecular weight exert influence over the excretion process.

In addition to the aforementioned routes, drugs may also be eliminated through other pathways, including the skin (via sweat), saliva, tears, hair, breast milk, and even meat (in cases where animals are slaughtered for consumption) (Brunton et al., 2011; Katzung et al., 2004).

4. Clinically important pharmacokinetic parameters

PK holds a crucial role in both the development of novel medications and the assessment of drug treatment effectiveness. For a drug to exert its intended effects, it must successfully reach its target

location within the body. The practical application of PK, not only in research but also in clinical settings, has significantly propelled advancements in the field of pharmacology. Alongside PK, PD represents another vital facet of pharmacology, assessing how a drug influences the body's responses and effects. Nevertheless, in a clinical context, the PK of a specific chosen drug can be precisely quantified, allowing for the establishment of an appropriate dosing regimen. This quantification involves the calculation of PK parameters that are applicable to the general population. These essential PK parameters include Cl, V_d, t1/2, and F. In addition to these parameters, the area under the curve is a crucial metric for both PK and PD analyses.

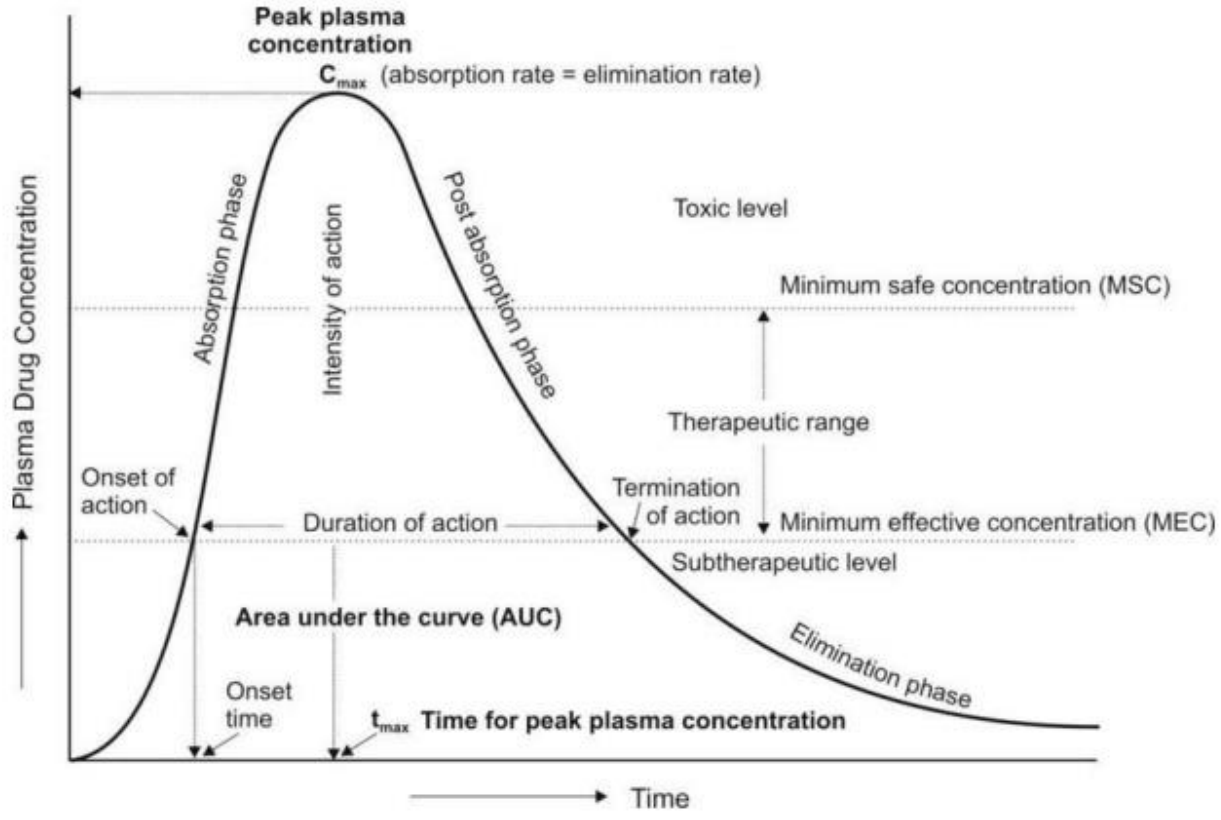


Figure 11: A typical plasma concentration-time profile showing pharmacokinetic and pharmacodynamic parameters, obtained after oral administration of single dose of a drug (Source: https://www.pharmacy180.com/article/plasma-drug-concentration-time-profile-2506/).

a. Area under the curve

It is the total area under the curve that describes the measured concentration of drug in the systemic circulation over time (Brunton et al., 2011). It reflects the actual body exposure to drug after administration of a dose of the drug and is expressed in mg*h/L. The area under the curve (AUC) is influenced by both the rate at which the body eliminates the drug and the administered dose. To calculate the total amount of drug eliminated by the body, one can sum or integrate the quantities eliminated during each time interval, starting from the moment of drug administration (time zero) and extending to infinity. This total amount corresponds to the fraction of the administered dose that ultimately enters the systemic circulation. In cases where a drug follows linear kinetics, the AUC exhibits a direct proportionality to the dose. Conversely, it demonstrates an inverse relationship with the drug's Cl. In essence, higher Cl results in reduced time that the drug remains in the systemic circulation, leading to a faster decline in plasma drug concentration. Consequently, in such scenarios, the body's exposure to the drug is diminished, resulting in a smaller area under the concentration-time curve.

Knowing the bioavailability and the dose, the Cl of the drug may be calculated by dividing the dose absorbed by the AUC. The Cl calculated is relatively independent on the shape of the concentration-time profile. This method gives precious information on the kinetic behavior of a drug on trial. It can also be used to study a change in the Cl of a drug in specific clinical conditions, such as disease or concomitant drug administration.

b. Clearance

The pivotal parameter in designing a drug dosing regimen is drug Cl (Brunton et al., 2011). Cl represents the volume of plasma from which the drug is entirely removed per unit of time (Brunton et al., 2011; Urso et al., 2002). For a more precise definition, it should be expressed as the ratio of two components: the rate of drug elimination (dE/dt) and the corresponding concentration of the drug

in the plasma (Cp) (Toutain and Bousquet-Melou, 2004a). Therefore, plasma Cl can be quantified in units involving volume, time, and body weight, typically expressed as mL/hr/kg:

Cl= Total body rate of drug elimination/Plasma concentration

This equation holds true for drugs exhibiting first-order kinetics, a scenario in which a consistent fraction of the drug within the body is eliminated per unit of time. Consequently, this represents a dose-independent reaction, and the majority of drugs conform to this first-order kinetics pattern. Conversely, for drugs that adhere to zero-order kinetics, where a constant amount of the drug in the body is eliminated per unit of time, indicating a dose-dependent process, Cl can be determined in units of volume per time as follows:

$$Cl = \frac{Vm}{Km+C}$$

Where, V_m= the maximal rate of elimination,

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

C= concentration of the drug in the plasma

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

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

Where, Cl = clearance of the drug from body/total systemic clearance; $Cl_{hepatic}$ = clearance of a drug from liver; Cl_{renal} = clearance of a drug from kidney; Cl_{other} = clearance from GI, skin, lung etc.

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

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

The interpretation of plasma Cl and inter-species comparisons are made easier by computing the overall body extraction ratio (from 0 to 1), which is the ratio of the body Cl divided by cardiac output.

Plasma Cl is the most important PK parameter because it is the only one which controls the overall drug exposure (for a given F %) and it is the parameter which allows computation of the dosage required to maintain an average steady-state plasma concentration. It is indeed the relevant parameter to compute the maintenance dose, whilst V_{ss} is the PK parameter to compute a loading dose (Toutain and Bousquet-Melou, 2004d). Moreover, plasma Cl holds paramount clinical importance in pharmacotherapy as it informs dosage adjustments to achieve optimal therapeutic levels. It aids in tailoring individualized treatment regimens. Monitoring Cl is particularly crucial for assessing renal and hepatic function, preventing drug accumulation and toxicity, and managing potential drug-drug interactions. By understanding its dynamics, healthcare professionals can adapt drug dosages based on the unique characteristics of patients, ensuring both safety and efficacy in clinical practice.

c. Volume of distribution

The V_d is a theoretical or apparent volume that would be needed to contain the same amount of drug in the body at the identical concentration as found in the plasma. In mathematical terms, it's defined as the ratio between the amount of drug in the body at a given time 't' and the drug's plasma concentration at that specific time (Toutain and Bousquet-Melou, 2004d; Benet and Galeazzi, 1979). V_d serves as a crucial parameter when considering drug distribution within the body and also when calculating the loading dose required to achieve the desired therapeutic plasma concentration of the drug. It is typically expressed in units of volume per mass, such as mL/kg or L/kg.

$$V_d = Dose/C_0$$

Where, V_d = volume of distribution; C_0 = concentration of drug in the plasma at time zero.

Volumes of distribution are proportionality constants between total amount of drug in the body and plasma concentrations. As snapshot plasma drug concentrations may be measured in different conditions (at equilibrium, under pseudo-equilibrium condition…), several volumes of distribution have been defined. The two most relevant are the V_d at equilibrium (V_{ss}), and the V_d during pseudo-equilibrium (V_{area}). Specifically, V_{ss} represents the hypothetical volume in which the total amount of drug would need to be uniformly distributed to achieve the observed plasma concentration at equilibrium. On the other hand, V_{area} reflects the volume required to account for the total amount of drug in the body during pseudo-equilibrium, considering the AUC. These volumes of distribution parameters play a pivotal role in determining the appropriate loading dose of a drug and understanding the residual drug amount in the body based on measured plasma concentrations, thereby guiding effective therapeutic dosing strategies.

Volumes of distribution may be interpreted in terms of drug distribution having recourse to physiological models involving drug binding to plasma and tissues. They should be determined early in drug development programs and those having a large V_d may be selected to obtain a long terminal t1/2 even for drugs having a relatively high Cl.

d. Terminal Half-life

t1/2 is the time required to divide the plasma concentration by two after reaching pseudo-equilibrium, and not the time required to eliminate half the administered dose. When the process of absorption is not a limiting factor, t1/2 is a hybrid parameter controlled by plasma Cl and extent of distribution. In contrast, when the process of absorption is a limiting factor, the t1/2 reflects rate and extent of absorption and not the elimination process (flip-flop kinetics). In flip-flop PK, t1/2 is determined by the interplay between absorption and elimination processes. This phenomenon is particularly evident when drugs exhibit slow or erratic absorption kinetics. Factors influencing flip-flop PK include the drug's physicochemical properties, formulation characteristics, and the physiology of the absorption site. For instance, drugs with poor solubility or permeability may experience delayed or incomplete

absorption, contributing to flip-flop kinetics. Failing to acknowledge flip-flop kinetics could result in a misinterpretation of PK data. This misinterpretation may then contribute to suboptimal dosing, jeopardizing therapeutic effectiveness, or, conversely, increasing the likelihood of adverse effects. Therefore, maintaining clinical awareness of flip-flop PK is essential for implementing a more accurate and individualized approach to drug therapy, ultimately improving the safety and efficacy of pharmacological interventions.

Indeed, after an extra-vascular (EV) drug administration, t1/2 can be more prolonged than after an IV administration. This is frequently the case in veterinary medicine where many long-acting formulations, obtained using slow sustained release dosage forms, subdermal implants and vaginal sponges are marketed to provide a prolonged duration of action by maintaining plasma concentration above a minimal therapeutic concentration.

The t1/2 is especially relevant to multiple dosing regimens, because it controls the degree of drug accumulation, concentration fluctuations and the time taken to reach equilibrium. Thus, the clinical utility of t1/2 is mainly to select an appropriate dosage regimen interval (Toutain and Bousquet-Melou, 2004b). This is because the relationship between t1/2 and dosing interval determines the amplitude of fluctuations in drug plasma concentrations during the dosing intervals.

It is expressed in the units of time as hours or minutes:

$t1/2 = 0.693 * V_d/Cl$ Or $t1/2 = 0.693 * K_{el}$

Where 0.693= log of 2; K_{el} = elimination rate constant of a drug (or λz).

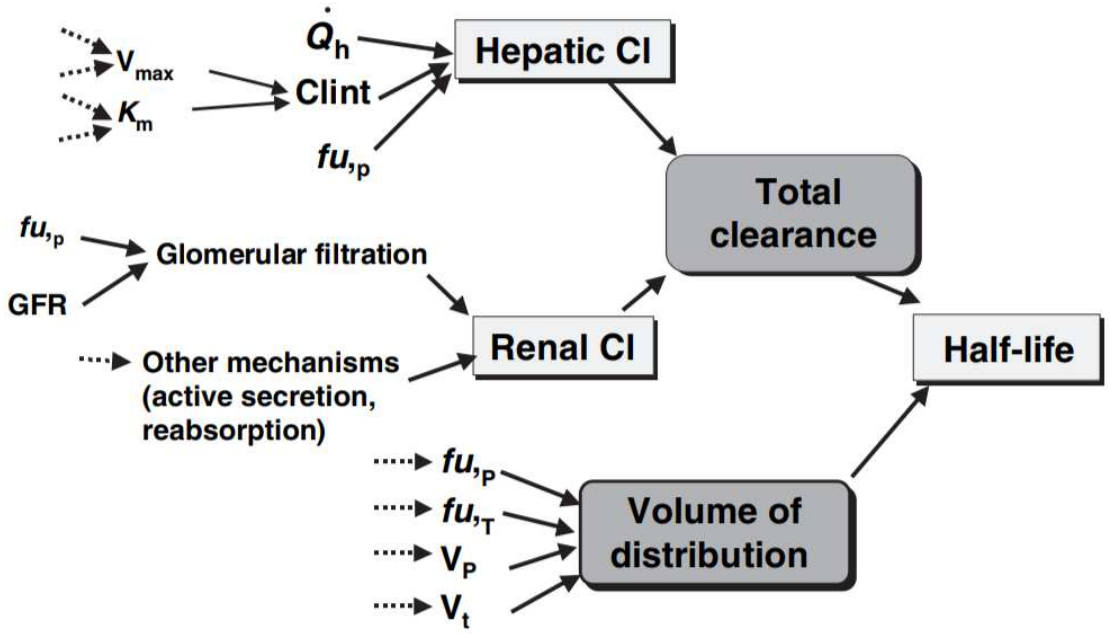


Figure 12: Physiological factors influencing the terminal half-life and giving it the status of a hybrid parameter (Source: Toutain and Bousquet-Melou, 2004b).

As seen in figure 12, and as mentioned earlier, t1/2 is intricately linked to Cl and V_d. Clearance from plasma is a composite measure involving various organ clearances, such as hepatic and renal clearances. Hepatic Cl, for instance, is influenced by factors like hepatic blood flow (Q_h), intrinsic hepatic Cl (Clint), and the free fraction in plasma ($fu_{,P}$). The intrinsic hepatic Cl is indicative of the maximum metabolic capacity (V_{max}) and is associated with the Michaelis Menten constant (K_M), reflecting the drug's affinity for the metabolic enzymatic system. Renal Cl, another integral component, is influenced by factors like GFR, active tubular secretion, reabsorption processes, pH-dependent ionization, and transporter interactions. Moreover, t1/2 is intricately connected to the drug's distribution within the body. This distribution is influenced by the drug's affinity for circulating proteins ($fu_{,P}$), tissues ($fu_{,T}$), and various volume-related factors, such as the volume of plasma (V_P) and tissues (V_t) (Toutain and Bousquet-Melou, 2004b).

e. Bioavailability

F % essentially signifies the proportion of a drug that reaches the systemic circulation to exert its therapeutic effects (Toutain and Bousquet-Melou, 2004a). This parameter is typically expressed as a percentage (%). When a drug is administered intravenously, its F % is at its maximum, reaching 100% (F = 1), as the entire drug is directly introduced into the systemic circulation. In contrast, for extravascular routes of administration such as oral, subcutaneous, or intramuscular, F % hinges on the rate of drug absorption relative to its elimination.

In these extravascular routes, a portion of the drug may undergo metabolism within the gastrointestinal tract or may be subject to absorption challenges, especially in the case of oral administration. Consequently, when evaluating bioavailability through these routes, it is more useful to calculate it relative to the intravenous dose, which provides a comparative or relative measure of F %.

$$F\% = 100 \times \frac{AUC(route) \times Dose\ (IV)}{AUC(IV) \times Dose\ (route)}$$

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

Understanding F % is crucial for tailoring drug doses, optimizing therapeutic outcomes, and ensuring the interchangeability of different formulations. The equivalence of generic drugs relies on demonstrating comparable F % to their brand-name counterparts. Moreover, the parameter is crucial for personalized medicine, considering individual patient variability, and plays a key role in determining the onset and duration of drug action. Additionally, optimal F % contributes to minimizing side effects by allowing the administration of lower doses while maintaining therapeutic efficacy.

f. Mean residence time

The mean residence time (MRT) of a drug represents the average duration during which the drug remains within the body. It can be described as the average time taken by intact drug molecules to traverse the body, encompassing all kinetic processes, such as the in vivo release from the dosage form, absorption into the body, and all subsequent disposition processes (Riegelman and Collier, 1980).

MRT is calculated using two important metrics: the AUC and the area under the moment curve (AUMC). The formula for calculating MRT involves these parameters and can be expressed as follows:

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

Clinically, MRT assists in optimizing drug dosing regimens by determining appropriate dosing intervals. It helps monitor how long a drug remains effective and aids in selecting the most suitable drug within a therapeutic class. MRT can be used to individualize treatment, particularly for drugs with varying MRTs among patients. It plays a role in therapeutic drug monitoring, ensuring drug levels stay within the desired range. In clinical research, MRT is pivotal for assessing new drugs' kinetics and safety. Overall, MRT guides drug dosing decisions, enhances treatment efficacy, and minimizes potential adverse effects.

5. Compartmental Vs Non-Compartmental pharmacokinetics

PK analyses are often simplified by modeling drug distribution within the body as a single compartment where drug concentrations are considered uniform. In clinical practice, the application of PK typically involves straightforward calculations (Atkinson et al., 2012). To derive the PK parameters of a drug, the primary measurement is the concentration of the drug in the plasma over time, known as the plasma concentration-time profile. This profile serves as the basis for applying standard PK equations (Baggot, 2008).

Within the plasma, drugs can exhibit varying degrees of binding to plasma proteins. Consequently, both bound and free drug concentrations are available for analysis. However, it is important to note that the biologically active form of the drug is the free drug concentration. Therefore, PK calculations are often performed using the free drug concentration, as it provides more clinically relevant information (Smith et al., 2006).

- Definition

Compartmental PK: This approach divides the body into multiple interconnected compartments, each representing a distinct physiological or anatomical space where drug concentrations are considered relatively uniform. It assumes that drugs move between these compartments and that drug disposition within each compartment follows first-order kinetics.

Non-Compartmental PK: Non-compartmental analysis does not rely on compartmentalization. Instead, it analyzes the entire concentration-time profile of a drug without specific reference to separate compartments. It often involves calculating PK parameters directly from the observed data without modeling (Gabrielsson and Weiner, 2001).

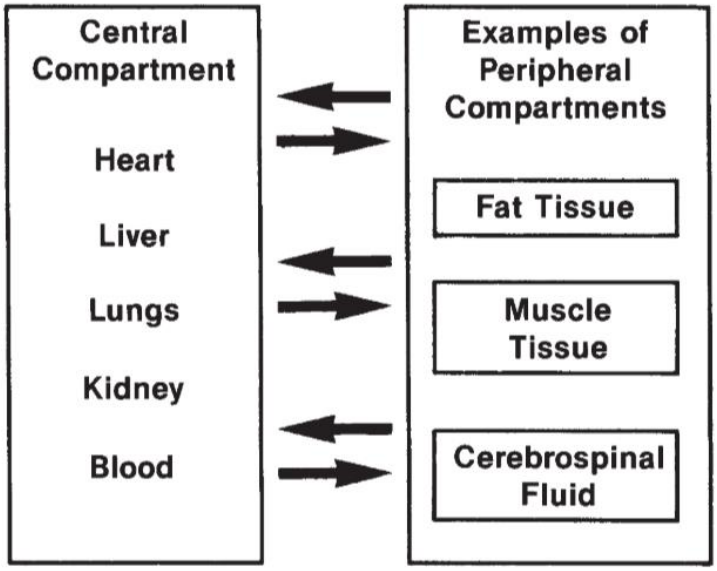


Figure 13: Typical organ groups for central and peripheral compartments (Source: Concepts in clinical pharmacokinetics, 7th edition, ASHP, 2018).

- Modeling:

Compartmental PK: In this approach, mathematical models (differential equations) are used to describe the rate of change in drug concentration within each compartment over time. These models involve parameters like Cl , V_d, and elimination rate constants, which can be estimated through curve-fitting techniques.

Non-Compartmental PK: Non-compartmental analysis does not involve modeling. Instead, it relies on simple mathematical formulas to compute PK parameters based on observed concentration-time data. Parameters commonly calculated include AUC, C_{max}, T_{max}, and t1/2.

- Data Requirement:

Compartmental PK: This approach typically requires multiple data points collected over time, especially for accurately estimating the parameters used in the compartmental models. More extensive data sets are often needed.

Non-Compartmental PK: Non-compartmental analysis can be performed with fewer data points and does not require a full concentration-time profile. It's particularly useful when limited sampling is available.

- Use Cases:

Compartmental PK: It is often used when detailed understanding of drug distribution within the body is necessary, especially for complex drugs or those with nonlinear kinetics. Compartmental models are suitable for predicting drug behavior under various dosing regimens.

Non-Compartmental PK: This approach is commonly employed in early-phase clinical trials or when a quick assessment of a drug's PK is needed. It provides a straightforward way to calculate basic PK parameters and assess F % (Cobelli and Toffolo, 1984).

- Complexity:

Compartmental PK: It involves more complex mathematical modeling and derive more accurate estimations if the assumption of the compartments is physiologically real and the non-linear models can also be systematically calculated (Cobelli and Toffolo, 1984).

Non-Compartmental PK: Non-compartmental analysis is simpler and more straightforward, making it accessible for routine PK evaluations, and more practical for clinicians (Cutler, 1978).

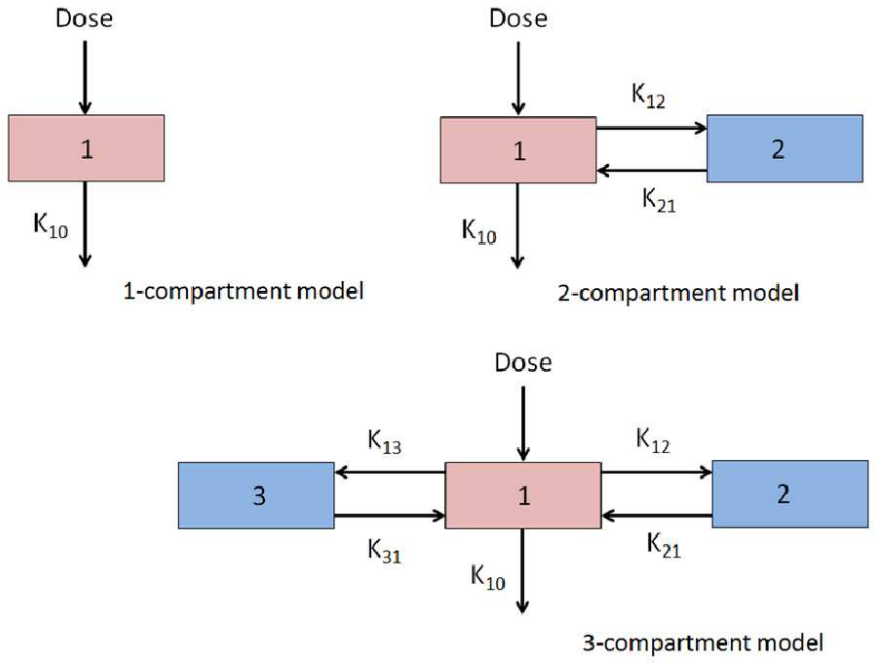


Figure 14: Compartmental models in pharmacokinetics (Source: Raymond M. Reilly, University of New Mexico Health Sciences Center, 2013).

Indeed, compartmental models include one, two, or three compartments. One-compartmental PK models simplify drug distribution within the body by considering it as a single, homogenous compartment. In this model, drugs are assumed to distribute uniformly, and elimination follows first-order kinetics. It's a fundamental concept for basic PK calculations.

In the two-compartmental models, drugs initially distribute rapidly into the central compartment before gradually moving into the peripheral compartment. This model accommodates more realistic distribution patterns, proving particularly useful for drugs with intricate tissue distribution. Unlike the simplicity of a one-compartment model, many drugs exhibit non-linear concentration-time

profiles, necessitating a more nuanced approach. In this scenario, the drug undergoes distribution in distinct body regions at varying rates, designating these regions as central and peripheral compartments based on instantaneous distribution. The assumption is that the drug initially distributes in the central compartment, from where it slowly disseminates into the peripheral compartment (remaining body parts) with a distribution rate constant typically denoted as K_{12}. Subsequently, it is redistributed back to the central compartment with another constant termed K_{21}. Elimination takes place from the central compartment at a constant rate (K_{10}), equivalent to K_{el} in a one-compartment model (Gabrielsson and Weiner, 2001). The constants K_{12} and K_{21} are considered slower than K_{10}. Therefore, in a two compartment model, the concentration versus time profile is an outcome of two PK processes: distribution phase (α) and elimination phase (β).

Three-compartmental models further refine the understanding of drug disposition. They include a central compartment, a peripheral compartment, and an additional compartment representing deep tissues or organs (adipose tissue, muscles, lymphatics…). These models are employed when a high degree of accuracy is needed to capture complex kinetic behavior, especially for drugs with deep tissue distribution and prolonged elimination.

In summary, compartmental PK relies on modeling and the concept of dividing the body into compartments to describe drug distribution, while non-compartmental PK involves direct calculation of PK parameters from observed data without modeling. The choice between these approaches depends on the specific research or clinical goals, the data available, and the complexity of the drug's kinetics.

6. PK-PD modelling

PK-PD (pharmacokinetic-pharmacodynamic) modelling is based on the dose response relationship over time and its application involves the identification of the effect of the drug in vivo under physiologic and pathologic conditions, determining dosing regimen and dosage form of the drug to

achieve the concentration to produce the desired effect (Pérez- Urizar et al., 2000). Figure 15 depicts the concept of PK-PD modelling.

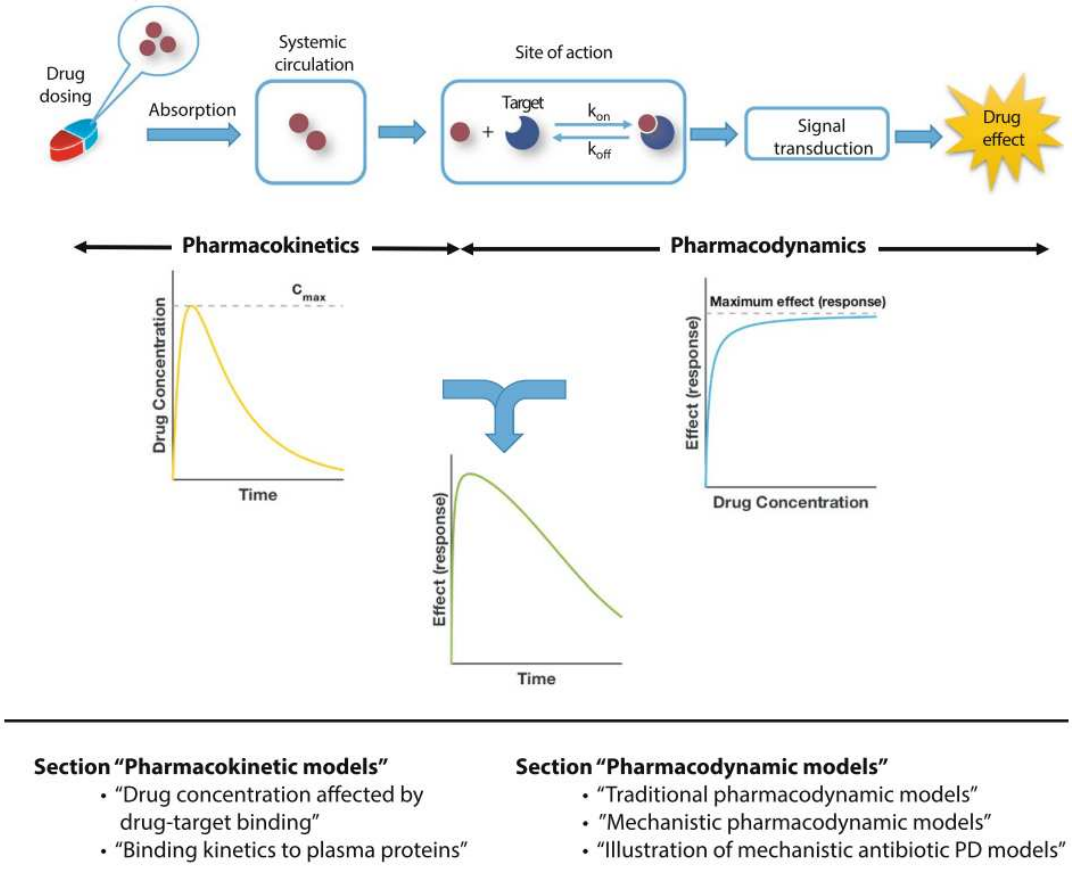


Figure 15: Schematic overview of PK/PD modeling which allows to investigate the drug efficacy over time under diferent dosing regimens (source: Clarelli et al., 2020).

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

- The linear pharmacodynamic model assumes a direct proportional relationship between drug concentration (C) and the intensity of drug effect (E):

  $E = S.\ C + E_0$

E= intensity of the effect, C = drug concentration, S = slope of the line, E_0 = value of the effect when no drug is present (E_0 can be dropped from the equation if there is no effect in the absence of drug).

This model is not appropriate when the drug concentration is to low or too high as the linearity follows the direct proportionality between drug concentration and drug effect only in the medium range of drug concentration (Pérez- Urizar et al., 2000).

- The log-linear model in PD involves the application of logarithmic transformations to the drug concentration-effect relationship. This model is an extension of the linear model and introduces the use of logarithms, allowing for the creation of a linear concentration-effect curve. Mathematically, it can be represented as:

  E= S. Log C + I, where I = imperic constant which has no physiologic or biological significance; rather, it is a parameter introduced in the mathematical model to account for the baseline effect when C is zero. In practical terms, 'I' represents the baseline effect or response that is present even when there is no drug in the system. It helps to establish the starting point of the concentration-effect curve.

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

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

$$E = \frac{Emax.C}{EC50 + C}$$

Where, C = concentration of the drug, E = effect produced by the concentration C, E_{max} = maximum possible response that can be attributed to the drug, EC_{50} = drug concentration that can produce 50 % of the maximum possible effect.

The E_{max} model is particularly useful for drugs that exhibit a graded dose-response relationship, meaning that the effect increases with increasing drug concentration until a maximum effect is reached. It also helps researchers and clinicians understand the PD of a drug, including its potency (EC_{50}) and efficacy (E_{max}). It is valuable for dose-response modeling, predicting therapeutic effects, and optimizing drug dosing regimens to achieve the desired therapeutic outcome while minimizing adverse effects.

- Other models such as Sigmoid E_{max} model and inhibitory are also used with some modified patterns of drug effect with respect to the concentration (Pérez-Urizar et al., 2000). While both the Sigmoid Emax model and inhibitory models are modifications of the E_{max} model, they cater to different nuances in drug concentration-effect relationships. While the basic E_{max} model describes a general dose-response relationship, the Sigmoid E_{max} model introduces the Hill coefficient for a more sigmoidal shape. The inhibitory model, on the other hand, is employed in situations where a drug exhibits both stimulatory and inhibitory effects. The choice between them depends on the nature of the drug's PD and the characteristics of the observed concentration-effect data.

As discussed in the introduction, it is essential to explore new analgesic drugs for farm animals. In the forthcoming chapters, we will delve into the specific applications and PK profiles of robenacoxib and DX in three distinct species: sheep, goats, and geese. Each species will be thoroughly examined in separate chapters, providing detailed insights into the utilization and PK behavior of these drugs in diverse veterinary contexts.

CHAPTER III: Pharmacokinetics of Robenacoxib in Sheep

1. INSIGHTS AND AIMS OF THE STUDY

The study's objective arises from the diverse applications of sheep, which often undergo painful procedures and are used as experimental models. Additionally, in advanced countries like the United States and Canada, there's a lack of approved pain management drugs for sheep or goats, necessitating off-label drug use. However, the knowledge gap in PK, efficacy, and residue depletion hampers this practice. Challenges such as injectable drug administration, time, cost, and limited expertise further complicate the situation. In sheep, the analgesic efficacy of NSAIDs has been frequently reported, such as for sheep suffering from footrot or undergoing castration and tail-docking (Welsh and Nolan, 1995; Small et al., 2014). Thus, in theory, animal species other than dogs and cats, such as sheep, could potentially benefit from RX. However, the PK and PD differences among animal species, especially between ruminants and monogastric species, require studies to elucidate the behavior of the drug in the target species. To the best of our knowledge, there are no reported RX studies in sheep. Hence, the aim of this study was to determine the PK of RX following a single oral (PO, 4 mg/kg), subcutaneous (SC, 4 mg/kg), and intravenous (IV, 2 mg/kg) dose.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

The pure powders of RX and diclofenac as internal standard with a standard purity of 99.0%, alongside the sodium chloride (NaCl), were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade acetonitrile (ACN), methanol (MeOH), and formic acid were obtained from VWR chemicals (Oud-Heverlee, Belgium). Deionized water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany). The aqueous and organic components of the mobile phase were degassed under pressure and mixed in the HPLC system. The mobile phases were filtered through 0.2 μm cellulose acetate membrane filters (Sartorius Stedim Biotech, Goettingen, Germany) with a solvent filtration apparatus.

2.2. Animals and experimental design:

The study employed five healthy adult female sheep (Wrzosówka breed) with body weights ranging from 18 to 26 kg (10–14 months of age). Based on a physical examination as well as complete chemical and hematological testing, the sheep were found to be clinically healthy. The health of the sheep was examined and certified by skilled veterinarians (C-F referring to me; B L-W), with confirmation of the absence of recent pharmacological treatment and the absence of parasites in the sheep. This experiment was carried out at the University of Life Sciences in Lublin, Poland.

The well-being and adaptability of the sheep to their new environment were diligently assessed as part of our rigorous animal care protocol. Daily monitoring involved a comprehensive evaluation of their behavior, which encompassed activities such as grazing habits, social interactions, and overall demeanor, along with a keen observation of their appetite, ensuring that their nutritional needs were met. To ensure the sheep's optimal acclimatization to the experimental conditions, a meticulous acclimatization process was implemented, involving their residence in a dedicated animal shed for a duration of 7 days leading up to the commencement of the trial. During this acclimatization period, the sheep enjoyed the convenience of ad libitum access to high-quality feed, specifically alfalfa hay, and a continuous supply of fresh water. Furthermore, to grant the animals a semblance of their natural grazing behavior, they were allowed to freely roam and graze during daylight hours. For the ease of individual identification and tracking, each sheep was thoughtfully equipped with unique ear tags, bearing an identity code that was securely affixed to the left ear.

The animal experiment was approved by the University of Lublin's animal welfare ethics committee and conducted in compliance with European law (Directive 2010/63/EU).

2.3. Drug, drug dosing, and sample collection

The commercial SC formulation containing 20 mg RX per mL (Onsior®, Elanco, Italy), and the oral tablets of 40 mg each (Onsior®, Elanco, Italy), were used in this study. The selected doses were based on RX data present in cats and dogs.

Animals underwent a three-phase parallel study design, with a washout period of four weeks to ensure an adequate Cl of the drug. The sheep were weighed each day before administration, and the doses were adjusted correspondingly. In phase l, a SC injection of 4 mg/kg RX was performed behind the right shoulder, above the ribs. In phase 2, The 4 mg/kg PO doses were prepared by carefully partitioning and weighing the grinded tablets of RX. The tablets were then dissolved in 20 mL of water and administered via an ororuminal tube, immediately after which the tube was flushed with 400 mL of water. In the third phase, sheep received a slow IV injection of RX at a dose of 2 mg/kg, in the right jugular vein.

Blood samples were collected using vacutainer lithium heparin tubes (BD, Vaud, Switzerland) from the left jugular vein at 0, 0.085 (for IV only), 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, and 48 h. Blood was centrifuged for 10 min at 1500 g immediately after collection. Then the plasma was harvested, transferred in crio-vials and stored at −20°C. It was analyzed within four weeks of each phase of the study.

2.4. Sample preparation

The procedure utilized in this study was adapted from a previously published method (Jung et al., 2009) and was further refined to meet our laboratory's requirements. In a nutshell, the process commenced by adding 50 mg of NaCl to 200 µL of plasma, a step designed to enhance the ionic strength of the aqueous medium. Following this, a solution containing 50 µL of the IS at a concentration of 50 µg/mL in MeOH was introduced into the plasma. Subsequently, to facilitate extraction and purification, 800 µL of ACN was incorporated into the mixture. This concoction was subjected to thorough vortex mixing, a process lasting 30 seconds, and was then placed in a controlled environment with continuous shaking at 60 oscillations per minute for 10 minutes. Afterward, the samples underwent centrifugation at 4000 x *g* for 10 minutes, leading to the separation of the upper layer. This upper layer was carefully transferred into a fresh, clean tube and subjected to a drying process at 45 °C, facilitated by a gentle nitrogen stream. The resulting residue was then reconstituted

in 120 µL of ACN:H_2O (60:40, *v/v*), subjected to vortex mixing for one minute, followed by a 10-minute sonication at 25°C. The final step involved centrifugation at 4000 x *g* for 2 minutes, facilitating the removal of any residual particulate matter. A precisely measured aliquot of 50 µL from the resulting upper layer was meticulously injected into the HPLC system for subsequent analysis. This comprehensive procedure was applied as well to the samples of plasma in geese and goats.

2.5. HPLC conditions:

The HPLC system was a LC Jasco consisting of a ternary gradient system (PU 980), in line degasser (DG-2080-53), autosampler (AS2055) and an UV multiple wavelength detector (MD-1510). The chromatographic separation assay was performed with a Luna C18 analytical column (150 × 4.6 mm inner diameter, 3 µm particle size, Phenomenex) maintained at 30 °C using a Peltier system (CO4062) (Jasco). The mobile phases were 0.1% v/v formic acid in H_2O:ACN 95:5 (v/v) (phase A) and ACN (phase B). The column was eluted isocratically using 38% A and 62% B at a flow rate of 1 mL/min. The preference for using a C18 column in HPLC to quantify RX and NSAIDs generally is grounded in the chemical properties of these compounds and the chromatographic principles. C18 columns, characterized by octadecyl (C_{18}) alkyl chains, offer a hydrophobic environment conducive to interactions with hydrophobic NSAIDs such as RX. Reversed-phase chromatography on C18 columns, where the stationary phase is more nonpolar than the mobile phase, facilitates effective separation and retention of these compounds. The method's wide acceptance and compatibility with UV detection make C18 columns a pragmatic choice for routine pharmaceutical analysis, ensuring reliable quantification of NSAIDs in various applications.

The optimal wavelength for the quantification was set at 275 nm. The detection of RX with UV light in HPLC relies on its specific chemical structure, which includes an aromatic ring system and functional groups that contribute to the presence of a chromophore. A chromophore is a chemical group that can absorb light in the ultraviolet or visible regions of the electromagnetic spectrum. In

the case of RX, its aromatic rings and conjugated double bonds create a chromophoric system that exhibits absorption of UV light.

2.6. Validation of the analytical method:

Before we delve into the specifics of our analytical method, it's crucial to acknowledge that we closely followed the stringent guidelines provided by EMA. Adherence to regulatory frameworks is not just a formality but a commitment to ensuring safety, efficacy, and precision in scientific research and pharmaceutical development. With the EMA's guidance as our foundation, we embarked on our analytical journey, confident that every step was taken to validate our methodology.

In line with the EMA's recommendations, we systematically assessed key parameters required for method validation, including precision, accuracy, specificity, linearity, and robustness. We approached this task with methodical precision, conducting experiments and applying rigorous statistical analysis to each parameter. Our primary objective was to guarantee that our analytical method consistently produced accurate and reproducible results.

In the upcoming sections, we will provide a detailed explanation of each of these analytical parameters as defined by the EMA, laying the groundwork for a comprehensive understanding of our methodology:

2.6.a. Reference standards

Reference standards or internal standards (IS) play a vital role in the process of method validation and the analysis of study samples. To create calibration standards, quality control samples, and stability samples, a blank biological matrix is enriched with the desired analyte using reference standard solution. Additionally, in chromatographic methods, appropriate IS may be introduced during sample processing, as outlined in the guideline. As mentioned, diclofenac was used as an IS in this study. The use of an IS in analytical methods is crucial for several reasons, primarily to enhance the accuracy and precision of measurements. Here are some key reasons why employing an IS is very important in analytical methods:

- Compensation for Variability: Analytical methods can be affected by various factors such as changes in instrument conditions, sample matrix effects, and environmental conditions. An IS, which is a known quantity of a substance added to the sample, helps compensate for these variations.
- Instrument Drift Correction: Instruments used in analytical methods may experience drift over time, leading to changes in sensitivity or baseline shifts. By including an IS, variations in instrument response can be monitored and corrected for, ensuring more accurate and reliable results.
- Matrix Effects Compensation: Sample matrices can differ widely, and these differences can affect the performance of analytical instruments. An IS with similar properties to the analyte of interest can help correct for matrix effects, ensuring that the measurement is not influenced by the specific characteristics of the sample.
- Calibration Integrity: An IS can be used during the calibration process to account for any losses or variations that may occur during sample preparation, extraction, or analysis. This helps maintain the integrity of the calibration curve and improves the accuracy of quantification.
- Enhanced Precision: The use of an IS allows for the normalization of analytical measurements. This normalization can significantly improve the precision of the method, reducing random errors associated with the analytical process.
- Quality Control and Validation: IS serve as an important tool for quality control and method validation. They provide a means to assess the accuracy, precision, and reliability of the analytical method throughout its use.
- Quantitative Accuracy: In quantitative analysis, IS are particularly important. They help correct for variations in sample preparation and analysis, ensuring that the final results accurately reflect the concentration of the analyte in the original sample.

- Method Robustness: The inclusion of an IS contributes to the robustness of an analytical method by making it less susceptible to changes in conditions or parameters that may affect the measurement process.

2.6.b.Selectivity

The analytical method must exhibit selectivity by effectively distinguishing the target analyte(s) and IS(s) from matrix-related or sample-specific components. This selectivity should be substantiated through the examination of at least six individual sources of the appropriate blank matrix, with each source independently analyzed to assess potential interference. Exceptions to this six-source requirement are acceptable for rare matrices. Generally, absence of interfering components is deemed acceptable when their response is below 20% of the lower limit of quantification for the analyte and 5% for the IS. Additionally, it is crucial to investigate potential interference arising from drug metabolites, degradation products during sample preparation, and co-administered medications. The latter should be considered during method validation, either on a study-specific or compound-specific basis. The potential for back-conversion of metabolites into parent analytes, especially for unstable metabolites like acidic metabolites to ester, unstable N-oxides, or glucuronide metabolites, should also be evaluated when relevant. Although this assessment may not be feasible in early stages of drug development, it is expected to be addressed as knowledge about the substance's metabolism advances. In cases where obtaining the target metabolites is challenging, back-conversion can be assessed through incurred sample reanalysis, albeit with the understanding that potential back-conversion during sample processing cannot be completely ruled out, as per the guideline.

2.6.c. Carry-over

Carry-over must be effectively managed during method development and should be minimized. In the validation phase, the assessment of carry-over involves injecting blank samples immediately after a high-concentration sample or a calibration standard at the upper limit of quantification. The level of carry-over observed in the blank sample following the high concentration standard should not

exceed 20% of the LLOQ, as specified below, and should be limited to 5% for the IS. In instances where it becomes apparent that carry-over is unavoidable, randomization of study samples should not be employed. Instead, specific measures should be devised, tested during validation, and implemented in the analysis of study samples to ensure that carry-over does not compromise accuracy and precision. This may entail the injection of blank samples following samples with expected high concentrations before proceeding with the analysis of the subsequent study sample.

2.6.d. Lower limit of quantification

The LLOQ is the lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision. The LLOQ is also considered being the lowest calibration standard (see Accuracy and Precision). In addition, the analyte signal of the LLOQ sample should be at least 5 times the signal of a blank sample. The LLOQ should be adapted to expected concentrations and to the aim of the study. As an example, for bioequivalence studies the LLOQ should be not higher than 5% of the Cmax, while such a low LLOQ may be not necessary for exploratory PK studies.

2.6.e. Calibration curve

In the process of method validation, calibration curves are essential and should be generated within the same matrix as the intended study samples. This is achieved by introducing known concentrations of the analyte into the blank matrix. Each analyte being studied requires its own calibration curve for each analytical run. Ideally, prior to validation, the expected concentration range should be determined and should fall within the calibration curve range, defined by the lowest (LLOQ) and the upper calibration standard (ULOQ). This range must be adequate for describing the PK of the analyte. Utilizing a minimum of six calibration concentration levels, including a blank sample and a zero sample, each calibration standard can be analyzed in replicates. A suitable relationship to describe the instrument's response concerning analyte concentration should be applied. Calibration curve parameters (slope and intercept for linear fit) must be reported along with back-calculated concentrations of the calibration standards and mean accuracy values. The back-calculated

concentrations of the calibration standards should typically be within ±15% of the nominal value, except for the LLOQ, which allows ±20%.

2.6.f. Accuracy

Accuracy in an analytical method signifies how closely the measured value aligns with the expected or nominal concentration of the analyte, expressed as a percentage. To assess accuracy, quality control (QC) samples, spiked with known analyte amounts, should be employed. These QC samples must be spiked independently from calibration standards, using separate stock solutions, unless stock solution nominal concentrations are established. These QC samples are analyzed against the calibration curve, and the resultant concentrations are compared to the expected values, reporting accuracy as a percentage of the nominal value. Accuracy evaluation entails within-run and between-run assessments. Within-run accuracy involves analyzing a minimum of 5 samples at four concentration levels within a single run, including the LLOQ, low QC, medium QC, and high QC. The mean concentration should generally fall within 15% of the nominal values, except for the LLOQ, which allows 20%. For between-run accuracy, LLOQ, low, medium, and high QC samples from at least three runs, conducted on two different days, should be evaluated, aiming for mean concentrations within 15% of the nominal values, except for the LLOQ, which permits 20%.

2.6.g. Precision

Precision in an analytical method reflects the consistency of repeated measurements of the analyte and is quantified as the coefficient of variation (CV, %). It's imperative to establish precision for the LLOQ, low, medium, and high QC samples, both within a single run and across different runs, utilizing the same data employed for accuracy assessment. Within-run precision necessitates a minimum of five samples at each concentration level (LLOQ, low QC, medium QC, and high QC) in a single run, with the within-run CV not surpassing 15%, except for the LLOQ, where it's permissible up to 20%. For between-run precision, evaluate LLOQ, low, medium, and high QC samples from at least three runs conducted on at least two different days, aiming to maintain a between-run CV below

15% for QC samples, except for the LLOQ, which permits 20%. These precision assessments ensure the reliability and consistency of the analytical method's measurements.

2.6.h. Dilution integrity

The accuracy and precision of samples must remain unaffected by the process of dilution. If relevant, the integrity of dilution should be confirmed by introducing an analyte concentration exceeding the ULOQ into the matrix and subsequently diluting this sample with a blank matrix (with a minimum of five determinations per dilution factor). Accuracy and precision should adhere to predetermined criteria, typically within ±15%. This assessment of dilution integrity should encompass the dilution levels applied to the study samples. The evaluation of dilution integrity can be included as part of a partial validation process. Alternatively, the use of a different matrix may be acceptable, provided it's demonstrated that it does not compromise precision and accuracy in the analytical method.

2.6.i. Recovery

Recovery means the amount of analyte determined by an analytical method in relation to the total quantity. Allows to determine losses of analyte during the analytical procedure, as well as being a way to express the accuracy. It was evaluated by comparison with the detector responses obtained for the extracted quality control samples and those for the pure standard dilutions. The recovery was expressed as mean (± SD).

2.6.j. Robustness

Robustness assessment entails a comprehensive examination of critical parameters within the analytical method to gauge its resilience and reliability. These parameters encompass a range of factors, including pH, temperature, analyte concentration, volatility, stability in solution, extraction time, composition of the extraction mixture, alterations in mobile phase composition, variations in flow rate, and the type of column used. By systematically varying these parameters within defined limits, the method's ability to consistently produce accurate and precise results under diverse conditions can be thoroughly evaluated.

Accordingly, in this study, stock solutions of the analyte RX (1 mg/mL) and the IS (1 mg/mL) were meticulously prepared in MeOH. Subsequently, these solutions were diluted to attain a concentration of 50 µg/mL and were carefully stored at -20°C to maintain stability. From this base concentration, further dilutions were prepared at 10, 5, 2.5, 1, 0.5, 0.1, and 0.05 µg/mL, forming the calibration curve for RX in plasma. These concentrations were employed to construct spiked curves, plotting RX concentrations against the ratio of IS peak areas. The linearity of the calibration curves, spanning the range of 0.05–50 µg/mL for plasma, was assessed through a thorough examination involving residual plots, fit tests, and back calculations.

To evaluate precision, intra-day and inter-day analyses were conducted using six plasma samples spiked with IS at three distinct concentration standards: high (10 µg/mL), middle (1 µg/mL), and low (0.05 µg/mL), serving as quality control (QC) samples. These assessments were carried out with the same instrument, by the same operator, on the same day for intra-day precision and on three different days for inter-day precision. Precision values were expressed as (CV, %).

The recovery of the drug was evaluated by comparing the detector responses obtained for the extracted quality control samples with those of the pure standard dilutions. Recovery was expressed as the mean (± SD). The Limit Of Detection (LOD) was determined as the plasma concentration producing a signal-to-noise ratio of 3, while the LLOQ was established as the lowest plasma concentration resulting in a signal-to-noise ratio of 5.

The identical validation method was applied to assess RX in both geese and goats' plasma and thus will not be explained in the following chapters.

2.7. Pharmacokinetic analysis:

The data were pharmacokinetically analyzed using a noncompartmental approach (ThothPro^{TMT} 4.3; ThothPro LLC, Poland). C_{max} and T_{max} were determined directly from the concentration vs. time curves. t1/2 was calculated using least squares regression analysis of the concentration-time curve. AUC was calculated by linear log trapezoidal (IV administration) and the linear-up log-down rule

(PO and SC administration). AUMC was calculated as $\int^{\infty}_{0}$ 0 C(t)dt. From these values, the volume of distribution at steady state (V_{ss} =dose x AUMC/AUC^2), MRT (MRT = AUMC/AUC), and Cl (Cl =dose/AUC) were calculated. The individual value of $AUC_{rest\%}$ was lower than 20% of $AUC_{(0-\infty)}$, and the square of coefficient of determination (R^2) of the terminal phase regression line was > 0.85. Values below the LLOQ were not considered for the PK analysis.

The PO and SC bioavailability were calculated using the following equation:

$$F\% = 100 \times \frac{AUC(SC\ or\ PO) \times Dose\ (IV)}{AUC(IV) \times Dose\ (SC\ or\ PO)}$$

The mean absorption time (MAT) was calculated using the following equation:

MAT(PO or SC)= MRT(PO or SC)-MRT(IV)

The body extraction ratio (E) for RX after IV administration was calculated for sheep as the Cl divided by cardiac output, where cardiac output (mL/kg/min) was calculated as body weight (kg) to the power of -0.19 multiplied by 180 (Toutain and Bousquet-Mélou, 2004c).

$$E\% = \frac{\text{Body clearance}}{\text{Cardiac output}} = \frac{\text{Body clearance}}{180 \times \text{Body weight}^{-0.19}}$$

2.8. Statistical analysis:

Bonferroni's multiple comparison test, a widely recognized statistical method within the framework of repeated measures ANOVA, was employed to meticulously assess and ascertain any statistically significant distinctions in PK variables among the three distinct treatment groups. Furthermore, to make a detailed comparison between the key parameters within the subcutaneous SC and PO administration groups, we utilized the paired t-test. In presenting the PK parameters, a comprehensive approach was adopted wherein geometric means and associated ranges were reported. However, for T_{max}, being a categorical variable, we provided the median value along with its corresponding range, as outlined in Julious and Debarnot's methodology (2000), while t1/2 ws experessed as the harmonic mean. To ascertain statistical significance, the conventional threshold of a *p-value* less than 0.05 was

applied. These rigorous analyses were conducted utilizing the robust statistical software, GraphPad InStat (version 5.3, GraphPad Software), which is esteemed for its reliability in scientific research.

3. RESULTS

3.1. Validation of the method

The quantitative HPLC method underwent a comprehensive validation process specifically tailored for sheep plasma, aligning with the rigorous criteria outlined in the EMA guidelines (Anonymous, 2012). The validation encompassed a thorough evaluation of various critical aspects. Selectivity, for instance, was diligently assessed to ensure the method's capacity to distinguish RX in both blank plasma and spiked samples, with the outcome revealing the absence of any interfering peaks, as seen in figure 16. Remarkably, the analytical method exhibited exceptional linearity, as evidenced by an impressive R2 value of 0.999, represented by the equation $y = 0.1223x + 0.003$.

Furthermore, the method's sensitivity was affirmed, with LODand LLOQ established at 0.01 and 0.05 µg/mL, respectively. In terms of recovery, the mean extraction recovery rate was found to be 95% ± 14%, underscoring the method's reliability in capturing the analyte accurately from plasma matrices.

Precision, both within the same day (intra-day) and across different days (inter-day), was meticulously examined. The results revealed a coefficient of variation lower than 14.3% for inter-day precision and an even more impressive 2.69% for intra-day precision. Additionally, the mean concentrations obtained for QC samples and LLOQ samples were well within the acceptable range, consistently measuring below 15% of the nominal values.

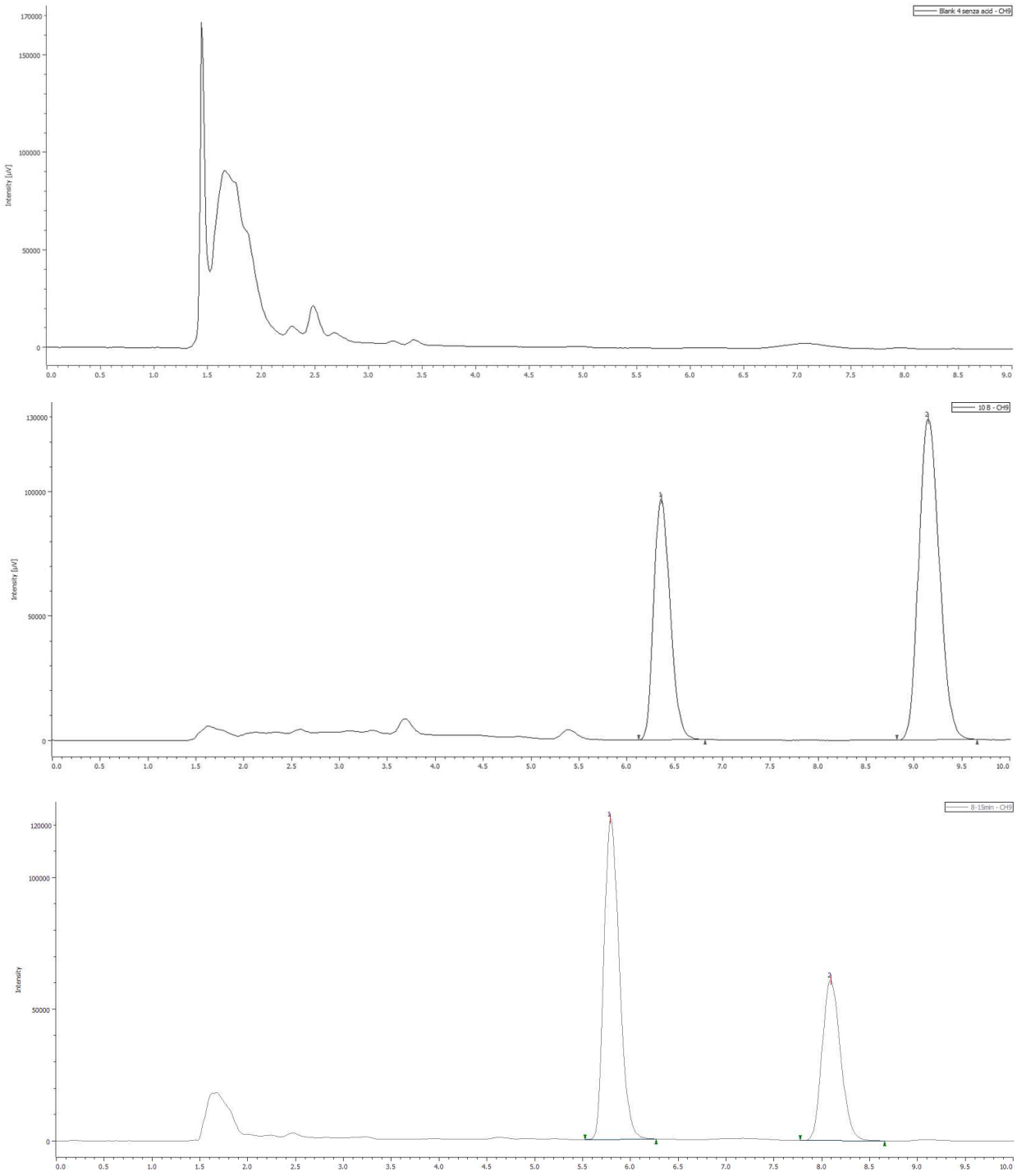


Figure 16: 1) chromatogram of control plasma; 2) Chromatogram of spiked plasma sample IS (50 ppm) and RX (10 ppm); 3) Chromatogram of the plasma sample collected from a treated goat at 15 minutes after IV administration.

This extensive validation process ensures the robustness, accuracy, and precision of the HPLC method, substantiating its suitability for the quantitative analysis of RX in sheep plasma. Indeed, in terms of robustness, the method demonstrated robust performance across various conditions, including stability in pH of mobile phase, its composition, temperature, stability in solution, no instrument variability, and other critical parameters.

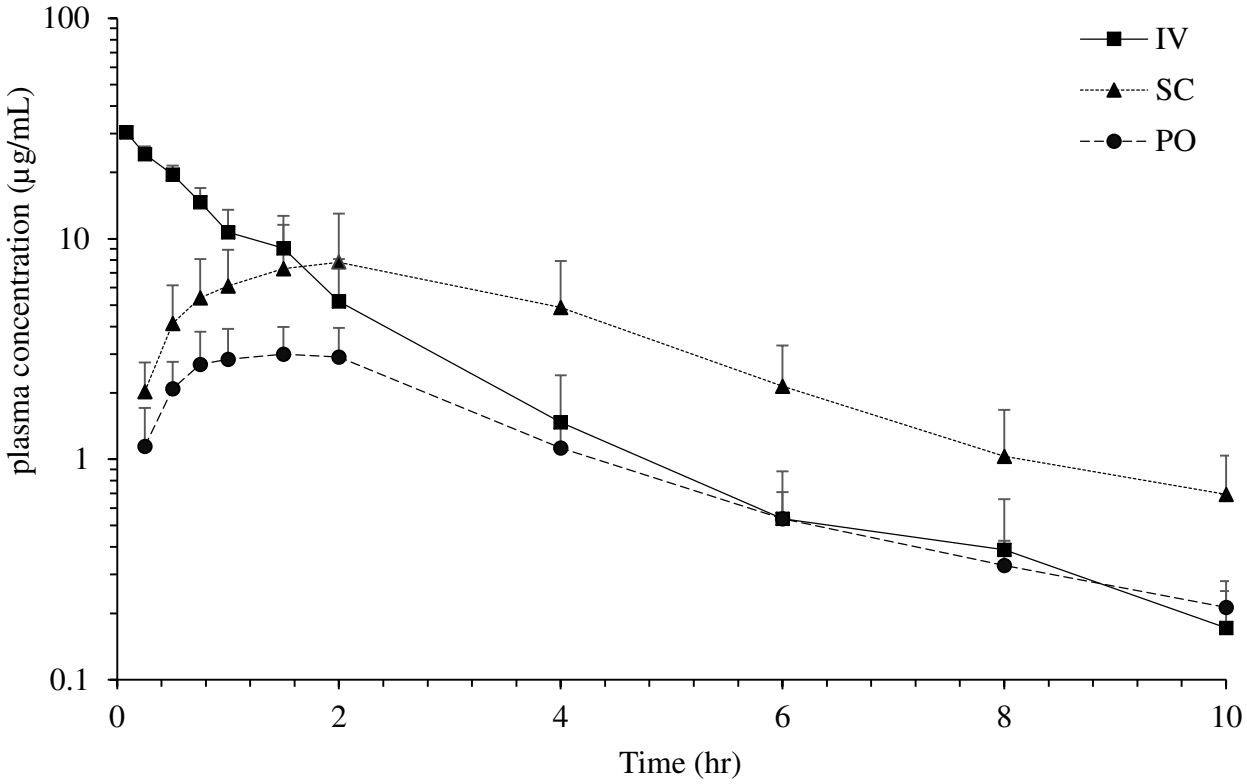
3.2. Animals

The sheep under observation did not manifest any discernible immediate or delayed adverse effects, neither at the local nor systemic level, during the entire monitoring period, extending up to a duration of 7 days. Such a notable absence of adverse effects serves as a vital testament to the safety and compatibility of the experimental interventions with the physiological systems of the sheep, further reinforcing the credibility of the study's outcomes and the welfare of the animal subjects involved in the research.

3.3. Pharmacokinetics

The study's findings are graphically represented in Figure 17, illustrating the mean plasma concentrations of RX (± SD) at various time points following IV, SC, and PO administrations. Comprehensive PK parameters, derived from non-compartmental PK analysis, are meticulously detailed in Table 3. Notably, RX was detected in plasma for an extended period of up to 24 hours across all routes of administration, albeit in trace amounts, while it remained quantifiable only up to the 10-hour mark.

The investigation into RX bioavailability revealed intriguing insights into its behavior based on the mode of administration. Specifically, following SC administration, a moderate bioavailability of 45.98% was observed, contrasting with the lower bioavailability of 16.58% observed following PO administration. This discernible disparity was further substantiated by significant alterations in the $AUC_{(0-\infty)}$ values, corrected for the dose, exhibiting an order of IV > SC > PO.



2291 Figure 17: Semi logarithmic mean plasma concentration–time curves of robenacoxib following
2292 intravenous (2 mg/kg), subcutaneous (4 mg/kg), and oral (4 mg/kg) administrations in sheep (n = 5).

Table 3: Mean pharmacokinetic parameters and range in sheep (n = 5) after single IV (2 mg/kg), SC (4 mg/kg), and PO (4 mg/kg) doses of robenacoxib.

		IV			SC			PO		
Parameter	Unit	Geo mean	min	max	Geo mean	min	max	Geo mean	min	max
$AUC_{(0-t)}$	mg hr/L	36.02[b,c]	25.43	52.9	31.81[a,c]	18.55	64.03	11.03[a,b]	7.95	15.73
$AUC_{(0-\infty)}$ normalized	mg hr/L	71.3[b,c]	50.06	103.1	33.63[a,c]	19.94	66.58	11.82[a,b]	8.61	16.43
λz	1/hr	0.259	0.181	0.352	0.318	0.263	0.401	0.258	0.222	0.292
t1/2[h]	hr	2.64	1.84	3.82	2.18	1.73	2.63	2.69	2.37	3.12
Cl	L/kg/hr	0.056	0.038	0.079	/	/	/	/	/	/
Vss	L/kg	0.077	0.065	0.088	/	/	/	/	/	/
$MRT_{(0-t)}$	hr	1.4[b,c]	0.96	1.78	3.27	2.76	3.78	3.02	2.78	3.22
$MRT_{(0-\infty)}$	hr	1.66[b,c]	1.14	1.97	3.79	2.99	4.55	3.75	3.24	4.03
Cmax	μg/ml	/	/	/	7.04	4.28	16.49	3.01	2.21	4.48
Tmax[§]	hr	/	/	/	2	1	2	1.5	0.75	2
F	%	/	/	/	45.98[c]	31.36	71.72	16.58	13.71	19.46
MAT	hr	/	/	/	1.87	1.8	2	1.62	1.82	1.44

Note: $AUC_{(0-t)}$, area under the curve from 0 h to last time collected samples; $AUC_{(0-\infty)}$, area under the curve from 0 h to infinity; λz, terminal phase rate constant; t1/2, terminal half-life; Cl, plasma clearance; V_{ss}, volume of distribution at a steady state; $MRT_{(0-t)}$, mean residence time from 0 hr to last time collected samples; $MRT_{(0-\infty)}$, mean residence time from 0 h to infinity; C_{max}, peak plasma concentration; T_{max}, time of peak concentration; F, bioavailability; MAT, mean absorption time.

[a], statistically different from IV; [b], statistically different from SC; [c], statistically different from PO; [§], Median value; [h], harmonic mean.

Upon IV administration, the calculated mean Cl was characterized by a relatively slow rate at 0.056 L/kg h, and the V_{ss} demonstrated a comparably low value of 0.077 L/kg. E maintained an average of 0.01.

Moreover, the MRT presented noteworthy distinctions among the routes of administration. Specifically, $MRT_{(0-\infty)}$ exhibited no statistically significant difference between SC and PO routes, yet starkly contrasted when compared to the IV route ($p < 0.05$).

4. DISCUSSION AND CONCLUSION

The pursuit of an optimal anti-inflammatory and pain medication tailored for the well-being of both companion pets and production animals encompasses several key attributes. Ideally, such a medication should exhibit a trifecta of characteristics: it must be inherently safe, facile to administer, boast efficient absorption properties, and boast a commendably protracted t1/2 and therapeutic effect, consequently permitting less frequent dosing intervals, as elucidated by Stuart et al. in their 2019 study.

To address this imperative, the current study was meticulously undertaken with the overarching aim of unraveling the intricate PK of RX when administered via three distinct routes: IV, SC, and PO. The dosage regimen of RX employed for each route of administration was thoughtfully extrapolated from existing data pertaining to feline and canine subjects, given the dearth of prior research specifically elucidating RX's PK in ruminants.

It's noteworthy that Onsior® tablets have earned regulatory approval within the European Union for surgical applications, prescribing a dosage of 2 mg/kg, complemented by a recommended range spanning from 2 to 4 mg/kg, as stipulated by the EMA in 2008. This empirical and scientific exploration, rooted in a paucity of data for ruminants, stands poised to illuminate crucial insights into the pharmacological behavior of RX, paving the way for informed and optimized therapeutic strategies that cater to the diverse needs of both companion animals and livestock. The dose of RX

2327 differed between IV and the extravascular routes in the present study. To avoid toxicity issues and
2328 collateral effects, the IV dose was purposefully chosen lower than for the other routes of
2329 administration. Furthermore, although IV is not an approved route of administration of RX, IV PK
2330 study was performed to establish disposition kinetic variables, such as V_{ss}, Cl and F. Although dose-
2331 dependent PK cannot be excluded, RX was found to be independent of dose with linear plasma RX
2332 concentrations in dogs (King et al., 2011; Schmid et al., 2010b; Borer et al., 2017). Additionally,
2333 despite administration of a higher PO dose, the peak plasma concentrations achieved were still less
2334 than those achieved after IV administration and the plasma concentrations on the terminal portions
2335 of the curves were similar for IV, PO, and SC administration. Given the observations in our study,
2336 and the linearity of RX concentrations observed in dogs, use of different doses for the determined PK
2337 parameters in sheep was justified.

2338 Our data indicated that RX has a moderate SC and low PO bioavailability, with mean values
2339 significantly different. Indeed, SC administration can evade drug metabolism (or hydrolysis) in the
2340 digestive tract, compared to oral administration (Benedetti et al., 2009). The reported F % values were
2341 higher in fasted cats (69% SC; 49% PO; King et al., 2013) and dogs (88% SC; 84% PO fasted; 60%
2342 PO fed; Jung et al., 2009). A decrease in the rate of absorption in sheep can be associated with the
2343 abundant fermentation system by the ruminal microflora (Baggot and Brown 1998), in addition to
2344 dilution and retention of the drug in the forestomach, compared to the diverse digestive system in
2345 monogastric species (Coetzee et al., 2011). Nevertheless, food is known to influence the absorption
2346 as well as binding of drugs reducing the total absorbed amount, especially for NSAIDs (Lees et al.,
2347 1998; Türck et al., 1996). It is also unknown whether RX binds to hay or digesta in ruminants,
2348 reducing furthermore F %, which is the case for several NSAIDs such as phenylbutazone and flunixin
2349 meglumine (Lees et al., 1998). However, because most sheep will not have had food withheld in
2350 clinical settings, the results for the present study may reflect the kinetics of orally administered RX

in a typical clinical setting. Although RX tablets provided either alone or with a minor amount of food might lead to a superior F % (King et al., 2013), more studies are needed to settle this in sheep.

Accordingly, the dose-normalized $AUC_{(0-\infty)}$ of RX following IV administration was statistically higher than $AUC_{(0-\infty)}$ of the SC and PO routes, as lower fraction of the doses was absorbed in these two routes. As for MRT_{IV} which is significantly different from MRT_{SC} and MRT_{PO}, the longer residence time for the extravascular routes may be elucidated by the sustained time for absorption following SC and PO administrations (Albarellos et al., 2016).

In sheep (1.5 hr), rats (1 hr, King et al., 2009), dogs (0.5 hr, Schmid et al., 2010b; Borer et al., 2017) and cats (0.5 hr; King et al., 2013), T_{max} was relatively short after oral administration. These data, alongside the relatively short half-life, are consistent with rapid absorption (or with a possible flip-flop phenomenon as discussed in the next chapter) (Lees et al., 2022). The expectation is that when RX is administered orally, it will undergo rapid absorption from the rumen. This anticipation is grounded in the substance's relatively high aqueous solubility, which measures at 0.17 g/L within a specific pH range, namely between 6.4 and 6.8. This level of solubility suggests that RX is well-suited for dissolution in bodily fluids, a critical step in the absorption process. Furthermore, RX exhibits a moderate lipid solubility, as indicated by its log partition coefficient in n-octanol/phosphate buffer at pH 6.8, which measures at 2.27. This aspect of RX's physicochemical properties facilitates its absorption in the intestinal tract, as noted by King et al. in their 2009 study.

In this study, the V_{ss} following IV administration of RX at a dose of 2 mg/kg in sheep was low with 0.077 L/kg, and lesser than that previously reported in dogs (0,24 L/kg; Schmid et al., 2010b), and in cats (0.19 L/kg; King et al., 2013). These variations in V_{ss} values across species highlight the potential influence of species-specific factors on the distribution of RX within the body. In the broader context of NSAIDs, a low V_d is often associated with a high degree of plasma protein binding, as described by King et al. in 2009. However, it is essential to note that the specific binding ratio of RX to plasma proteins in sheep remains undisclosed. Nevertheless, it is worth noting that in dogs and cats, at a

concentration of 2 μg/mL, RX exhibited a substantial degree of protein binding, exceeding 98%, as reported by Jung et al. in 2009. Understanding the extent of protein binding in sheep is crucial for comprehending the drug's distribution within the body, as it can influence its therapeutic efficacy and PK profile. Furthermore, the V_{ss} value in sheep was observed to be in close proximity to the estimated blood volume of sheep, which is approximately 0.075 L/kg, as reported by Luethy et al. (2017). This observation underscores the significance of investigating RX's binding to plasma proteins, as it could shed light on whether the drug tends to remain within the extracellular or intracellular compartments. Such insights are pivotal for assessing the drug's effectiveness, as emphasized by Lees et al. in 2022.

Additionally, previous studies have demonstrated the selective distribution of RX to sites of inflammation in various animal species, including rats, dogs, and cats. This unique distribution pattern is attributed to RX's physicochemical properties, particularly its characteristic as a weak acid with a pKa of 4.7. Importantly, these studies have also highlighted a prolonged residence time of RX in inflammatory exudates, lasting for more than 24 hours. This extended duration of action has significant clinical implications for the drug's therapeutic utility, as demonstrated in previous studies (King et al., 2009; Pelligand et al., 2012; Pelligand et al., 2014). However, it is imperative to investigate whether this prolonged residence time and extended duration of action hold true in sheep, as this could have substantial clinical relevance for the use of RX in this specific animal population. In summary, the study's findings regarding the V_{ss} of RX in sheep raise intriguing questions about species-specific PK and the potential impact of plasma protein binding. Moreover, the unique distribution pattern of RX to inflammatory sites and its extended duration of action warrant further investigation in sheep to assess their clinical significance and potential implications for therapeutic applications in this species.

In this study, the slow Cl (0.056 L/hr/kg) of RX in sheep was slower than that previously reported in dogs (0.81 L/hr/kg; Schmid et al., 2010b) and cats (0.44 L/hr/kg; King et al., 2013). The differences in Cl of RX between species can be attributed to variances in cardiac output. Indeed, the low estimated

E for RX in sheep found in the present study (0.01) (Toutain and Bousquet-Melou, 2004c) was lower than that found in cats and dogs, for which the range was between 0.05 and 0.15 (King and Jung, 2021; classified as low to moderate; Toutain and Bousquet-Melou, 2004c). The reduced capacity for RX elimination in sheep may arise from several underlying factors. One potential contributor could be a lower hepatic extraction ratio in sheep. This discrepancy may be linked to differences in the composition, expression levels, and enzymatic activities of biotransformation enzymes across species. Additionally, variations in renal Cl and its proportion as a percentage of the overall Cl process may also play a role in these inter-species distinctions, as elucidated by Toutain and Bousquet-Melou (2004c), and Dantzler (2016).

The t1/2 values did not exhibit statistically significant differences across the three routes of administration examined in this study. Notably, these values were observed to be longer than those reported for cats (1.49 hr, Schmid et al., 2010a) and dogs (0.81 hr, King et al., 2013). Despite the relatively slow Cl observed in the sheep, it's noteworthy that the t1/2 values, while not exceptionally prolonged, still fall within a range that could be considered relatively short. It's worth highlighting an intriguing finding from studies conducted in dogs. In cases involving peripheral inflammation, RX has exhibited a remarkably extended duration of action, surpassing 24 hours. This extended effect duration can be attributed to RX's selectivity for inflammatory sites. This unique attribute has rendered RX suitable for once-daily administration in dogs, despite the seemingly short half-life in the bloodstream, as discussed by Lees et al. in 2022. Thus, as previously stated, similar studies in diseased sheep are required to study this, because a possible prolonged duration of action, independently of t1/2, can considerably extend the dosage interval and lower the frequency of administration.

The limitation of this study (and the following two studies) is that no PD study was established. Circulating concentrations of NSAIDs required to provide good analgesia and anti-inflammatory effect should be of the IC_{80} value for COX-2 inhibition (Warner et al., 1999; Lees et al., 2004). The

reported IC_{80} for COX-2 by RX was 0.1049 µg/mL in cats, and 0.163 µg/mL in dogs, and RX doses used in these studies provided analgesia. Regarding this study, in all sheep, RX concentrations were maintained above the mentioned IC_{80} of dogs for at least 10 hours, for the three routes of administration. If it is assumed that sheep and dogs have a similar inhibitory concentration of COX-2, the doses experimentally tested in this study lead to plasma concentrations that might provide clinical effects (Giorgi et al., 2016; Sartini et al., 2021). This is also supported by the calculated mean AUC, which was at least 5 times higher in sheep than in dogs and cats (when doses normalized).

The PK-PD relationship of most analgesic and anti-inflammatory drugs is characterized by indirect effects in biological systems (Sharma and Jusko, 1998). However, it remains uncertain whether a hysteresis effect exists in sheep and should be carefully considered. It's worth noting that in previous studies, RX exhibited negative hysteresis in cats (Pelligand et al., 2012; Pelligand et al., 2014). It was attributed to several biological factors, including unique patterns of drug accumulation in deep tissues, slow binding and release dynamics from the target receptor, and the drug's high potency in inhibiting COX-2 in peripheral tissues (Pelligand et al., 2012). In such instances, the effect of a drug persists after its concentration has declined. In other words, the onset of the PD effect lags behind the peak concentration of the drug in the body. Indeed, this delay in the effect-response relationship can have implications for dosing regimens and treatment strategies, as the timing of drug administration and the persistence of effects need to be carefully considered to achieve optimal therapeutic outcomes. These factors may contribute to variations in the PK-PD relationship between species, emphasizing the importance of thorough investigation and consideration of species-specific effects in pharmaceutical research.

Another constraint worth mentioning pertains to the absence of an evaluation regarding the establishment of a maximum residue limit (MRL) for RX in food products derived from sheep. This particular aspect holds significant importance in ensuring the safety of human consumers. Without comprehensive data on the elimination of RX from various tissues, the potential application of this

drug in sheep intended for human consumption is hindered. Consequently, its use may be primarily restricted to experimental animals and sheep engaged in wool production, as outlined in Di Salvo et al. (2017). To address this limitation and propose a preliminary withdrawal interval in food-producing animal species like sheep, an alternative approach can be considered. This involves multiplying the t1/2 of RX by a factor of 10, as advocated by Riviere and Sundolf (2009) and Smith (2013). As a result, a cautious estimate of a meat withdrawal interval of approximately 4 days may be tentatively suggested. However, it is crucial to note that further research and thorough investigations into RX's tissue elimination kinetics are imperative. This is critical for establishing precise and safe withdrawal periods for food products originating from RX-treated sheep, as the actual scenario of tissue residues may significantly differ from the theoretical calculated withdrawal period.

To sum up our findings, it is evident that the SC route of administration, specifically at a dosage of 4 mg/kg, offers a notable advantage in terms of F % when contrasted with a single PO administration in sheep. This suggests that the SC route is a more favorable choice for delivering RX in these animals. Additionally, the SC route appears to be a practical option for occasional use of RX, especially in peri-operative scenarios. In light of these promising outcomes, we emphasize the significance of further research to explore the efficacy and safety profile of RX in sheep. Moreover, if relevant, comprehensive investigations into the drug's tissue kinetics should be pursued to establish a dependable withdrawal interval. This is crucial not only for the welfare of the animals but also to ensure the safety of consumers, particularly if sheep treated with RX are intended for human consumption. In conclusion, RX warrants thorough consideration and investigation for its potential applications in sheep, both in veterinary and agricultural contexts.

CHAPTER IV: Pharmacokinetics of Robenacoxib in Goats

1. INSIGHTS, IMPORTANCE OF GOATS IN AGRICULTURE, AND AIM OF THE STUDY

Human populations are significantly impacted by the socioeconomics of goat rearing, particularly in rural and economically underdeveloped areas. Due to its traits, including strong environmental adaptability and the capacity to utilize low-quality natural resources, the goat—whose meat, milk, and skin are used by humans—is a significant livestock species around the world (Skapetas and Bampidis, 2016).

There are approximately 2.2 billion sheep and goats in the world. In 2017, it was projected that there were at least 218 million dairy goats in the world. Dairy goat populations have been rising progressively all throughout the world, with massive increases in the 1990s (FAO, 2019). Both established and emerging countries are seeing an increase in demand for dairy goat products. In fact, goat milk and its products are becoming more and more popular due to their healthy and nutritional advantages, which include greater digestibility and lipid metabolism, in addition to their taste, compared to cow milk (Haenlein, 2003). The majority of goats are raised by small-scale farmers outside of specialized production systems. The production of goat milk is notably significant in the Mediterranean region, the Middle East, Eastern Europe, and portions of South America, whereas India, Bangladesh, Pakistan, and Turkey produce and consume the majority of the world's goat milk (Ribeiro and Ribeiro, 2010). In Lebanon, for instance, more than 6000 families depend on goat herd products including milk, meat, and fur for their livelihood (MOA, 2009), with this herd being represented in large part by the local caprine population known as Baladi (95%) and, to a smaller extent, by the Damascus breed (Hajj, 1999; Nehme and Abi Saab, 2003).

As the numbers of goats and the significance of their role as production animals increase, the need to improve and extend the quality of life of these animals is also growing in parallel, especially given the current public pressure for better agricultural practices and enhanced animal welfare (Stuart et al., 2019). Indeed, goats experience varying degrees of pain, resulting either from husbandry operations

such as castration, vasectomy, and tail docking, or from painful pathologies, whether acute or chronic, such as lameness, mastitis, vaginal prolapse, penis deviation, osteoarthritis, spondylitis, and other painful conditions (Plummer and Schleining, 2013; Galatos, 2011). Similarly, as elaborated in the preceding section concerning sheep, and taking into account factors such as the lack of approved pain management medications for these animals in the majority of countries, pain management in goats continues to be largely inefficient up to the present day. Another motive for the use of COX-2 selective drugs would be the rising occurrence of abomasal ulceration in sheep and goats due to the use of non-slective NSAIDs and other factors.

As a result, the goal of this study was to establish the PK of RX after single intravenous (IV) (2 mg/kg), subcutaneous (SC) (4 mg/kg), and oral (PO) (4 mg/kg) administrations.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

The pure powders of RX and diclofenac as the IS with a standard purity of 99.0%, alongside the sodium chloride (NaCl), were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade ACN, MeOH, and formic acid were obtained from VWR chemicals (Oud-Heverlee, Belgium). Deionized water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany). The mobile phase's aqueous and organic components were combined in the HPLC apparatus after being degassed under pressure. With the aid of a solvent filtration device, the mobile phases were filtered through 0.2 μm cellulose acetate membrane filters (Sartorius Stedim Biotech, Goettingen, Germany).

2.2. Animals and experimental design

Eight, 5-month old, healthy adult female Baladi goats, with body weights ranging from 16 to 25 kg, were used in the study. In 10 by 10 meters' stalls with 10 x 30 meters' outdoor runs attached, animals were group-housed. Bedded on straw, they were provided with feed (alfalfa hay) and water *ad libitum*. Goats were declared healthy before being enrolled in the study based on a physical examination, hemogram, and serum chemical profile, all of which were completed within 3 days of the study's

initiation. No recent pharmacological treatment had been administered (2 months), and the goats were parasite-free. To determine the dose to administer, body weights were measured 24 hr prior to the drug's administration. The animal experiment was approved by the Lebanese ministry of Agriculture ethical committee, verifying that this study complies with European standards for animal welfare guidelines (study protocol number 1120221).

2.3. Drug, drug dosing, administration and blood sample collection

In this trial, we utilized two different formulations of RX: a commercial SC formulation with a concentration of 20 mg of RX per mL (Onsior®, Elanco, Italy), and oral tablets containing 40 mg each (Onsior®, Elanco, Italy). The choice of these doses for ruminants was made in the absence of established recommendations, and instead, we relied on RX dosage data from cats and dogs, where Onsior® tablets are authorized for surgical use in the European Union at a recommended dose of 2 mg/kg, within a range of 2-4 mg/kg (EMA, 2008).

The study followed a meticulous three-phase, two-dose design conducted in an unblinded, parallel manner. A four-month washout period separated the IV and SC treatments, and a one-week interval separated the SC and PO treatments. In the first phase, goats received an IV injection lasting one minute, administered in the right jugular vein, with RX dosed at 2 mg/kg. In the second phase, a SC injection of RX at 4 mg/kg was administered behind the right shoulder and above the ribs. The third phase involved a precise weighing and division of the crushed RX tablets to create individual 4 mg/kg PO doses. These doses were then administered through an oro-ruminal tube after dissolving the crushed tablets in 20 mL of water, followed by a flush with 100 mL of water.

Throughout the study, blood samples were collected via the left jugular vein at specific time intervals: 0, 0.085 (for IV administration only), 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, and 24 hours after administration. The selection of these blood sampling time points was based on the prior PK data obtained in sheep in the previous chapter. Subsequently, the collected blood samples were centrifuged for 10 minutes at 1500 x *g* to separate the plasma, which was then transferred into cryo-vials and

stored at a temperature of -20° C. Within one week following the conclusion of the final phase, the plasma samples underwent analysis.

2.4. Plasma robenacoxib determination

The sample preparation was determined using a published method (Jung et al., 2009), and it was modified according to the previous chapter. To increase the ionic power of water, 50 mg of NaCl was added to 200 µL of plasma. The plasma was then spiked with 50 µL of an IS solution in MeOH (50 µg/mL). 800 mL of ACN was then added. The samples were shaken at 60 oscillations per minute for 10 minutes after vigorous vortex mixing (30 sec) and then centrifuged at 4000 x *g* for 10 minutes. The upper layer was transferred into a clean tube and dried at 45 °C while being gently streamed with nitrogen. The residue was dissolved in 120 µL of ACN:H_2O 60:40 (*v/v*), vortexed for 1 minute, sonicated at 25 °C for 10 minutes, and then finally centrifuged at 4000 x *g* for 2 minutes. An aliquot of 50 µL of the upper layer was injected onto the HPLC system for analysis.

The LC Jasco HPLC system included an autosampler (AS2055), ternary gradient system (PU 980), in-line degasser (DG-2080-53), and a UV multiple wavelength detector (MD-1510). Utilizing a Peltier device (CO4062) to maintain the column temperature at 30 °C, the chromatographic separation experiment was carried out using a Luna C18 analytical column (150 × 4.6 mm inner diameter, 3 µm particle size, Phenomenex). The mobile phases were formic acid 0.1% in H_2O:ACN 95:5 (*v/v*) (phase A) and ACN (phase B). Using 38% A and 62% B with a flow rate of 1 mL per minute, the column was isocratically eluted. 275 nm was chosen as the ideal wavelength for the RX quantification.

2.5. Validation of the analytical method

RX and IS singular stock solutions were prepared in MeOH at the concentration of 1000 µg/mL, and then diluted to reach a final concentration of 100 µg/mL and stored at −20 °C. This last concentration was then diluted to the following concentrations: 10, 5, 2.5, 1, 0.5, 0.1, and 0.05 µg/mL, in order to prepare the calibration curve of RX in plasma. These RX concentrations *vs* the ratio of IS peak areas were used to create spiked curves. Based on the residual plot, fit test, and back calculation, the

linearity of the calibration curves in the 0.05–50 µg/mL for plasma range was evaluated. Six plasma samples spiked with IS at high (10 µg/mL), middle (1 µg/mL), and low (0.05 µg/mL) concentration standards were analysed using the same instrument and operator on the same day and three different days, respectively, to determine the intra-day and inter-day precision. These precision values were expressed as the (CV, %). Comparing the detector responses (in terms of areas) obtained for the extracted quality control samples and those for the pure standards dilutions allowed us to assess the drug recoveries. The recovery was expressed as mean (± SD). The LLOQ was established as the lowest plasma concentration that produced a signal to noise ratio of 5. The LOD was estimated as the plasma concentration that produced a signal to noise ratio of 3.

2.6. Pharmacokinetic and statistical analysis

The data were pharmacokinetically evaluated using a non-compartmental method (ThothProTM 4.3; ThothPro LLC, Poland). C_{max} and T_{max} were calculated directly from the concentration *vs* time curves. t1/2 was estimated using least squares regression analysis of the concentration-time curve. Using the linear trapezoidal rule, AUC_{last} was calculated. AUMC was calculated as $\int^{\infty}_{0}$ 0 C(t)dt. From these values, MRT (MRT = AUMC/AUC), and Cl (Cl =dose/AUC) were calculated. The individual value of AUC_{rest} was lower than 20% of $AUC_{(0-\infty)}$, and the R^2 of the terminal phase regression line was > 0.85. Values below the LLOQ were not considered for the PK analysis.

The PO and SC bioavailability were calculated using the following equation:

$$F\% = 100 \times \frac{AUC(SC\ or\ PO) \times Dose\ (IV)}{AUC(IV) \times Dose\ (SC\ or\ PO)}$$

MAT was calculated using the following equation:

$$MAT_{(PO\ or\ SC)} = MRT_{(PO\ or\ SC)} - MRT_{(IV)}$$

The extraction ratio for RX after IV administration was calculated for goats as the Cl divided by cardiac output, where cardiac output (mL/kg/min) was calculated as body weight (kg) to the power of -0.19 multiplied by 180 (Toutain and Bousquet-Mélou, 2004b).

$$E\% = \frac{\text{Body clearance}}{\text{Cardiac output}} = \frac{\text{Body clearance}}{180 \times \text{Body weight}^{-0.19}}$$

To determine statistically significant differences in PK variables between the three treatment groups, Bonferroni's multiple comparison test (repeated measures ANOVA) was used. The paired t-test was used to compare T_{max}, C_{max}, F%, and MAT between the SC and PO groups. A *p-value* $\prec$ 0.05 was considered statistically significant. GraphPad InStat was used for the analyses (GraphPad Software 5.3v).

3. RESULTS

3.1. Validation of the method

According to the EMA guidelines, the quantitative HPLC method was fully validated for goat's plasma in terms of linearity, intra-day and inter-day precision, selectivity, recovery, LOD, and LLOQ (Anonymous, 2012). The method's selectivity was tested for interference with blank plasma and spiked samples, and no peaks interfering with RX were found. With an R^2 of 0.999 (y = 0.1681x + 0.0113), the analytical method demonstrated optimal linearity. The mean extraction recovery was 89% ± 8% and the LOD and LLOQ were 0.01 and 0.05 µg/mL, respectively. A CV% lower than 14.9 and 3.72% was seen for the intra- and inter-day precision, respectively. The mean concentrations for the QCs and LLOQ samples were less than 15% of the nominal values.

3.2. Animals

Qualified veterinarians (B L-W; C-F) evaluated the health of the goats before, during, and after the study. Throughout the entire study period, the goats did not exhibit any noticeable immediate or delayed (up to 7 days) adverse effects, either locally or systemically.

3.3. Pharmacokinetics

The PK Analysis, as depicted in Figure 18, provides a semi-logarithmic representation of the mean (± SD) plasma concentrations of RX over time following IV, SC, and PO administrations. Notably,

RX remained quantifiable in plasma for up to 2 hours after IV administration and up to 6 hours following both SC and PO administrations.

Table 4 furnishes a comprehensive overview of the mean PK parameters, employing a non-compartmental model. The presented PK parameters are expressed as geometric means and ranges, with the exception of T_{max} (a categorical variable), which is denoted as the median value along with its range (Julious and Debarnot, 2000).

Upon IV administration, the mean calculated Cl of RX was relatively slow, measuring 0.52 L/h/kg, and the V_d was notably low at 0.24 L/kg. Remarkably, when considering the $AUC_{(0-\infty)}$ corrected for the dose, there were no statistically significant differences observed among the three administration routes.

The assessment of bioavailability revealed high values following both SC (98.02%) and PO (91.73%) administrations. The EV V_d values, when corrected for the calculated F %, were substantially greater in the SC (0.95 L/kg) and PO (1.71 L/kg) groups compared to the IV group (0.24 L/kg). Furthermore, the t1/2 was significantly shorter after IV administration (0.32 hours) compared to the extravascular routes (1.37 hours for SC and 1.63 hours for PO).

It is worth noting that MAT_{SC} and MAT_{PO} were higher than their respective t1/2 values. This discrepancy may indicate the presence of a flip-flop phenomenon for the extravascular routes, suggesting a complex interplay between absorption and elimination processes. Finally, The E ratio displayed an average of 8%.

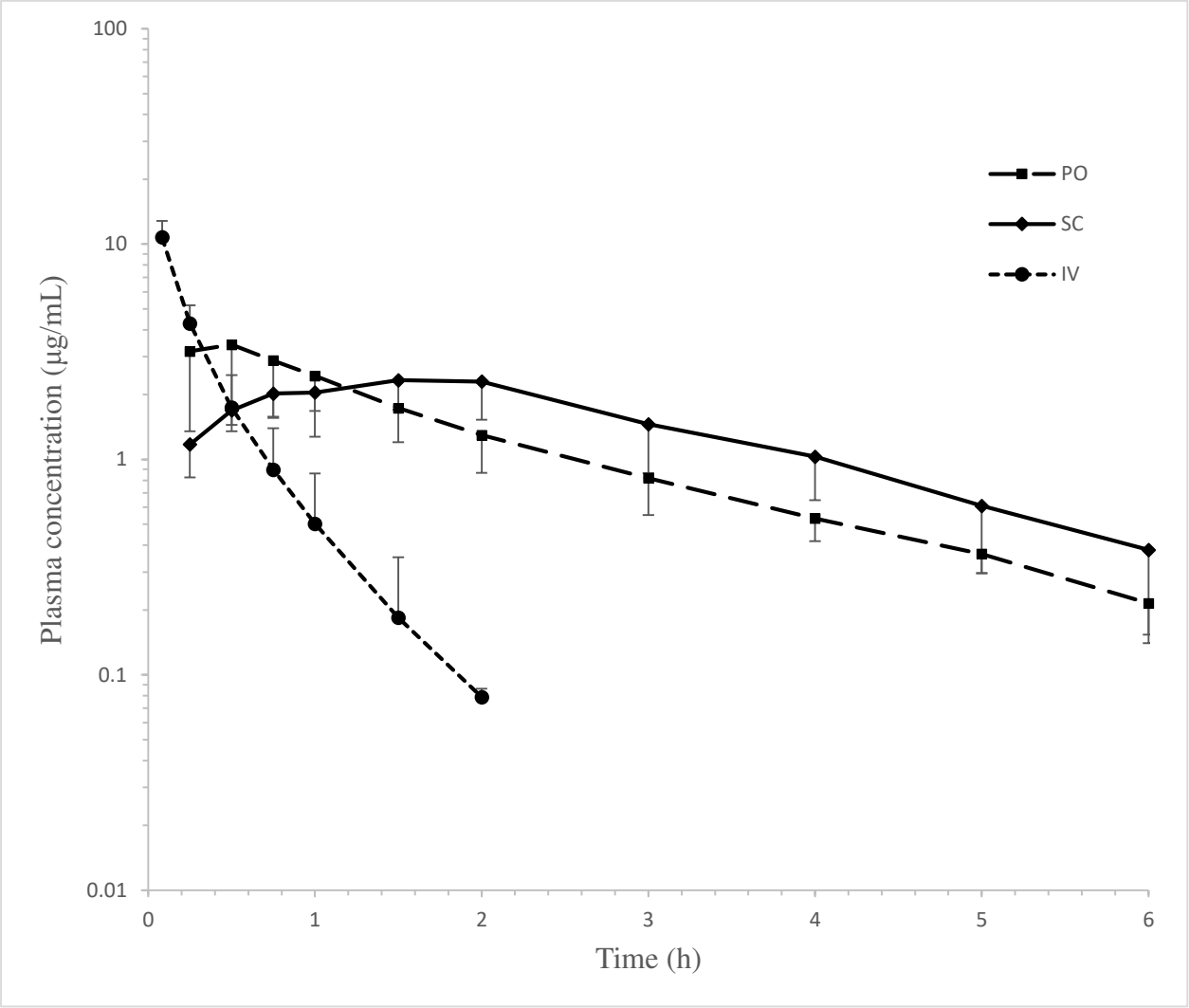


Figure 18: Semi logarithmic mean plasma concentration–time curves and standard deviations of robenacoxib following intravenous (2 mg/kg), subcutaneous (4 mg/kg) and oral (4 mg/kg) administrations in goats (n = 8).

2641 Table 4: Mean pharmacokinetic parameters and range of robenacoxib after single IV (2 mg/kg), SC

2642 (4 mg/kg), and PO (4 mg/kg) doses in goats (n = 8).

		IV			SC			PO		
Parameter	Unit	Geo mean	max	min	Geo mean	max	min	Geo mean	max	min
$AUC_{(0–t)}$	hr*ug/mL	3.78 [b,c]	5.97	2.46	7.75	10.09	6.23	6.42	9.88	4.11
$AUC_{(0–\infty)}$ D	hr*ug/mL	7.64	12.20	4.96	8.71	11.21	6.41	7.02	10.19	4.58
λz	1/hr	2.11[b,c]	3.43	1.32	0.50	0.86	0.25	0.42	0.62	0.31
t1/2	hr	0.32[b,c]	0.53	0.20	1.37	2.77	0.79	1.63	2.19	1.10
Cl[d]	L/hr/kg	0.52	0.8	0.32	0.49	0.69	0.31	0.7	0.15	0.42
V_d[d]	L/kg	0.24[b,c]	0.39	0.17	0.95	2.22	0.51	1.71	4.78	0.67
$MRT_{(0–t)}$	hr	0.25[b,c]	0.36	0.21	2.32[a,c]	2.84	1.80	1.81[a,b]	2.13	1.26
$MRT_{(0–\infty)}$	hr	0.28[b,c]	0.41	0.22	2.89	5.01	1.96	2.33	3.24	1.46
Cmax	µg/mL	_	_	_	2.34	2.95	1.35	3.34	7.47	2.15
Tmax[m]	hr	_	_	_	1.5[c]	2.00	0.75	0.50	0.75	0.25
F	%	_	_	_	98.02	120.46	76.73	91.73	123.00	57.70
MAT	hr	_	_	_	2.60	4.60	1.73	2.01	3.00	1.05

Note: $AUC_{(0–t)}$, area under the curve from 0 h to last time collected samples; $AUC_{(0–\infty)}$ D, area under the curve from 0 h to infinity normalized for the dose; λz, terminal phase rate constant; t1/2, terminal half-life; Cl, plasma clearance; V_d, volume of distribution; $MRT_{(0–t)}$, mean residence time from 0 h to last time collected samples; $MRT_{(0–\infty)}$, mean residence time from 0 h to infinity; C_{max}, peak plasma concentration; T_{max}, time of peak concentration; F, bioavailability; MAT, mean absorption time.

[a], statistically different from IV; [b], statistically different from SC; [c], statistically different from PO; [d], extravascular routes corrected for bioavailability; [m], Median value;

3. DISCUSSION AND CONCLUSION

To the best of the authors' knowledge, this is the first study which reports the PK of RX in goats. The current research aimed to investigate the pk of RX when administered IV, SC, and PO. Even though the IV route for RX is not recommended, it was critical to evaluate this route in order to determine true Cl, V_d, and absolute bioavailability for the EV administrations. As in the previous chapter, the IV dose was purposefully chosen lower than for the other routes of administration to reduce potential systemic toxicity and collateral effects. Although dose-independent PK cannot be completely ruled out in goats, RX PK was found to be dose-dependent with linear plasma drug concentrations in dogs (King et al., 2011; Schmid et al., 2010; Borer et al., 2017). No systemic or local adverse effects were observed following the various routes of administration of RX at a dose of 2-4 mg/kg in goats. It was the case as well in sheep (Fadel et al., 2022), dogs (Jung et al., 2019), cats (King et al., 2013), rabbits (Jeffrey et al., 2022), rats (King et al., 2009), and rainbow trout (Raulic et al., 2021).

After administering RX intravenously to goats, the observed V_d was relatively low, measuring 0.24 L/kg. This value is comparable to the V_d found in other animal species such as dogs (0.24 L/kg), cats (0.19 L/kg), and rats (0.3 L/kg), but notably higher than that reported in sheep (0.077 L/kg). In the context of NSAIDs, a low V_d is typically associated with a high degree of plasma protein binding (King et al., 2009; Sakai, 2009). Although the specific binding ratio of RX to plasma proteins in goats was not determined in this study, it is worth noting that at an RX concentration of 2 μg/mL, protein binding exceeded 98% in dogs and cats (Jung et al., 2009). While it is possible that a similar pattern exists in goats, further research is necessary to confirm this hypothesis. The differences in V_d values between goats and sheep could potentially stem from variations in the extent of plasma protein binding, the presence or absence of an enterohepatic drug cycle, or differences in body composition. Also, sheep and goats have distinct body sizes, with sheep typically being larger, which can impact the distribution of drugs within their bodies. Additionally, differences in tissue perfusion and the

extent of plasma protein binding in their respective circulatory systems can influence how RX is distributed throughout their tissues.

Despite the relatively low V_d in goats, it remained higher than the average blood volume in these animals, which typically ranges from 0.05 to 0.06 L/kg. This observation aligns with the notion that a significant portion of the drug tends to reside in the extracellular compartment (Lees et al., 2022), even though it is generally accepted that intra-cellular drug concentrations are important for drug efficacy and toxicity, as well as for predicting drug interactions and inter-subject variability in drug response (Chu et al., 2013). In any case, the selective distribution of RX to sites of inflammation has been shown in rats, dogs, and cats, and is due to its physicochemical nature as a weak acid (pKa 4.7). RX has a lengthy residence period in exudates (> 24 h), with a long duration of activity (King et al., 2009; Pelligand et al., 2012; Pelligand et al., 2014), which is unquestionably beneficial in clinical situations. This phenomenon may contribute to the observed differences in V_d and underscores the complexity of RX's PK behavior in different animal species.

In this study, Cl following IV administration of RX in goats was low (0.52 L/h/kg), comparable to that found in cats (0.44 L/kg/h; King et al., 2013), lower than that found in dogs (moderate; 0.81 L/kg/h; Schmid et al., 2010b), and substantially higher than that found in sheep (0.056 L/h/kg) and rats (0.14 L/h/kg). Species differences in the isoform composition, expression, and activities of biotransformation enzymes and the functions of excretory organs (Dantzler, 2016) may be the main reason behind the differences in Cl of RX in the different animal species. Additionally, the different cardiac output among species can cause to the species differences in Cl of RX (Toutain and Bousquet-Melou, 2004b). In fact, the estimated E for RX in goats found in the present study (8%) was similar to that found in cats and dogs, for which the range was between 5 and 15% (King and Jung, 2021; classified as low to moderate; Toutain and Bousquet-Melou, 2004b). In sheep, however, E was considerably lower (1 %). It would be interesting in our case to discuss such different results between small-ruminants. Indeed, goats have a more active metabolism and a higher elimination capacity than

sheep (and cattle…) (Wells, 2010; Aksit et al., 2015). This is linked to their respective feeding behavior; goats are natural browsers that can stand on their hind legs or even climb trees. They choose the most nutritious available food but also the portions of plants containing many toxic alkaloids that need to be metabolized by a heavy hepatic first pass effect. Whereas sheep are known as selective grazers, preferring to feed on grass and forbs. Thus, goats are better adapted to tolerate and detoxify plant toxins and exogenous compounds (such as drugs), compared with sheep. As a result, for the same dose of RX, sheep had substantially lower values of t1/2, $AUC_{(0-\infty)}$, and $MRT_{(0-\infty)}$, than goats. This was also demonstrated for a variety of different drugs when administered to both species, including albendazole (Aksit et al., 2015), oxfendazole (Bogan et al., 1987), levamisole-oxyclozanide combination (Gokbulut et al., 2014), ivermectine (Gokbulut et al., 2009a; Gokbulut et al., 2011), closantel (Hennessy et al., 1993). In these mentioned studies, it was assumed that phase I and phase II hepatic reactions were more prominent in goats. Nonetheless, knowing that RX is extensively metabolized by the liver in cats and dogs (Anonymous, 2008), it may be presumed that the higher rate of hepatic metabolism and a higher hepatic extraction ratio in goats resulted in the faster Cl of RX than in sheep, and therefore the lower t1/2 (0.32 hr vs 2.64 hr). In cats and dogs, RX is excreted predominantly via the biliary route (70%) rather than via the kidneys (30%), suggesting that the hepatic extraction ratio may be the main contributor to the overall extraction ratio rather than the renal extraction ratio. However, further research on the excretion and metabolism of RX in goats is required to confirm this.

The EV routes exhibited a 4-fold higher t1/2 than IV (1.37 hr SC; 1.63 hr PO; 0.32 hr IV), suggesting the occurrence of a flip-flop phenomenon. It refers to a scenario where the drug absorption rate is slower than its elimination rate, resulting in a longer duration of drug presence in the body despite a relatively short half-life. This can occur when drugs are delivered in sustained release dose forms, when they have a low intrinsic first-order absorption rate constant (k_a), or when they have a formulation with poor solubility, such as RX (Zornoza et al., 2006). If MAT is significantly longer

than MRT_{IV}, as it was in our case, this would confirm a flip-flop situation (Toutain and Bousquet-Mélou, 2004b). This is supported by the visual comparison of the terminal phase of the EV curves (λz) in figure 18, which are substantially lower than those of the IV plasma level (EV curves have a flatter decline), exhibiting significantly statistical differences ($p < 0.0001$; table 4) (Winter et al., 2022; Zornoza et al., 2006). Indeed, these higher t1/2 values for the EV routes reflect drug absorption and the absorption constant k_a rather than drug elimination (Cl and V_d) (Yáñez et al., 2011). The comparison of the terminal exponential phase after EV and IV administration also provides an easy way to detect a flip-flop phenomenon (Winter et al., 2022; Zornoza et al., 2006). However, referring back to the t1/2 differences, the significant difference in V_d values between IV and EV routes might have also triggered the t1/2 difference.

This inter-occasion variability in V_d for the same individuals can be caused by a variety of factors and was previously evidenced in several studies. First, due to technical circumstances, the washout interval between the IV and EV phases was four months. This period is lengthy, especially in the case of 5-month-old goats that are constantly growing and consequently undergoing physiological changes. The increase in V_d with age could be related to the different proportion of water and fat in the body and the development of the forestomachs (Waxman et al., 2004). In mammals, the proportion of body water is higher in young animals, while the proportion of fat increases with age. Consequently, a higher V_d for liposoluble drugs like RX would be anticipated in older goats, as was the case for marbofloxacin in goats (Bregante et al., 2000; Lüders et al., 2010). In fact, the distribution of most drugs in the body is influenced by many age-related factors including protein binding (plasma and tissue protein), fluid compartment sizes, the percentage of body fat, as well as hemodynamic factors such as cardiac output, regional blood flow, and membrane permeability (Eltom et al., 1993).

Second, the environmental changes might have influenced the values as well. There was a significant environmental temperature difference between the first phase (held in August at 35 °C) and the second and third phases (held in December at -15 °C). Large temperature differences have been reported to

affect the PK and PD of a drug (Johansson, 2001; Nies et al., 2012). Indeed, hypothermia and hyperthermia can directly affect the kinetics of a drug, which could have a major clinical relevance (Johansson, 2001). For instance, hepatic blood flow can vary over about a 4-fold range from half normal flow to twice normal flow (Nies et al., 2012). Vasoconstriction and vasodilation, which occur as well in reaction to changes in ambient temperature, can also affect the V_d.

To note, the t1/2 of RX in goats (0.32 hr) following IV treatment was significantly lower than in sheep (2.64 hr). This lower t1/2 might have been attributed to either a smaller distribution volume, which is not the case, or to faster Cl, which explains the situation as previously indicated.

The F values observed in this study were high (98.02% SC and 91.73% PO), above those of sheep (46% SC and 17% PO), dogs (88% SC and 84% PO), and cats (69% SC and 49% PO). This disparity between the values is thought to be due to species-specific differences (Toutain et al., 2010), such as: digestive tract physiology (differences in pH, transit times, and enzymatic activity), metabolism (first pass effect…), drug transporters (in the intestine, influx and efflux transporters), dietary habits, gut microbiota, and absorption sites.

The study's limitation is that no PD study was conducted. The reported IC_{80} for COX-2 by RX was 0.1049 µg/mL in cats, and 0.163 µg/mL in dogs, and RX doses used in these studies provided analgesia. In this study, RX plasma concentrations were maintained above the top mentioned IC_{80} for 1.5-2 hr IV, and 6 hr SC and PO. If it is presumed that goats and dogs have comparable COX-2 inhibitory concentrations, the doses studied in this research result in plasma concentrations that might provide therapeutic effects. The conversion of AUC to average plasma concentration, and thus the calculation of mean AUC (per hr), lends support to this supposition, as it was at least six times greater in goats than in dogs (Jung et al., 2009) and cats (Giraudel et al., 2009) (when time and doses were normalized).

The design of a parallel study rather than a cross-over study would be another limitation. Given the prolonged washout period and the impact on parameters, such as on V_d, a cross-over study would

have lessened the inter-individual variability. Moreover, the assessment of the MRL is crucial before widespread use of RX in goats intended for human consumption. Without tissue elimination data, one alternative for calculation of a preliminary withdrawal interval in food animal species is to multiply the terminal plasma t1/2 by 10 (Riviere and Sundolf, 2009; Smith, 2013). Thus, a conservative meat withdrawal interval of 2 days may be suggested.

4. Comparative PK of robenacoxib between goats and sheep, with a highlight on the possible indications

In summary, in comparison to the previous chapter, this study highlights a significant finding in the context of treating sheep and goats. Despite the common assumption that these two species share similarities, the PK parameters of a single drug can exhibit substantial differences between them. Recognizing these distinctions is essential for optimizing drug dosing and therapeutic outcomes. It underscores the importance of conducting PK investigations specifically in the target animal species, rather than relying on extrapolation from one species to another, which can yield inaccurate or unreliable results.

Regarding the suitability of RX for use in goats, especially for chronic treatments, it may pose challenges due to its relatively short half-life. Nonetheless, SC and PO routes offer practical options for occasional, one-time applications, such as peri-operative use. The reported prolonged duration of RX's effect in peripheral tissues (exceeding 24 hours) lends credibility to its peri-operative application, although further research is needed in this regard. If considering the EV routes of RX in goats, comprehensive investigations into its efficacy, safety profile, and tissue kinetics are imperative.

For both sheep and goats, the utilization of coxibs in sheep and goats may represent a crucial step forward in veterinary medicine, particularly given the escalating incidence of abomasal ulceration in these species. Abomasal ulceration in goats and sheep can arise from a multitude of factors, including the administration of non-selective NSAIDs, stress, dietary imbalances, infections, parasites, and genetic predispositions. To mitigate and prevent the risks associated with its occurrence, a

multifaceted approach is essential. When NSAID therapy is necessary, opting for a COX-2 selective NSAID, such as RX, becomes crucial. Unlike traditional NSAIDs, which can disrupt the protective lining of the abomasum and increase the risk of ulceration, COX-2 selective NSAIDs specifically target the enzymes responsible for inflammation, reducing the likelihood of gastrointestinal side effects. Nevertheless, judicious use, proper dosing, and veterinary oversight are paramount to ensure the safe and effective utilization of RX and minimize the risk of abomasal ulceration in these valuable livestock species.

CHAPTER V: Pharmacokinetics of Robenacoxib in Geese

1. INSIGHTS, IMPORTANCE OF GEESE IN AGRICULTURE, AND AIM OF THE STUDY

The avian industry, with a prominent focus on poultry, stands as one of the most extensive sectors within the global food industry. Despite the longstanding history of domesticated geese for commercial purposes, geese have traditionally been considered a minor species within this industry. This is primarily because their production rates have historically lagged behind other avian species like chickens and turkeys (Cilavdaroglu et al., 2020; Kozák et al., 2010).

Nevertheless, recent years have witnessed a significant expansion in goose production on a global scale, primarily driven by an increasing demand for goose products, especially in countries such as China, Hungary, Ukraine, Egypt, and Poland (Cilavdaroglu et al., 2020; Kozák et al., 2010). This surge in popularity can be attributed to several factors. Geese have emerged as a preferred choice among avian species due to their exceptional growth intensity and their remarkable ability to efficiently utilize green forages (Romanov, 1999).

Geese serve as valuable resources in various aspects of agriculture and industry. They are selectively bred to yield high-value products such as meat, fatty liver, eggs, and feathers (Hugo, 1995; Romanov, 1999). Additionally, geese play a crucial role in integrated farming systems by aiding in weed and pest control, thereby contributing to sustainable agricultural practices (Hugo, 1995).

As mentioned in the introduction, avian pain management is characterized by multiple challenges. Behaviour associated with painful stimuli is often subtle and not very specific in birds. Thus, the farmer's appreciation of the intensity of pain, as well as his familiarity with the normal behaviour of both animal species and individual birds in order to recognize signs of pain, is critical for the selection of an analgesic drug and its dosing regimen (Hawkins, 2006). According to numerous studies (Proudfoot and Hulan, 1983; Shlosberg et al., 1996; Thomas et al., 1966; McGeown et al., 1999), NSAIDs are effective for a wide range of clinical treatments in avian medicine and are used to reduce pain and inflammation of various origins, including musculoskeletal, visceral and postoperative pain.

Arthritis and degenerative joint disease are two of the most serious illnesses affecting waterfowl, particularly young geese (Degernes et al., 2011). The drug's PK processes, differ significantly between mammals and birds, as well as between different avian species. Some NSAIDs exhibit significant species differences in their primary PK properties, demonstrating that it is difficult to extrapolate PK data and posology from mammals to birds and between different bird species. Furthermore, different animal species, including in-between birds, may have very different NSAID safety profiles (Hawkins, 2006; Baert and De Backer, 2003).

A range of NSAIDs, including meloxicam, piroxicam, carprofen, ketoprofen, celecoxib, and mavacoxib, have been employed in the avian domain to manage pain and inflammation (Dhondt et al., 2017). However, it is important to note that their use in avian species goes beyond the approved labels for these drugs. The administration of this class of medications can have adverse effects on various physiological systems, notably impacting the gastrointestinal, renal, and hematopoietic systems. Among the deleterious effects associated with NSAID use in birds, nephrotoxicity emerges as the most frequently reported side effect (Jayakumar et al., 2010; Pereira and Werther, 2007; Zollinger et al., 2011). This issue has been particularly evident in some countries where the vulture population has experienced a decline. The decline has been attributed to the presence of NSAIDs like diclofenac and flunixin, which leave behind renal residues and ultimately lead to kidney failure in vultures (Toutain et al., 2010; Zorrilla et al., 2015).

However, it's noteworthy that certain NSAIDs, specifically tolfenamic acid and meloxicam, have demonstrated a higher level of safety in vultures, likely due to their selective inhibition of COX-2 (Turk et al., 2021). In light of this, coxibs may represent a potentially safer alternative for use in avian species and could be a more suitable option for managing pain and inflammation in birds while minimizing the risk of adverse effects. Thus, considering the well-established safety record of RX in various other species and the limited availability of PK data for NSAIDs in geese, often extrapolated

from different animal models, the primary objective of this study was to evaluate the PK of RX after both PO and IV administration.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

NaCl and pure powders of RX and diclofenac used as the IS with a standard purity of 99.0% were purchased from Sigma-Aldrich (Milan, Italy). ACN, MeOH, and formic acid were purchased from VWR chemicals (Oud-Heverlee, Belgium) in HPLC grade. With the aid of a Milli-Q Millipore Water System, deionized water was produced (Millipore, Darmstadt, Germany). The aqueous and organic components of the mobile phase were degassed under pressure and combined in the HPLC system. The mobile phases were filtered through 0.2 μm cellulose acetate membrane filters using a solvent filtration apparatus (Sartorius Stedim Biotech, Goettingen, Germany).

2.2. Animals and experimental design

In this research, a cohort of eight female geese, all four months of age, was randomly selected from a larger population. To ensure their eligibility for the study, comprehensive evaluations encompassing serum chemistry, physical examinations, and hematological analyses were conducted, confirming their good health status. Prior to commencing the study, these geese underwent a one-week acclimatization period in a spacious enclosure measuring 60 m^2, complete with an indoor shelter spanning 9 m^2.

Throughout the study, the geese were provided with a drug-free pelleted diet twice daily, and access to water was provided without restriction. Continuous monitoring of the geese's daily behavior and appetite was carried out to assess their well-being and adaptability to the study conditions. Notably, it's important to underline that the animal experiment adhered to ethical standards and was granted approval by the ethical committee of the Lebanese Ministry of Agriculture, as evidenced by the study protocol number 1120222. This ensured full compliance with applicable regulations and international animal welfare guidelines.

A meticulously structured two-phase research study was undertaken, involving two distinct dosage forms (2 mg/kg IV and 4 mg/kg PO), following an open, parallel design, with a washout period of four months.

In the initial phase, conducted in September 2022, a group of eight four-month-old geese was subjected to intravenous administration of 2 mg/kg of RX (Onsior®, concentration: 20 mg/mL). The injection was skillfully performed using a sterile 20-gauge needle measuring 3.75 cm, targeting the left-wing vein. During this phase, the geese displayed a range of body weight spanning from 3.40 to 4.30 kg, with an average of 3.72 kg.

Subsequently, in the second phase, which took place in December 2022, the geese were administered RX orally at a dosage of 4 mg/kg (Onsior®, tablet concentration: 20 mg/tablet) via crop gavage. The procedure involved the use of a rounded tip metal catheter. The RX tablets were diligently crushed, weighed, and divided to create the precise 4 mg/kg PO doses. Following dosage administration, the catheter was promptly flushed with 5 mL of water to ensure proper delivery. During this phase, the geese exhibited a range of body weights from 4.55 to 5.43 kg, with an average body weight of 5.10 kg.

To collect vital data for the study, blood samples (approximately 2 mL each) were obtained at specific time intervals: 0, 0.085 (exclusive to IV administration), 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, and 24 hours' post-administration. These blood samples were meticulously drawn from the right-wing vein via direct venipuncture. Heparinized tubes were used for the collection, followed by centrifugation at 1500 x g. The resultant plasma specimens were meticulously stored at a temperature of -20 °C and analyzed within a time frame of 10 days from the moment of collection.

2.3. Plasma robenacoxib determination

The sample preparation was determined using a published method (Jung et al., 2009), and it was modified according to the previous chapters. 50 mg of NaCl was added to 200 µL of plasma. The plasma was then spiked with 50 µL of an IS solution in MeOH (50 µg/mL). 800 mL of ACN was

then added. The samples were shaken at 60 oscillations per minute for 10 minutes after vigorous vortex mixing (30 sec) and then centrifuged at 4000 x *g* for 10 minutes. The upper layer was transferred into a clean tube and dried at 45 °C while being gently streamed with nitrogen. The residue was dissolved in 120 µL of ACN:H_2O 60:40 (*v/v*), vortexed for 1 minute, sonicated at 25 °C for 10 minutes, and then finally centrifuged at 4000 x *g* for 2 minutes. An aliquot of 50 µL of the upper layer was injected onto the HPLC system for analysis.

The LC Jasco HPLC system included an autosampler (AS2055), ternary gradient system (PU 980), in-line degasser (DG-2080-53), and a UV multiple wavelength detector (MD-1510). Utilizing a Peltier device (CO4062) to maintain the column temperature at 30 °C, the chromatographic separation experiment was carried out using a Luna C18 analytical column (150 × 4.6 mm inner diameter, 3 µm particle size, Phenomenex). The mobile phases were formic acid 0.1% in H_2O:ACN 95:5 (*v/v*) (phase A) and ACN (phase B). Using 38% A and 62% B with a flow rate of 1 mL per minute, the column was isocratically eluted. 275 nm was chosen as the ideal wavelength for the RX quantification.

2.4. Validation of the analytical method

RX and IS singular stock solutions were prepared in MeOH at 1000 µg/mL concentration, then diluted to a final concentration of 100 µg/mL and stored at -20 °C. This final concentration was then diluted to the following concentrations: 10, 5, 2.5, 1, 0.5, 0.1, and 0.05 µg/mL in order to prepare the calibration curve of RX in plasma. Spiked curves were created using these RX concentrations *vs* the ratio of IS peak areas. The linearity of the calibration curves in the range of 0.05-50 µg/mL for plasma was evaluated using the residual plot, fit test, and back calculation. Six plasma samples spiked with IS at high (10 µg/mL), middle (1 µg/mL), and low (0.05 µg/mL) concentration standards were analysed using the same instrument and operator on the same day and three different days, respectively, to determine the intra-day and inter-day precision. These precision values were expressed as the (CV %). We were able to assess drug recoveries by comparing the detector responses (in terms of areas) for the extracted quality control samples and those for the pure standards dilutions.

The recovery was calculated using the mean and ± SD. The LLOQ was established as the lowest plasma concentration that produced a signal to noise ratio of 5. The LOD was estimated as the plasma concentration that produced a signal to noise ratio of 3 (EMA, 2009).

2.5. Pharamcokinetic and statistical analysis

Using a non-compartmental method, the PK evaluation of the data was performed (ThothPro^{TM} 4.3; ThothPro LLC, Poland). The concentration *vs* time curves were used to directly calculate C_{max} and the T_{max}. By analysing the concentration-time curve using least squares regression, the t1/2 was calculated. The AUC was calculated by linear log trapezoidal for the IV administration and by the linear-up log-down rule for the oral administration. AUMC was calculated as $\int^{\infty}_{0} 0\ C(t)dt$. From these values, MRT (MRT = AUMC/AUC), and Cl (Cl =dose/AUC) were calculated. The individual value of AUC_{rest} was lower than 20% of $AUC_{(0-\infty)}$, and the square of coefficient of determination of the terminal phase regression line was > 0.85. Values below the LLOQ were not considered for the PK analysis.

The PO bioavailability were calculated using the following equation:

$$F\% = 100 \times \frac{AUC(PO) \times Dose\ (IV)}{AUC(IV) \times Dose\ (PO)}$$

For random inter-occasion Cl variability, the formula was corrected by the t1/2 (Wagner, 1967) using the following equation:

$$F\% = 100 \times \frac{AUC\ (PO) \times t1/2(IV)}{AUV\ (IV) \times \mathrm{t1/2(PO)}}$$

The MAT was calculated using the following equation:

$$\mathrm{MAT(PO)} = \mathrm{MRT(PO)\text{-}MRT(IV)}$$

The body extraction ratio for RX after IV administration was calculated using Cl/CO (Toutain and Bousquet-Melou, 2004b), where CO (mL/kg/min) was the cardiac output calculated according to the allometric equation in birds: $290.7 \times \text{body weight (in kg)}^{0.69}$ (Grubb, 1983; Waxman et al., 2019).

To determine statistically significant differences in PK variables between the two treatment groups, the paired t-test was used. A *p-value* < 0.05 was considered statistically significant. GraphPad InStat was used for the analyses (GraphPad Software 5.3v).

3. RESULTS

3.1. Analytical method validation

The analytical method exhibited excellent linearity, as indicated by an R-squared value of 0.99 and the equation y= 0.1817x+0.0121, over the concentration range of 0.05 – 50 µg/mL. The recovery rate was determined to be 87±8.2%. The LOD and LLOQ were established at 0.01 and 0.05 µg/mL, respectively. Impressively, the (CV, %) for both intra-day and inter-day precision was found to be below 13.8% and 3.19%, respectively. Furthermore, the mean concentrations of the QC samples and the LLOQ samples deviated by less than 15% from their nominal values, underscoring the method's reliability and accuracy. Furthermore, the HPLC method's reliability, accuracy, and precision are firmly established, affirming its appropriateness for quantitatively analysing RX in geese plasma. Notably, the method displayed remarkable robustness, maintaining consistent performance across a spectrum of conditions. These conditions encompassed factors such as the stability of the mobile phase's pH, its composition, temperature variations, solution stability, absence of instrument-related variability, and other pivotal parameters.

3.2.Animals

Qualified veterinarians (C-F; B L-W;) evaluated the health of the geese before, during, and after the study. Throughout the entire study period, the geese did not exhibit any noticeable immediate or delayed (up to 7 days) adverse effects, either locally or systemically.

3.3.Pharmacokinetics

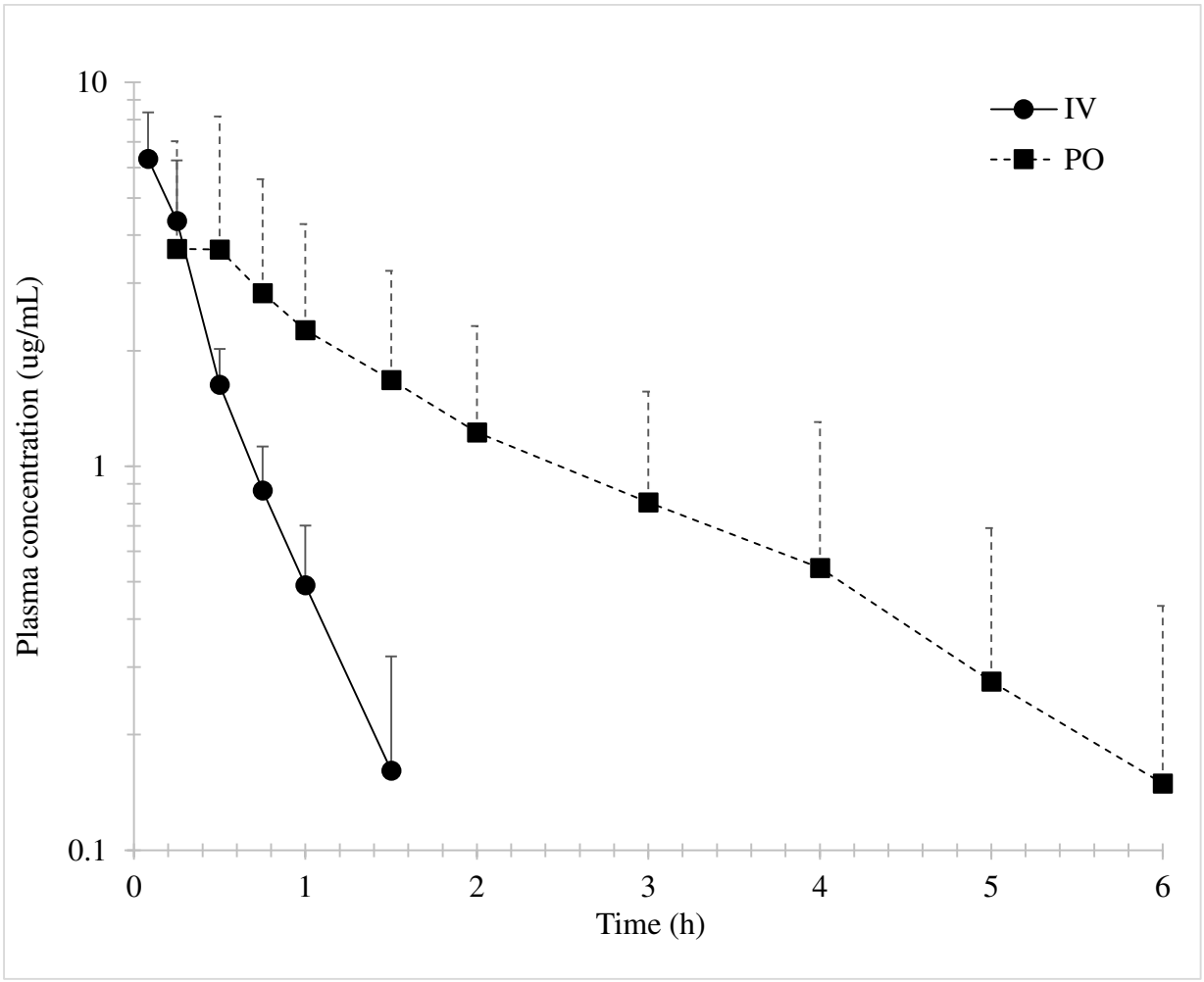
Figure 19 illustrates the semi-logarithmic representation of the mean plasma concentrations of RX (± SD) over time following single IV and PO administration. Quantifiable RX levels were observed up to 1.5 hours following IV administration and up to 6 hours following PO administration. In Table 5,

2971 we present the mean PK parameters derived from a non-compartmental PK model. With the exception
2972 of T_{max} (expressed as a median value and range), and t1/2 as a harmonic mean, the PK parameters of
2973 RX are depicted as geometric means and corresponding ranges, following the approach outlined by
2974 Julious and Debarnot (2000).

2975 Following IV administration, the mean Cl value was found to be moderate at 0.68 L/h/kg, while the
2976 V_d value was relatively low at 0.34 mL/kg. Notably, peak RX plasma concentration, reaching 6.78
2977 μg/mL, was achieved rapidly at 0.5 hours.

2978 In contrast, oral Cl (0.14 L/hr/kg), corrected for the fraction absorbed (F%), was significantly lower
2979 compared to the IV route (0.68 L/hr/kg). The oral bioavailability, as assessed through AUC
2980 calculations, exceeded 150%, whereas it was determined to be 46.44% using the t1/2 corrected
2981 formula.

2982 Additionally, the MAT following oral administration of 1.45 hours exceeded the oral t1/2 of 0.99
2983 hours. Moreover, the MRT for the PO route, at 1.86 hours, was notably higher than that observed for
2984 IV administration (0.37 hours), suggestive of the presence of a flip-flop phenomenon. Furthermore,
2985 the Ebody was determined to be low, with a geometric mean value of 1%.



2986 Figure 19: Semi logarithmic mean plasma concentration–time curves of robenacoxib following

2987 intravenous (2 mg/kg) and oral (4 mg/kg) administration in geese (n = 8).

Following IV administration, the mean Cl value was found to be moderate at 0.68 L/h/kg, while the V_d value was relatively low at 0.34 mL/kg. Notably, peak RX plasma concentration, reaching 6.78 µg/mL, was achieved rapidly at 0.5 hours.

In contrast, oral Cl (0.14 L/hr/kg), corrected for the fraction absorbed (F%), was significantly lower compared to the IV route (0.68 L/hr/kg). The oral bioavailability, as assessed through AUC calculations, exceeded 150%, whereas it was determined to be 46.44% using the t1/2 corrected formula.

Additionally, the MAT following oral administration of 1.45 hours exceeded the t1/2 of 0.99 hours. Moreover, the MRT for the PO route, at 1.86 hours, was notably higher than that observed for IV administration (0.37 hours), suggestive of the presence of a flip-flop phenomenon. Furthermore, the Ebody was determined to be low, with a geometric mean value of 1%.

Table 5: Mean pharmacokinetic parameters and range after single IV (2 mg/kg) and PO (4 mg/kg) doses of robenacoxib in geese (n = 8)

			IV			PO	
Parameter	Unit	Geo mean	max	min	Geo mean	max	min
$AUC_{(0–t)}$	hr*ug/mL	2.8*	3.86	1.89	12.12	25.93	3.92
$AUC_{(0–\infty)}$ D	hr*ug/mL	5.85*	8.22	4.14	12.6	27.62	4.5
λz	1/hr	1.96*	2.63	0.89	0.74	1.163	0.44
$t1/2^h$	hr	0.35*	0.77	0.26	0.99	1.55	0.75
Cl^c	L/hr/kg	0.68*	0.96	0.48	0.14	0.11	0.41
$V_d{}^c$	L/kg	0.34	0.59	0.21	0.19	0.26	0.03
$MRT_{(0–t)}$	hr	0.3*	0.45	0.18	1.66	1.86	1.41
$MRT_{(0–\infty)}$	hr	0.37*	0.71	0.28	1.86	2.46	1.54
C_{max}	µg/mL	_	_	_	6.78	15.94	2.23
$T_{max}{}^m$	hr	_	_	_	0.5	1	0.25
F	%	_	_	_	46.44	133.72	21.1
MAT	hr	_	_	_	1.45	2.13	1.01

Note: $AUC_{(0–t)}$, area under the curve from 0 h to last time collected samples; $AUC_{(0–\infty)}$ D, area under the curve from 0 h to infinity normalized for the dose; λz, terminal phase rate constant; t1/2, terminal half-life; Cl, plasma clearance; V_d, volume of distribution; $MRT_{(0–t)}$, mean residence time from 0 h to last time collected samples; $MRT_{(0–\infty)}$, mean residence time from 0 h to infinity; C_{max}, peak plasma concentration; T_{max}, time of peak concentration; F, bioavailability; MAT, mean absorption time.

*, statistically significant from PO; ^{m}, Median value; ^{c}, oral route corrected for bioavailability; ^{h}, harmonic mean.

4. DISCUSSION AND CONCLUSION

No systemic or local adverse effects were observed following IV and PO administrations at a dose of 2-4 mg/kg in geese, as it was the case in sheep, goats, dogs (Jung et al., 2019), cats (King et al., 2013), rabbits (Jeffrey et al., 2022), rats (King et al., 2009), and rainbow trouts (Raulic et al., 2021).

In the context of avian species, drug administration can take various forms, with individual and flock therapy being common approaches. Among these, the utilization of drinking water and feed medication techniques has traditionally been prevalent. However, it's noteworthy that for this specific study, these methods were not deemed suitable due to several limitations. These limitations encompassed variations in drug intake among geese, imprecise dosing accuracy, and solubility challenges (Powers, 2006; Turk et al., 2021; Vermeulen et al., 2002). In contrast, parenteral medication represents an alternative route of drug delivery, offering the advantage of rapid onset of action, especially in cases involving critically ill birds. Indeed, when precision in dosing and stability are critical considerations, oral gavage emerges as a preferred choice (Flammer, 1994; Powers, 2006; Vermeulen et al., 2002).

It's worth noting that although the IV route for administering RX is generally discouraged, it played a pivotal role in this study. This choice was driven by the necessity to accurately determine essential PK parameters such as Cl, V_d, and the absolute fraction absorbed for the oral route. To mitigate the risk of systemic toxicity and potential side effects, the IV dose was deliberately set at a lower level compared to the oral dose (King et al., 2011; Schmid et al., 2010; Borer et al., 2017). It's important to emphasize that both the IV and PO dosages fell within the therapeutic ranges recommended for cats and dogs, as per the guidelines established by the European Medicines Agency or EMA (EMA, 2008).

Following IV administration, V_d was low (0.34 L/kg), and was comparable to that in dogs (0.24 L/kg), cats (0.19 L/kg), goats (0.24 L/kg), rats (0.3 L/kg), and higher than that in sheep (0.077 L/kg). As discussed in the previous chapters, NSAIDs are characterized by a small V_d, due to the high binding

to serum albumin. Indeed, RX (2 μg/mL) protein binding exceeded 98% in dogs and cats (Jung et al., 2009). Given the similar V_d, it may be the case as well in geese. Inopportunely, plasma protein binding was not assessed in this study. The discrepancies in V_d values between geese and sheep may be explained by differences in body temperature and body components (fat/water partition) (Dorrestein, 1991; Toutain et al., 2010). Additional factors contributing to the observed differences in V_d values between geese and sheep may include variations in organ perfusion rates, metabolic activities, and tissue composition. Organ-specific characteristics, such as blood flow to adipose tissue and the affinity of the drug for specific tissues, could also play a role in influencing the distribution patterns.

The Cl of RX following IV administration in geese was determined to be at a moderate level, specifically measuring 0.68 L/hr/kg. This Cl value was notably higher compared to several other animal species, such as sheep (0.056 L/hr/kg; Fadel et al., 2022), rats (0.14 L/hr/kg; King et al., 2009), and slightly exceeded that observed in cats (0.44 L/hr/kg; King et al., 2013) and goats (0.52 L/hr/kg). These variations in RX Cl across different animal species can be attributed to inherent species-specific differences in isoform composition, expression, and enzymatic activities of biotransformation enzymes, as well as differences in excretory organ functions (Dantzler, 2016).

Birds, in general, are recognized for their relatively rapid Cl rates compared to larger-sized mammals, primarily due to their elevated rate-specific metabolic rate. Moreover, birds possess proportionally larger excretory organs relative to their body size (Frazier et al., 1995). However, it's noteworthy that the extent of RX distribution within the body (Eb_{ody}) in geese was determined to be notably low at 1%. This observation might suggest a limited capacity for geese to efficiently eliminate RX (Toutain and Bousquet-Melou, 2004b). Additionally, comparing E_{body} with mammals might not be the best strategy considering the species differences between birds (allometric growth curve) and mammals.

Furthermore, the extensive metabolism of RX by the liver in cats and dogs (EMA, 2008) may not necessarily apply to geese. Although biotransformation enzymes are widely distributed among avian species, our knowledge regarding their specific functions remains limited, and avian excretory organs

exhibit distinct physiological and anatomical differences compared to those in mammals (Dorrestein, 1991; Toutain et al., 2010; Vermeulen et al., 2002). Consequently, further in-depth investigations are warranted to explore and elucidate the specific mechanisms of RX metabolism and elimination in geese and other avian species.

Regarding the t1/2, a notable difference was observed between the oral and IV routes of RX administration. Specifically, the t1/2 was significantly longer following oral administration (0.99 hours) compared to the IV route (0.35 hours). This difference may be attributed to the presence of a flip-flop phenomenon, which can occur in formulations with limited solubility, such as RX (Zornoza et al., 2006). The occurrence of a flip-flop phenomenon can be confirmed when the MAT is greater than the MRT following IV administration, which was indeed the case in this study (MAT of 1.45 hours > MRT_{IV} of 0.37 hours) (Yáñez et al., 2011). This phenomenon has also been suggested to occur in previous studies involving cats, dogs, and rats, given their short T_{max} values ranging from 0.25 to 1.5 hours, similar to the T_{max} observed in this study (0.5 hours) (Lees et al., 2022).

Another significant factor that may directly impact the t1/2 is the Cl, which was found to be significantly different between the IV and oral routes in the same individuals. This inter-individual variability in Cl could potentially be attributed to the extended washout interval period of four months, necessitated by technical constraints. This period is particularly extensive for four-month-old geese, which are in a phase of continuous growth and physiological changes. In fact, the growth-dependent decrease in drug elimination has been well-documented in animals. Ontogeny and maturation of drug-metabolizing pathways in the livers of young animals may have contributed to the faster Cl. Raidal et al. (2013) proposed that Cl was increased in younger animals because they have a relatively higher abundance of biotransforming enzymes when liver volume was normalized to body weight (Blanco et al., 2000; Burgos-Vargas et al., 2004). Additionally, in younger animals characterized by lower plasma protein concentrations, a higher proportion of the drug exists in a free, unbound form, rendering it more readily available for renal excretion (Toutain and Bousquet-Melou,

2004b). Furthermore, distinct urinary pH conditions favorable for drug excretion were observed in younger animals, and this was supported by an augmented GFR in comparison to adults (Gonda et al., 2003; Savage, 2008; Fadel et al., 2023).

Comparing the t1/2 values in this study after IV administration (0.35 hours) with other species revealed variation. It was similar to that observed in goats (0.32 hours) but lower than values reported in dogs (0.69 hours), cats (1.49 hours), sheep (2.64 hours), and rats (1.9 hours). It's well-established that the t1/2 for many NSAIDs can vary significantly between species (Hawkins, 2006). Indeed, in line with previous research on meloxicam, celecoxib, and mavacoxib, this study reaffirms that dose extrapolation is not a suitable method for determining dosage and posology in avian species due to inter-species differences in PK values (Baert and De Backer, 2003; Dhondt et al., 2017).

When the fraction absorbed (F%) was calculated using the conventional equation, the resulting values were found to be abnormal, exceeding 150%. It's crucial to note that when determining absolute F, a substantial error can occur if the concentration curves for the IV and PO routes correspond to different Cl rates (Rescigno, 2000). This discrepancy arises because the AUC is directly proportional to the fraction absorbed only under the condition of constant Cl and uniform concentration. In cases where these conditions are not met, determining F% solely through AUC comparisons becomes impractical (Rescigno, 2000).

Indeed, the $AUC_{(0-\infty)}$ for the oral route was significantly higher than that for the IV route, even after normalizing for the dose administered. To account for the inherent random variability in inter-occasion Cl, it has been suggested to adjust the computed F% by considering the elimination half-life (Wagner, 1967; Toutain and Bousquet-Melou, 2004a). Consequently, the calculated oral F% was determined to be moderate at 46%, closely resembling the F% observed in cats (49%), substantially exceeding that in sheep (16%), yet falling below the range observed in dogs (62-84%) and rats (80%). It's important to consider that anatomical and physiological distinctions in the digestive tract, as well

as variations in the levels of efflux proteins contributing to intestinal barrier function, may contribute to these differences, in addition to species-specific factors (Turk et al., 2021).

While the T_{max} of 0.5 hours may initially suggest rapid absorption, it's important to highlight that this short T_{max} could be more indicative of a flip-flop PK profile, as previously noted (Lees et al., 2022).

This study acknowledges several noteworthy limitations that should be considered when interpreting its findings. Firstly, it's important to recognize that the extended duration of the washout period, while necessary for practical and technical reasons, introduced certain constraints. This extended washout period was primarily dictated by the study's longitudinal design, which was chosen due to technical limitations. A cross-over study design, while reducing both intra- and inter-individual variability, was not feasible under the given circumstances. Consequently, this prolonged washout period may have potentially impacted the study's outcomes.

A second limitation to note is the absence of a PD investigation within the scope of the study. The lack of a comprehensive assessment of PD effects represents a notable drawback. Specifically, the determination of the IC_{80} for COX-2 inhibition would have provided valuable insights into the relationship between RX plasma concentrations in geese and their potential to elicit analgesic and anti-inflammatory effects, a crucial aspect of the drug's pharmacological profile (Warner et al., 1999).

In the context of geese, achieving effective therapeutic outcomes with RX may present some challenges, primarily owing to its relatively short t1/2. Unless administered frequently, the drug's efficacy in this avian species could be limited. However, it's worth noting that the oral form of RX may emerge as a compelling option for occasional use. This consideration stems from observations in other animal species where, despite exhibiting short elimination half-life values similar to those observed in geese, RX has proven to be well-suited for once-daily administration. As discussed in previous chapters, this is due to the prolonged residence time/accumulation of RX in inflammatory exudates, and to some specific PD characteristics, such as receptor binding kinetics, target engagement, and negative hysteresis effect, that could contribute to the observed once-daily

suitability despite a short t1/2. This allows RX to provide an extended duration of peripheral action, allowing it to maintain therapeutic effectiveness over longer intervals between doses.

CHAPTER VII: Pharmacokinetics of Deracoxib in Sheep and Goats

1. INSIGHTS AND AIM OF THE STUDY

Breeding goats and sheep carries significant socio-economic implications for human populations, particularly in rural and developing areas. While these animals have been utilized for milk, meat, coat, and skin for millennia, their popularity has surged further more in recent times. Currently, there are more than 2.2 billion sheep and goats in the world (FAO, 2019). In the Middle East, the production of small ruminants plays a crucial role in the livelihoods of many farmers, contributing to 28-58% of agricultural output (Hosri et al., 2016). As an example, in Lebanon, the responsibility for this production lies predominantly with small-scale farmers operating in marginal lands (Hosri and El Khoury, 2004; MOA, 2009). Similarly, in most parts of the world, small ruminants are primarily raised by small-scale farmers, outside specialized production systems. These animals are considered minor species in Western countries and North America, resulting in a limited number of licensed drugs for them. Thus, many drugs, particularly NSAIDs, are used in an off-label manner (Clark, 2013; Matthews, 2016).

Given the high event of adverse effects caused by non-selective NSAIDs, compounds were developed that would reduce pain and inflammation while posing less risk to the patient. These drugs, the coxibs, selectively inhibit COX-2 while sparing COX-1. Among coxibs, DX is a highly COX-2 selective drug, approved for use in dogs to treat musculoskeletal and post-operative pain and inflammation. Recent literature has explored the use of some coxibs in ruminants and small-ruminants, such as RX and firocoxib (Fadel et al., 2023; Fadel et al., 2022; Stuart et al., 2019; Wilson et al., 2017; Wasfi et al., 2015; Stock et al., 2014). One potential advantage of employing coxibs may be the prospective to prevent the occurrence of abomasal ulceration.

Abomasal ulceration is a multifactorial disease of many ruminant species, as well as a common cause of morbidity and mortality. Abomasal ulcers can be found in ruminants of all ages and production systems (Hund and Wittek, 2018; Vatn and Ulvund, 2000). Clinical signs, ranging from mild (anorexia/hyporexia) to severe (acute death), are often vague and challenging to definitively interpret

as indicators of abomasal ulceration (Fladung et al., 2022). In goats and sheep, it can arise from a multitude of factors, including the administration of NSAIDs, stress, dietary imbalances, infections, parasites, and genetic predispositions. To address and prevent the associated risks, a multifaceted approach is essential. One strategy recently gaining prominence involves the administration of proton pump inhibitors, a practice increasingly observed in ruminants. Recent literature has focused on investigating the PK and PD of proton pump inhibitors such as pantoprazole, esomeprazole, and omeprazole in sheep, goats, and cattle, elucidating their potential in treating abomasal ulceration (Smith et al., 2021; Fladung et al., 2022; Olivarez et al., 2020; Morgado et al., 2022; Smith et al., 2023). Simultaneously, substituting non-selective NSAIDs with coxibs, with the potential to reduce the likelihood of gastrointestinal side effects, may be an effective measure to prevent the occurrence of ulcers.

To the authors' knowledge, no prior studies have investigated DX in small-ruminants. Therefore, considering the limited availability of medications for pain management in small ruminants, this study seeks to characterize the PK of DX after a single oral dose in sheep and goats.

2. MATERIALS AND METHODS

2.1.Chemicals and Reagents

The pure standard powders of DX and tolbutamide as the IS with a purity of 99.0%, alongside NaCl, were purchased from Sigma-Aldrich (Milan, Italy). HPLC-grade ACN, MeOH, and formic acid were obtained from VWR chemicals (Oud-Heverlee, Belgium). Deionized water was produced using a Milli-Q Millipore Water System (Millipore, Darmstadt, Germany). The mobile phase's aqueous and organic components were combined in the HPLC apparatus after being degassed under pressure. With the aid of a solvent filtration device, the mobile phases were filtered through 0.2 μm cellulose acetate membrane filters (Sartorius Stedim Biotech, Goettingen, Germany).

2.2.Animals and Experimental Design

Five healthy male goats and five healthy male sheep, aged between 12 and 16 months and weighing 25–30 kg, were included in the study. They were housed in stalls with straw bedding and had *ad libitum* access to feed and water. The health status of the goats and sheep was confirmed through a physical examination, hemogram, and serum chemical profile conducted within three days of initiating the study. The animals had not received any recent pharmacological treatments within the last two months, and they were free from parasites. The animal experiment was approved by the Lebanese Ministry of Agriculture ethical committee, verifying that this study complies with European standards for animal welfare guidelines (study protocol number 0920233).

2.3. Drug Dosing, Administration and Blood Sample Collection

This trial employed the commercial oral tablets formulation of 75 mg DX each (Deramaxx®, Novartis, Switzerland). Sheep and goats were administered two tablets each, totalling 150 mg, followed by the sequential administration of 10 mL of water to facilitate tablet swallowing. Blood samples were obtained from the left jugular vein at specific time points (0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, and 48 hr) using vacutainer lithium heparin tubes (BD, Vaud, Switzerland). Subsequently, the collected blood was subjected to centrifugation at 1500 *x g* for 10 min. The resulting plasma was separated, transferred into cryovials, and stored at -20 °C. Plasma samples were analysed within a two-week time frame.

2.4. Plasma Deracoxib Determination

The analytical methodology was developed in our lab. It underwent comprehensive and extensive validation in accordance with the guidelines set forth by the EMA (EMA, 2012). To enhance the ionic strength of water, 50 mg of NaCl was introduced into 700 µL of plasma. Subsequently, the plasma was fortified with 70 µL of an IS solution in MeOH at a concentration of 50 µg/mL. Extraction was achieved by adding 3 mL of ACN. Following vigorous vortex mixing (30 sec) and subsequent shaking at 60 oscillations per minute for 10 min, the samples underwent centrifugation at 4000 *x g* for 13 min.

The resulting upper layers were carefully transferred to clean tubes and subjected to drying at 45 °C under gentle nitrogen stream. The resultant residue was reconstituted in 350 µL of mobile phase and vortexed for 30 sec. An 80 µL aliquot was then injected onto HPLC system for subsequent analysis.

The LC Jasco HPLC system included an autosampler (AS2055), ternary gradient system (PU 980), in-line degasser (DG-2080-53), and a UV multiple wavelength detector (MD-1510). Utilizing a Peltier device (CO4062) to maintain the column temperature at 30 °C, the chromatographic separation experiment was carried out using a Luna C18 analytical column (150 × 4.6 mm inner diameter, 3 µm particle size, Phenomenex). The mobile phase consisted of dihydrogen potassium phosphate 10 mM adjusted to pH 4.0 and ACN (45:55 *v/v*). With a flow rate of 0.8 mL/min, the column was isocratically eluted. DX (and NSAIDs generally) is commonly quantified using C18 columns in HPLC due to the favorable properties of these columns for separating and analyzing non-polar and moderately polar compounds like DX. C18 refers to the stationary phase of the column, which is composed of octadecylsilane-bonded silica particles. This phase is known for its hydrophobic interactions, making it suitable for drugs with lipophilic characteristics, such as DX. The reversed-phase C18 column allows for effective separation and retention of DX based on differences in hydrophobicity, aiding in accurate and reliable quantification during HPLC analysis.

For the DX quantification, 252 nm was chosen as the optimal wavelength. DX's detectability with UV light in HPLC is attributed to its intrinsic chemical properties and the presence of a chromophore. DX contains a diazenyl substituent that acts as a chromophore, capable of absorbing UV light at specific wavelengths, allowing for its quantification based on the intensity of the absorbed light. This characteristic makes UV detection a suitable method for assessing the concentration of DX in pharmaceutical formulations or biological samples, providing a reliable means for PK and PD studies.

2.5.Validation of the Analytical Method

Singular stock solutions of DX and IS were initially prepared in MeOH at a concentration of 1000 µg/mL, which were subsequently diluted to achieve a final concentration of 100 µg/mL and stored at

−20 °C. Further dilutions were performed to obtain concentrations of 10, 5, 1, 0.5, 0.1, 0.05 and 0.025 µg/mL, facilitating the creation of a calibration curve for DX in plasma. These concentrations of DX, in conjunction with the ratio of IS peak areas, were employed to generate spiked curves. The linearity of the calibration curves within the 0.025–2.5 µg/mL range for plasma was assessed through residual plot analysis, fit testing, and back calculation.

For precision evaluation, six plasma samples spiked with IS at high (5 µg/mL), middle (1 µg/mL), and low (0.025 µg/mL) concentrations were analyzed using the same instrument and operator on both the same day and three different days to determine intra-day and inter-day precision. The precision values were expressed as the (CV, %). Drug recoveries were assessed by comparing detector responses (in terms of areas) from the extracted quality control samples to those from pure standards dilutions, and the recovery was presented as mean (± SD).

The LLOQ was defined as the lowest plasma concentration producing a signal-to-noise ratio of 5, while the LOD was estimated as the plasma concentration resulting in a signal-to-noise ratio of 3 (EMA, 2012).

2.6. Pharmacokinetic Analysis

Using a non-compartmental method, the PK evaluation of the data was performed (PKanalix™ R1; 2023). The concentration *vs* time curves were used to directly calculate the C_{max} and T_{max}. By analysing the concentration-time curve using least squares regression, the t1/2 was calculated. The AUC was calculated by the linear-up log-down rule. The AUC_{rest} for each individual was less than 20% of $AUC_{(0-\infty)}$, and the R^2 of the terminal phase regression line was greater than 95 %.

Statistical analysis for significant differences in PK variables between the two animal groups employed the unpaired t-test, with statistical significance set at a *p-value* < 0.05. GraphPad InStat was used for the analyses (GraphPad Software 5.3v).

3. RESULTS

3.1. Validation of the Method:

The method's selectivity was confirmed through analysis of blank plasma and spiked samples, with chromatograms revealing no observed peaks interfering with DX or IS, as seen in figure 20. Stability, robustness, precision, and accuracy were also demonstrated, attesting to the method's reliability and performance across various parameters.

The analytical method demonstrated optimal linearity, with R^2 of 0.997 ($y = 0.2951x + 0.0793$). The LOD and LLOQ were 0.01 and 0.025 µg/mL, respectively, and the mean extraction recovery was 89.35 ± 5.82%. The inter- and intra-day precision showed a CV% lower than 10.67 and 5.89 %, respectively. The mean concentrations of the quality control and LLOQ samples were less than 15% and 20 % of the nominal values, respectively.

3.2.Animals

Qualified veterinarians (C F; B L-W) evaluated the health of the animals before, during, and after the study. Throughout the entire study period, the small-ruminants did not exhibit any noticeable immediate or delayed (up to 7 days) adverse effects.

3.3. Pharmacokinetics

The plasma concentrations of DX in sheep and goats following oral administration are presented in Figure 21, depicting the mean (± SD) values at the respective sampling time points. The presence of DX was quantifiable in plasma up to 48 hr in all goats and in four out of five sheep. In that one remaining sheep, DX was detectable, below the LLOQ however.

Table 6 displays the mean PK parameters based on non-compartmental method. Apart from T_{max}, which was expressed as the median value and range, and t1/2 which was expressed as the harmonic mean, the PK parameters of DX have been presented as geometric means and ranges.

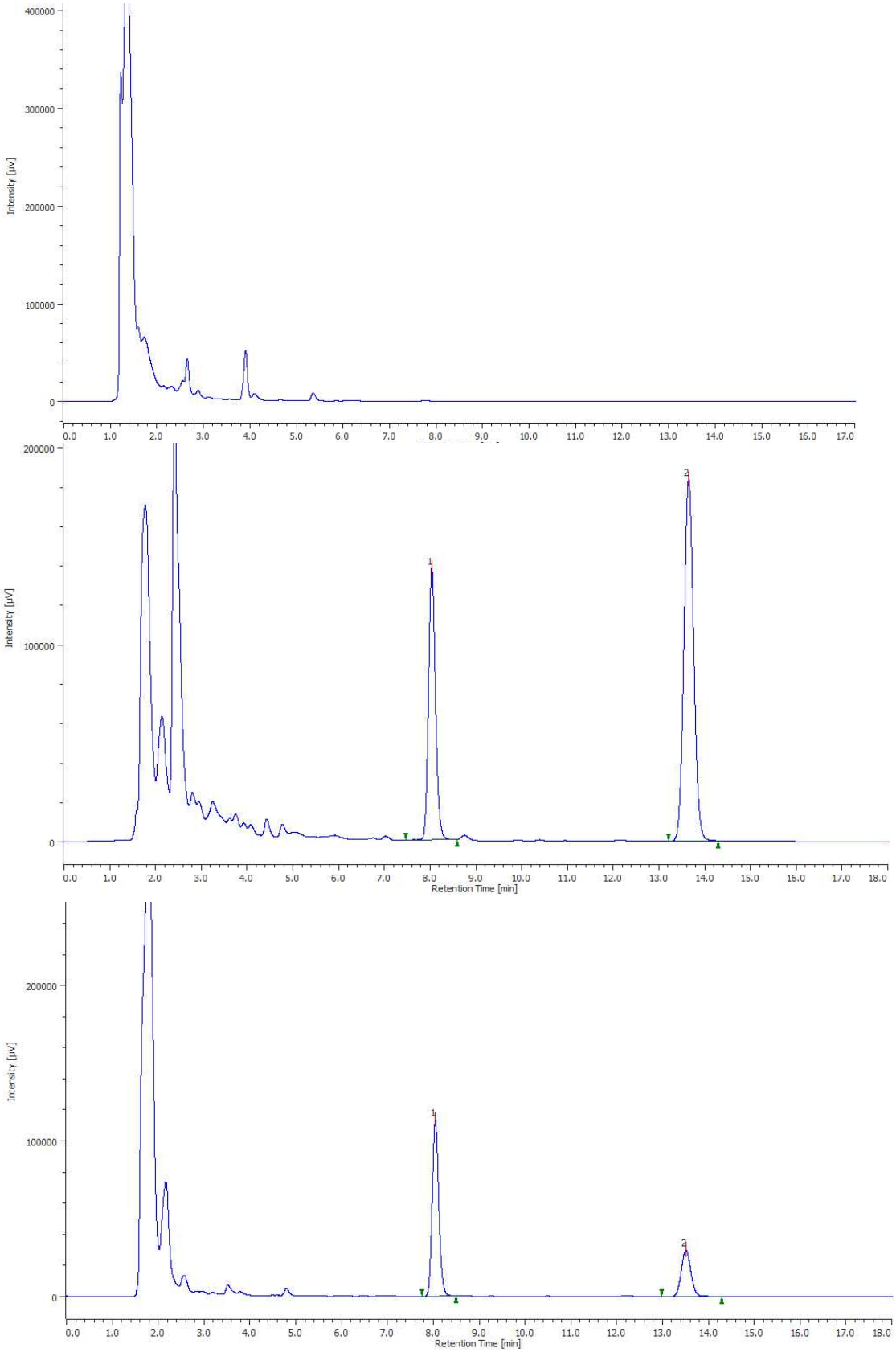


Figure 20: 1) chromatogram of control plasma (blank); 2) Chromatogram of spiked plasma sample IS (50 ppm) and DX (10 ppm); 3) Chromatogram of the plasma sample collected from a treated goat at 4 hours after oral administration.

No statistically significant differences were observed in any of the PK parameters between sheep and goats. Notably, there was considerable individual variability within both species.

4. DISCUSSION AND CONCLUSION

This investigation represents the inaugural exploration into the PK of DX in sheep and goats, aiming to elucidate the disposition kinetics, systemic exposure, and safety of this NSAID in these species. The study design incorporated a free-grazing regimen with *ad libitum* access to food and water, mirroring natural feeding patterns in farm settings (Stuart et al., 2019). This approach ensures the relevance of research findings to practical agricultural scenarios. Although the effectiveness of DX tablets under fed and fasted conditions has been established in dogs (Deramaxx® package insert), extrapolating these findings to ruminants requires a delicate exploration.

An ideal anti-inflammatory and analgesic medication for both companion animals and production livestock necessitates attributes such as safety, ease of administration, efficient absorption, and a prolonged half-life, allowing for less frequent dosing (Stuart et al., 2019). Notably, the administered DX dose of two tablets of 75 mg each orally induced no immediate or delayed adverse effects in either sheep or goats (Deramaxx® package insert; Gassel et al., 2006; Davis et al., 2011; Fadel et al., in press). Similar or lower DX doses have been deemed safe in various species, including dogs (Deramaxx® package insert), cats (Gassel et al., 2006), horses (Davis et al., 2011), and geese (Fadel et al., in press).

In this study, the dose normalized per body weight fluctuated around 5 to 6 mg/kg per animal, remaining below 8 mg/kg. Doses surpassing this threshold in dogs have been associated with non-linear kinetics and potential competitive inhibition of COX-1 (Deramaxx® package insert). Thus, the justification for administering such a dose in this study could be warranted within its specific context.

Despite inherent physiological differences between sheep and goats that have manifested significantly different PK profiles for many drugs in previous literature, both exhibited comparable

disposition kinetics of DX. This is evident in similar systemic drug exposures, elimination rates, and analogous C_{max} and T_{max} values (Toutain et al., 2010). While a non-statistically significant difference in the t1/2 values existed between sheep (16.66 hr) and goats (22.86 hr), this variance was attributed to individual variability within each group. Recognizing the potential impact of the study's limited sample size on statistical robustness is crucial, as biological diversity in drug disposition is inherent even within a single species (Gassel et al., 2006).

The relatively long t1/2 values observed in sheep and goats, 16.66 hr and 22.86 hr, respectively, exceeded those reported for other species, including dogs (3 hr; Deramaxx® package insert), cats (7.9 hr; Gassel et al., 2006), geese (6.3 hr; Fadel et al., in press), and horses (12.49 hr; Davis et al., 2011). Potential explanations for these variations encompass differences in V_d or Cl, requiring further exploration through IV studies. Consideration of the predominant hepatic biotransformation route for DX in canines raises the possibility of enzyme saturation and contributes to the previously reported non-linear kinetics. Enzyme concentration variations among species, lower in other animals than in dogs, may lead to saturation at lower concentrations, extending the half-life (Kim and Giorgi, 2013; Davis et al., 2011). Additionally, species differences in the expression of biotransformation enzymes and the functions of excretory organs could contribute to these differences (Dantzler, 2016). Another plausible explanation might be a prolonged absorption phase, possibly indicative of a flip-flop phenomenon, as suggested by Davis et al. (2011), due the relatively longer T_{max} in horses (6.33 hr) compared to cats and dogs. Once again, this theory remains unproven without IV data.

In an in vitro canine whole blood assay, the IC_{50} and IC_{80} values of COX-2 by DX were determined as 0.16 µg/mL and 0.39 µg/mL, respectively (McCann et al., 2004). In the present study, mean plasma concentrations remained below the IC_{80} but consistently above the IC_{50} for at least 10 hr in both sheep and goats. Assuming a comparable COX-2 inhibitory concentration in sheep, goats, and dogs, the experimentally tested doses in this study may not result in plasma concentrations potentially yielding optimal clinical effects (Giorgi et al., 2016). It is important to note that while whole blood assays are

3321 valuable, they may not completely mimic *in vivo* physiological traits. In addition, COX-2 inhibitory
3322 concentrations can vary between species (Kim and Giorgi, 2013). Further PD assessments specific to
3323 sheep and goats are warranted.

3324 In summary, this study explored oral DX PK in sheep and goats, revealing a well-tolerated dose and
3325 a lack of adverse effects (Deramaxx® package insert; Gassel et al., 2006; Davis et al., 2011; Fadel et
3326 al., in press). DX exhibited comparable disposition kinetics, reflected in a comparable systemic drug
3327 exposure in both species. DX manifested a relatively long t1/2, a favorable asset for reducing
3328 frequency of administration. Notably, a consistent pattern of high individual variability was observed
3329 within both species, mirroring findings in cats and dogs (Gassel et al., 2006). This observation
3330 emphasizes the significance of acknowledging and accommodating individual variations in response
3331 to DX treatment in sheep and goats rather than relying solely on species-based considerations, as if
3332 the subjects belonged to the same species, a perspective supported by both comparable PK parameters
3333 and uniform individual variability (Giorgi et al., 2016). While the extended half-life of DX may
3334 appear promising in practical applications, a comprehensive evaluation of its profile necessitates
3335 further investigations, including PD assessments and multiple-dose studies in these species.

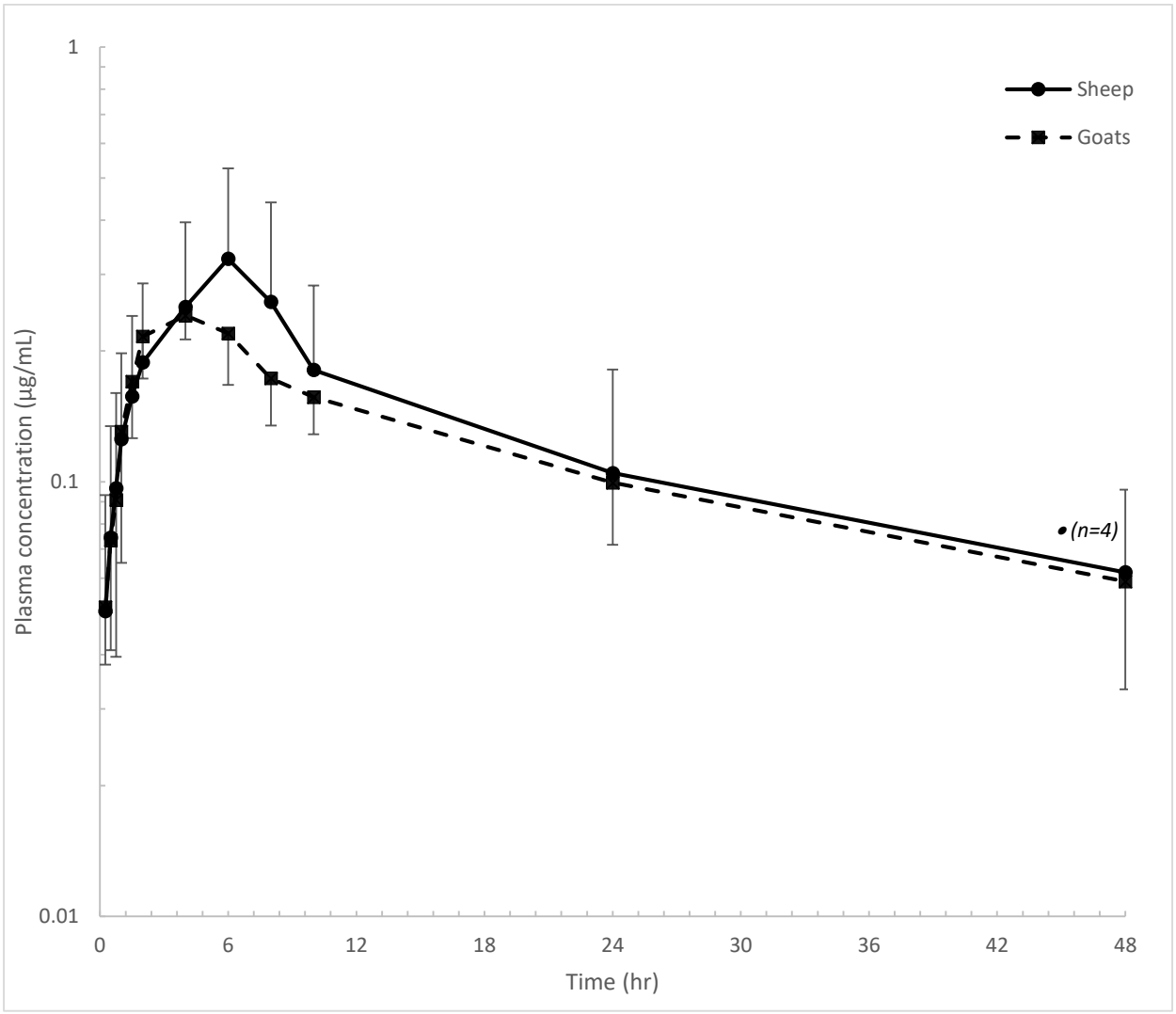


Figure 21: Semi-logarithmic mean plasma concentration–time curves and standard deviation of deracoxib (150 mg/animal) following oral administration in sheep (n=5; n=4 at 48 hr) and goats (n=5).

Table 6: Mean geometric pharmacokinetic parameters of deracoxib and range in sheep (n=5) and goats (n=5) after a single oral dose of 150 mg/animal.

		Sheep			Goats		
Parameter	Unit	Geo mean	max	min	Geo mean	max	min
$AUC_{(0–t)\ D}$	kg·h/mL	0.93	0.21	0.53	0.87	0.11	0.68
$AUC_{(0–\infty)\ D}$	kg·h/mL	1.2	2.6	0.7	1.3	1.8	0.78
λz	1/hr	0.042	0.068	0.03	0.03	0.042	0.02
$t1/2^{h}$	hr	16.66	22.83	10.18	22.86	33.82	16.37
$MRT_{(0–t)}$	hr	14.28	18.24	10.69	16.39	20.28	11.34
$MRT_{(0–\infty)}$	hr	24.85	32.22	17.45	33.37	48.96	23.05
$C_{max\ D}$	µg/mL	0.06	0.11	0.039	0.046	0.05	0.043
T_{max}^{m}	hr	6	6	4	4	6	2

Note: $AUC_{(0–t)\ D}$, area under the curve from 0 hr to last time collected samples normalized per dose; $AUC_{(0–\infty)\ D}$, area under the curve from 0 hr to infinity normalized per dose; λz, terminal phase rate constant; t1/2, terminal half-life; $MRT_{(0–t)}$, mean residence time from 0 hr to last time point of samples collection; $MRT_{(0–\infty)}$, mean residence time from 0 hr to infinity; $C_{max\ D}$, peak plasma concentration normalized per administered dose; T_{max}, time of peak concentration; ^{h}, harmonic mean; ^{m}, Median value.

CHAPTER VIII: Pharmacokinetics of Deracoxib in Geese

1. INSIGHTS AND AIM OF THE STUDY

In contemporary veterinary practice, the integration of pain management has arisen in response to heightened animal welfare imperatives and societal pressures. This extends to avian species as well, where pain management presents challenges due to subtle and non-specific behavioral signs. Studies suggest NSAIDs effectively treat avian inflammation and pain of various origins, including musculo-skeletal, visceral and post-operative pain (Proudfoot and Hulan, 1983; Shlosberg et al., 1996; McGeown et al., 1999; Fadel et al., 2023). In young geese for instance, arthritis and degenerative joint disease are two of the most serious illnesses (Degernes et al., 2011). While numerous drug profiles have been primarily established in poultry and other bird species, the PK of NSAIDs not only differ between mammals and birds but also among various bird species (Baert and De Backer, 2003). This variability poses challenges for extrapolation. Moreover, notable differences in safety profiles exist among different animal species (Hawkins, 2006).

Various classical NSAIDs, including meloxicam, piroxicam, carprofen, and ketoprofen, have been used off-label in birds for inflammation and pain (Dhondt et al., 2017). Despite the reported efficacy, the gastro-intestinal, renal, and hematopoietic systems are all affected by the toxic effects of this class of medications. Nephrotoxicity is the most frequently reported classical NSAID side effect in birds (Pereira and Werther, 2007; Zollinger et al., 2011). The decline in some bird populations in some regions is attributed to some classical NSAIDs such as diclofenac and flunixin, causing renal failure. On the contrary, tolfenamic acid and meloxicam, demonstrating COX-2 preferential selectivity, have shown safety in birds (Zorrilla et al., 2015) Hence, more specifically targeted drugs, such as coxibs, might offer an even more secure alternative in avian species compared to other classical NSAIDs.

DX, as mentioned in the previous chapters, is a highly COX-2 selective coxib (COX-1/COX-2 = 1275), approved for use in dogs to treat musculo-skeletal and post-operative pain and inflammation (Kim and Giorgi, 2013). Due to its favorable safety profile, and limited or extrapolated NSAIDs' PK

data in geese from other species, this study aimed to evaluate the PK of DX after a single oral administration in geese.

The animal experiment was approved by the Lebanese ministry of Agriculture ethical committee, verifying that this study complies with appropriate regulations and animal welfare international guidelines (study protocol number 0920233).

2. Materials and Methods

2.1. Animals, Drug Administration, and Blood Collection

A cohort of six healthy female geese, displaying weights within the range of 4.1 to 4.9 kg, was selectively chosen for this experiment. These geese were thoughtfully provided with a diet comprising drug-free pelleted feed and granted unrestricted access to water ad libitum, mirroring natural feeding conditions. The administration of DX at a dosage of 4 mg/kg was carried out through oral means, utilizing a meticulous approach involving crop gavage with a rounded-tip metal cannula. The DX tablets, each containing 25 mg of the active substance, underwent a thorough preparation process, involving grinding, precise weighing, partitioning, and suspension in water to achieve a concentration of 20 mg/mL.

To ensure accuracy, each goose received a specific volume of this suspension tailored to attain the desired 4 mg/kg dose. Following the administration, the cannula was thoroughly flushed with 3 mL of water to guarantee the complete delivery of the dosage. Blood samples were systematically collected from the right-wing vein via direct venipuncture at various time points, specifically at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, and 24 hr post-administration. The blood was carefully collected in heparinized tubes and subjected to centrifugation at 1500 *x g*. The resulting plasma was judiciously stored at -20 °C and analyzed within a span of 10 days, ensuring the integrity of the collected samples for subsequent PK assessments. This comprehensive methodological approach aimed to capture a detailed profile of the PK of DX in the geese population under investigation.

2.2. Plasma Deracoxib Determination, Pharmacokinetics Analysis and Statistics

As described in the previous chapter, plasma concentrations of DX were meticulously quantified using a HPLC system coupled to a UV detector, specifically set at 252 nm to ensure optimal sensitivity. The chromatographic separation process employed a Luna C18 analytical column with dimensions of 150 x 4.6 mm and a particle size of 3 µm, providing an ideal platform for effective analyte separation. The mobile phase, a critical component of the analytical setup, comprised dihydrogen potassium phosphate at a concentration of 10 mM, adjusted to a pH of 4.0, and ACN in a volumetric ratio of 45:55 (*v/v*). This chromatographic method adhered to stringent validation criteria in accordance with the EMA guidelines.

In the preparation of the plasma samples for analysis, a systematic and validated protocol was followed. Specifically, 50 mg of NaCl, 5 µL of formic acid, and 70 µL of a 500 µg/mL IS solution were meticulously added to 700 µL of plasma. The subsequent drug extraction process involved the addition of 3 mL of ACN, followed by a sequence of vortex mixing, oscillation, and centrifugation. The upper layer, containing the extracted analyte, was carefully transferred, subjected to a drying process under nitrogen, and then reconstituted in 350 µL of the mobile phase.

The analytical method exhibited notable linearity and reproducibility within the concentration range of 0.01 to 2.5 µg/mL. The LLOQ was determined to be 0.01 µg/mL, ensuring the sensitivity of the assay in detecting low concentrations of DX. To ascertain the precision and accuracy of the assay, five replicates spanning various analyte concentrations were meticulously examined. The accuracy consistently demonstrated values below 10.70%, except at the LLOQ (0.01 µg/mL), where it measured 18.23%. Precision values were consistently below 9.48%, attesting to the reliability and robustness of the analytical method.

For the PK evaluation, a non-compartmental approach utilizing PKanalix^{TM} R1 software (2023) was employed. Direct calculations of maximum plasma concentration and the corresponding time to reach it were derived from concentration vs. time curves. The t1/2 was determined through the application

of least squares regression, providing insights into the drug's persistence in the systemic circulation. The AUC was calculated using the linear-up log-down rule, providing a comprehensive measure of the drug exposure over time. Ensuring the robustness of the analysis, the AUC_{rest} for each individual was consistently below 20% of $AUC_{(0-\infty)}$, and the R^2 for the terminal phase regression line, calculated on at least three time points, surpassed 95%. These stringent criteria underscored the reliability and accuracy of the PK assessments, ensuring the fidelity of the obtained results.

3. RESULTS AND DISCUSSION

Figure 22 provides a comprehensive visual representation in the form of a semi-logarithmic plot, detailing the mean (± SD) plasma concentrations of DX over time subsequent to a single oral administration. DX was consistently quantifiable at all time-points, offering a robust dataset for subsequent PK analysis. Apart from Tmax, which was expressed as the median value and range, and t1/2 which was expressed as the harmonic mean, the PK parameters of DX have been presented as geometric means and ranges, in table 7.

This pioneering study stands as the inaugural report on the PK properties of oral DX in geese. Notably, the administration of DX at a dose of 4 mg/kg orally in geese demonstrated an absence of both systemic and local adverse effects, not only during the immediate post-administration period but also throughout the subsequent 7-day observational period. This robust safety profile aligns with the favorable safety characteristics established in other animal species, particularly in dogs, at the recommended doses.

It is indeed imperative to underscore the alignment of the administered dose in this study with the recommended range in dogs, reinforcing the scientific justification for its application in geese. The observed prolonged half-life in dogs at doses exceeding 8 mg/kg, as indicated in the Deramaxx® package insert, underscores the potential for dose-dependent variations in t1/2 across species. Additionally, the potential risk of competitive inhibition of COX-1, associated with higher-than-

recommended doses, is a concern that warrants attention in geese, mirroring considerations in other animal species (Deramaxx® package insert).

The determined t1/2 in geese from this study was approximately 6.3 hours, presenting a distinctive temporal characteristic. Notably, this duration surpassed that reported for dogs (3 hours, Deramaxx® package insert) and was comparatively shorter than values reported in cats (7.9 hours; Gassel et al., 2006) and horses (12.49 hours; Davis et al., 2011). The observed variations in t1/2 may be indicative of differences in the V_d or Cl among these species. Nevertheless, a definitive assessment necessitates the implementation of an intravenous study to comprehensively elucidate the drug's PK behavior.

The median T_{max} in this study was notably 1 hour, in contrast to 2 hours in dogs, 3.6 hours in cats, and 6.3 hours in horses (Deramaxx® package insert; Gassel et al., 2006; Davis et al., 2011). This variance may be attributed to several factors, including distinct feeding conditions, site of absorption, gastric pH, transit time through the gastrointestinal tract, and other related variables, as postulated by Baert and De Backer (2003).

An exploration of the peak plasma concentration revealed intriguing patterns. Dogs achieved a C_{max} of 1.33 µg/mL with an oral dose of 3–4 mg/kg, surpassing reported Cmax values for horses (0.54 µg/mL) at 2 mg/kg and cats (0.28 µg/mL) at 1 mg/kg (Davis et al., 2011; Gassel et al., 2006). The closely aligned C_{max} values in dogs and the present study (1.29 µg/mL) suggest consistent drug behavior across these species.

Moreover, in an in vitro canine whole blood assay, the IC_{50} and IC_{80} values of COX-2 by DX were determined as 0.16 µg/mL and 0.39 µg/mL, respectively (McCann et al., 2004). In the present study, mean plasma concentrations remained consistently above the IC_{80} for more than 10 hr, and above the IC_{50} for at least 24 hr. Assuming a comparable COX-2 inhibitory concentration in geese and dogs, the experimentally tested doses in this study may result in plasma concentrations yielding optimal clinical effects (Giorgi et al., 2016).

In conclusion, the comprehensive investigation into the administration of DX at an oral dose of 4 mg/kg in geese yielded a noteworthy absence of both systemic and local adverse effects. The findings presented in this study elucidate a PK profile in geese characterized by a discerned t1/2 of approximately 6.3 hours, complemented by a median T_{max} of 1 hour. These observed temporal parameters suggest a moderated and relatively swift elimination of DX in geese, emphasizing its potential suitability for occasional, peri-operative use, in the context of geese veterinary care. The implications of these PK characteristics underscore the significance of further research endeavors to holistically comprehend the efficacy and suitability of DX for various applications in geese. Specifically, a nuanced understanding of its COX-2 selectivity and protein binding characteristics, tailored to the unique physiology of geese, is imperative for informed decision-making in veterinary practices before approval for use.

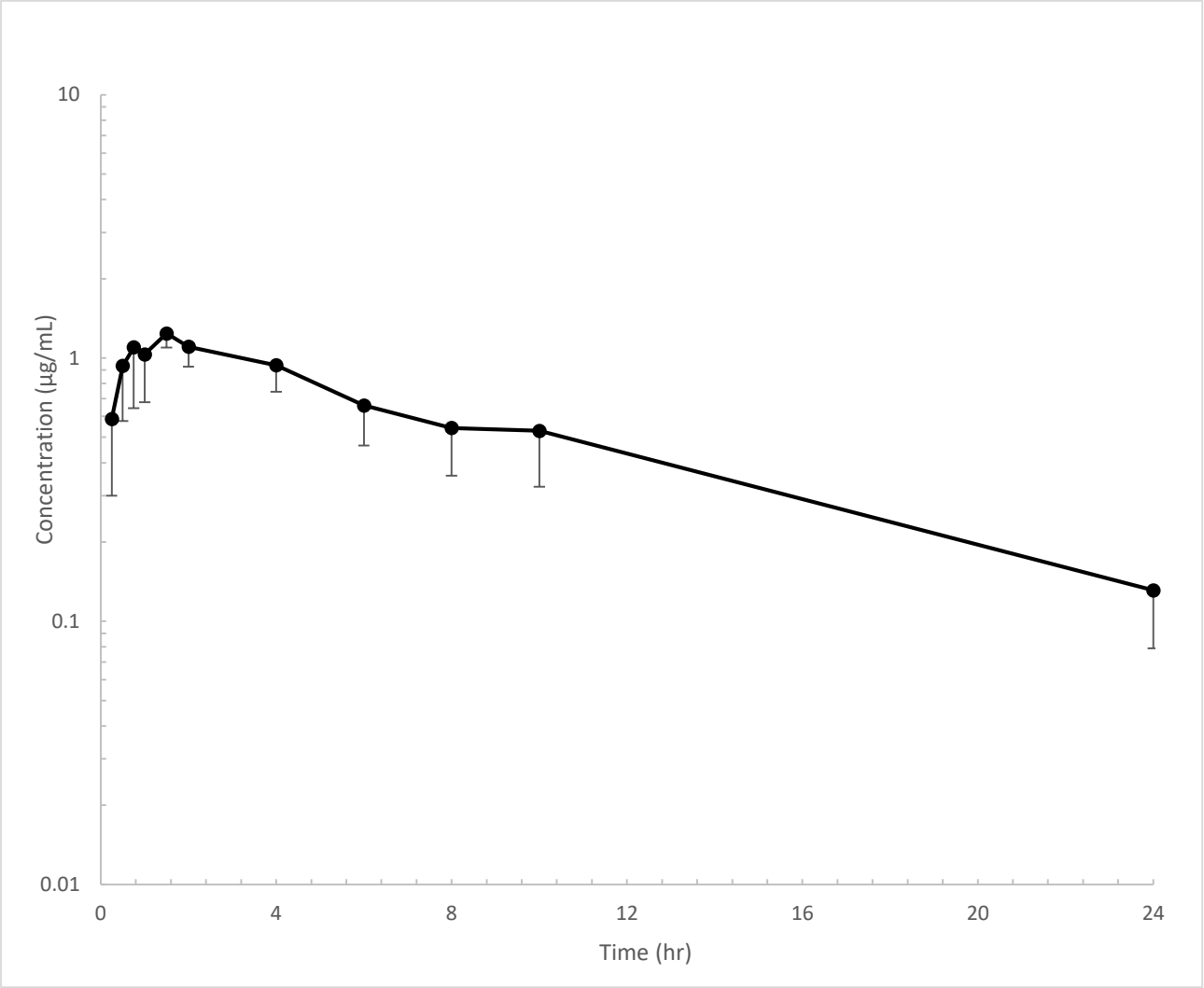


Figure 22: Semi-logarithmic mean plasma concentration–time curves and standard deviation (bars) of deracoxib (4 mg/kg) following oral administration in geese (n = 6).

Table 7: Mean pharmacokinetic parameters of deracoxib and range in geese (n = 6) after a single oral dose (4 mg/kg).

Parameter	Unit	Geometric mean	max	min
$AUC_{(0–t)}$	hr*ug/mL	10.90	13.81	5.25
$AUC_{(0–\infty)}$	hr*ug/mL	12.27	16.32	6.22
λz	1/hr	0.110	0.180	0.078
$t1/2^{h}$	hr	6.30	8.92	3.90
$MRT_{(0–t)}$	hr	7.05	8.69	3.61
$MRT_{(0–\infty)}$	hr	9.52	13.11	5.48
C_{max}	µg/mL	1.29	1.62	1.11
T_{max}^{m}	hr	1.00	2.00	0.75

Note: $AUC_{(0–t)}$, area under the curve from 0 hr to last time collected samples; $AUC_{(0–\infty)}$, area under the curve from 0 hr to infinity; λz, terminal phase rate constant; t1/2, terminal half-life; $MRT_{(0–t)}$, mean residence time from 0 hr to last time point of samples collection; $MRT_{(0–\infty)}$, mean residence time from 0 hr to infinity; C_{max}, peak plasma concentration; T_{max}, time of peak concentration; ^{h}, harmonic mean; ^{m}, Median value.

Prior to delving into the examination of projects beyond the scope of the thesis topic, I would like to take a moment to showcase captivating images from the farm setting. The gratification of working with animals is priceless; it brings a big joy, and our drive to advance the field of pharmacology for these creatures serves as our primary motivation.

Figure 23: Experimental field settings in the farm.

The second acknowledgment is dedicated to all my wonderful colleagues and friends, whose exceptional collaboration made even the most challenging days feel manageable**.**

OVERVIEW OF SUPPLEMENTARY PROJECTS UNDERTAKEN

Aside from my thesis research on coxibs, various other projects, encompassing both original research articles and reviews, were initiated. These projects delved into diverse drugs and drug categories, spanning a wide spectrum of animal species. While the primary focus was on advancing the field of pain management strategies, we also ventured into other areas of veterinary pharmacology. These endeavors aim to contribute significantly to the field of veterinary pharmacology, with the ultimate goal of enhancing the well-being and welfare of animals. Thoroughly gathered and analyzed data from PK studies have the potential to revolutionize veterinary medicine, offering tailored treatments for various species and improving outcomes for farm and companion animals. These studies play a critical role in obtaining regulatory approval for drugs. Additionally, PK studies also encourage the development of drugs tailored for minor species by pharmaceutical companies, addressing gaps in treatment options for exotic pets, wildlife, and agricultural animals. In summary, the meticulous approach to PK studies not only advances scientific understanding but also accelerates regulatory approval processes and encourages the creation of a more diverse range of effective veterinary drugs. The projects listed below include some that have been previously published, with others scheduled for publication in the near future (2024):

1- Paracetamol: A Focus on Dogs

Reference: **Fadel, C.**, Sartini, I., & Giorgi, M. (2021). Paracetamol: A focus on dogs. *American Journal of Animal and Veterinary Sciences*, *16*(4), 247-262. https://doi.org/10.3844/ajavsp.2021.247.262

Paracetamol (APAP), known as an aniline analgesic, antipyretic, and non-narcotic, holds a significant place in human medicine, being widely used and recognized as an essential drug. In veterinary medicine, its application extends to many countries under extra label use, with exclusive usage in certain animals, including dogs. The mechanism of action of APAP mirrors that of NSAIDs, but it

also possesses unique characteristics setting it apart from other medications in its class. Numerous studies focusing on APAP in dogs have been published since its introduction into clinical practices. These studies have delved into various aspects, such as PK, PD, effectiveness, and toxicity, especially in cases of inadvertent or accidental overdosing. When administered at therapeutic doses, APAP has demonstrated its potency as a powerful analgesic and antipyretic agent in dogs, even showcasing some anti-inflammatory effects. However, it necessitates careful handling and cautious usage.

At doses below 100 mg/kg, APAP exhibits no side effects, making it relatively safe within this range. Typically recommended therapeutic levels, falling between 10 and 20 mg/kg, have proven effective in managing postoperative pain in dogs. Interestingly, APAP can serve as an alternative to NSAIDs, particularly when NSAIDs are contraindicated. Additionally, it finds utility in combination with opioids and in opioid-free anesthesia surgery protocols, showcasing its versatility in pain management strategies. Moreover, research has revealed that APAP exhibits cardioprotective and anti-arrhythmic effects in dogs, although these effects require further detailed exploration for a comprehensive understanding. This multifaceted nature of APAP highlights its potential as a valuable tool in veterinary medicine, albeit one that demands careful consideration and ongoing investigation to fully uncover its range of applications and ensure safe usage in canine patients.

2- Gabapentin in Cattle: A Pharmacology Snapshot

Reference: **Fadel, C**., Sartini, I. & Giorgi, M. (2022). Gabapentin in Cattle: A Pharmacology Snapshot. American Journal of Animal and Veterinary Sciences, 17(3), 187-197. https://doi.org/10.3844/ajavsp.2022.187.197

Gabapentin (GBP), a medication derived from gamma-aminobutyric acid, serves as a versatile antiepileptic and analgesic drug. Its multifaceted properties have made it an essential component in multimodal pain management strategies. Additionally, GBP is employed off-label as an anticonvulsant and anxiolytic in veterinary medicine, particularly gaining popularity in oral prescriptions for cattle.

Since its integration into cattle farm practices, extensive research efforts have been dedicated to understanding the effects of GBP in bovine species. These studies, spanning pharmacokinetics and safety assessments, have provided valuable insights into its application in veterinary settings. Notably, recent research endeavors have explored the synergistic effects of GBP and meloxicam, a nonsteroidal anti-inflammatory drug (NSAID), in specific procedures such as dehorning and managing lameness in cattle.

Combining GBP with meloxicam has proven to be highly effective, leading to significant therapeutic outcomes. The co-administration of these medications not only enhances pain relief but also showcases a notable potential in enabling veterinarians to perform various surgical procedures on cattle without causing undue discomfort to the animals. This development is particularly significant in the context of animal well-being in veterinary medicine, where managing pain and preventing animal suffering are fundamental principles. The optimal administration of oral GBP doses, typically falling within the range of 10 to 20 mg/kg, has been identified as both safe and efficacious, especially when combined with meloxicam. To maximize its benefits, veterinarians are advised to administer these doses approximately 8 hours before any planned procedure, as part of a preemptive therapy approach. This preemptive strategy has demonstrated remarkable success in enhancing the overall well-being of cattle undergoing various farming practices and surgical interventions.

In this comprehensive review, our focus delves into the clinical applications and therapeutic effects of GBP in cattle. By highlighting its significance in both farming practices and surgical interventions, this exploration aims to provide practical insights for veterinarians, paving the way for improved pain management protocols and enhanced animal welfare standards in the field of veterinary medicine.

3- Synopsis of the pharmacokinetics, pharmacodynamics, applications, and safety of firocoxib in horses

Reference: **Fadel, C.**, & Giorgi, M. (2023). Synopsis of the pharmacokinetics, pharmacodynamics, applications, and safety of firocoxib in horses. *Veterinary and animal science*, *19*, 100286. https://doi.org/10.1016/j.vas.2023.100286

According Based on both in vitro and in vivo studies, firocoxib (FX), a second-generation coxib, has demonstrated remarkable selectivity as a COX-2 inhibitor in horses. It possesses a COX-1/COX-2 IC50 ratio of 643 in equines, indicating its high specificity for COX-2 while sparing the inhibitory effects on COX-1. This unique characteristic has led to its approval for treating musculoskeletal issues and lameness in horses, as well as osteoarthritis in both horses and dogs.

In the realm of equine osteoarthritis treatment, firocoxib offers two licensed formulations: an injectable version for IV administration at a dose of 0.09 mg/kg for five days and an oral paste formulation at a dose of 0.1 mg/kg for 14 days. Various analytical methods, notably utilizing HPLC and LC-MS, have been developed to quantify FX levels in biological fluids, enhancing its clinical monitoring and usage precision. Firocoxib exhibits exceptional pharmacokinetic and pharmacodynamic properties compared to other coxibs. It boasts an oral bioavailability exceeding 80% and is efficiently absorbed by horses. With a V_d approximately at 2 L/kg and slow elimination, it maintains prolonged presence within the equine system. Its extended elimination half-life of around 2 days facilitates convenient once-daily dosing. A recommended loading dose of 0.3 mg/kg ensures swift establishment of steady-state drug concentrations within 24 hours, making it suitable for acute treatments as well.

Notably, FX's potency is underscored by its IC_{80}, measuring at 103 ng/mL in whole blood, indicating a substantial receptor affinity. Compared to other commonly administered nonsteroidal anti-inflammatory drugs (NSAIDs) in horses, FX stands out with its EC_{50} of 27 ng/mL, further emphasizing its superior binding capability. These distinctive features position firocoxib as a highly

effective and promising therapeutic option for equine osteoarthritis, reflecting its profound impact on the field of equine medicine.

4- Single and multiple oral amoxicillin treatment in geese: a pharmacokinetic evaluation

Reference: Sartini, I., Łebkowska-Wieruszewska, B., **Fadel, C.**, Lisowski, A., Poapolathep, A., & Giorgi, M. (2022). Single and multiple oral amoxicillin treatment in geese: a pharmacokinetic evaluation. *British poultry science*, *63*(4), 493–498. https://doi.org/10.1080/00071668.2022.2036699

Although amoxicillin has broad-spectrum antibiotic activity and is extensively used in poultry, its use has never been investigated in geese. This study aimed to evaluate the pharmacokinetics of amoxicillin after a single and multiple oral doses in geese. A total of 20 geese were enrolled in this study and randomly pooled in two groups (n = 10). In group I, animals were treated with a single oral 20 mg/kg dose of amoxicillin, while geese in group II were administered multiple doses (20 mg/kg/day for 4 d). Concentrations of amoxicillin in plasma were analysed using a validated HPLC-UV method and drug plasma concentrations were modelled for each subject using a non-compartmental approach. Amoxicillin showed rapid absorption after a single-dose treatment, with a t1/2 of approximately 1 h. C_{max}, T_{max} and AUC values differed statistically between groups I and II (after the first dose administered). A large variability was observed in the pharmacokinetic profiles and drug accumulation may occur after the multiple administration. No accumulation in plasma was predicted from an in-silico simulation performed using the same multiple dosage schedule. The in-silico simulation does not seem to accurately predict in-field conditions.

5- Intoxication of dogs and cats with common stimulating, hallucinogenic and dissociative recreational drugs

Reference: Oster, E., Čudina, N., Pavasović, H., Prevendar Crnić, A., Božić, F., **Fadel, C**., & Giorgi, M. (2023). Intoxication of dogs and cats with common stimulating, hallucinogenic and dissociative

recreational drugs. *Veterinary and animal science*, *19*, 100288. https://doi.org/10.1016/j.vas.2023.100288

The issue of pets being exposed to illicit drugs, whether accidentally, intentionally, or maliciously, has become increasingly concerning over the past decade. This growing concern is primarily attributed to the rise in illicit drug usage among humans, posing challenges in diagnosis and management. Owners often remain unaware of their pets' exposure, either due to their lack of knowledge or reluctance to admit the presence of recreational drugs in their households, fearing legal consequences. Furthermore, drugs sold on the black market are frequently adulterated with other substances, leading to nonspecific clinical symptoms and complicating accurate diagnosis.

To address this problem, there are affordable onsite diagnostic tests available in the market that could aid in identifying intoxication caused by illicit drugs. However, these tests often yield false positive results due to their low specificity. Consequently, reliable and accurate diagnosis remains a challenge. In this research paper, we have meticulously compiled information about the most common recreational drugs, including amphetamines, methamphetamine, 3,4-methylenedioxy-methamphetamine (MDMA), phencyclidine (PCP), lysergic acid diethylamide (LSD), psilocybin mushrooms, and cocaine. Our focus has been on exploring their toxicokinetic properties, mechanisms of toxic action, clinical presentations, and treatment methods specifically concerning dogs and cats. By delving into these details, our aim is to enhance the understanding of these substances' effects on pets and contribute valuable knowledge to the field, ultimately aiding in their appropriate diagnosis and treatment.

6- Pharmacokinetics and pharmacodynamics of tiamulin after single and multiple oral administrations in geese

Reference: Sartini, I., Vercelli, C., Lebkowska-Wieruszewska, B., Lisowski, A., **Fadel, C.**, Poapolathep, A., Dessì, F., & Giorgi, M. (2023). Pharmacokinetics and antibacterial activity of

3640 tiamulin after single and multiple oral administrations in geese. *Veterinary and animal science*, *22*,
3641 100317. https://doi.org/10.1016/j.vas.2023.100317

3642 The objective of this study was to investigate the pharmacokinetic properties of tiamulin, a semi-
3643 synthetic antibiotic exclusively approved for veterinary use. Tiamulin is effective against Gram-
3644 positive bacteria, Mycoplasma spp., and Leptospirae spp. In this in vivo experimental trial involving
3645 geese, eight healthy individuals were subjected to a longitudinal study conducted in two phases: a
3646 single oral administration of 60 mg/kg versus 60 mg/kg/day for four days, with a two-week wash-out
3647 period. Blood samples and cloacal swabs were collected at predetermined intervals. The study
3648 employed a fully validated HPLC method to quantify tiamulin concentrations in goose plasma.
3649 Cloacal swabs were utilized to identify bacterial strains using specific methods tailored to each
3650 species, with confirmatory tests conducted. The minimal inhibitory concentration (MIC) was
3651 determined for each isolated bacterial species. Remarkably, tiamulin remained quantifiable and
3652 significantly above the LLOQ even 10 hours after a single dose treatment and throughout the initial
3653 day of multiple treatments. Comparative analysis revealed significant differences in various
3654 pharmacokinetic parameters between the groups, including C_{max} (p=0.024), $AUC_{(0-t)}$ (p=0.031),
3655 $AUC_{(0-inf)}$ (*p*=0.038), t1/2 (*p*=0.021), Cl/F (*p*=0.036), and V_d/F (*p*=0.012). Tiamulin demonstrated a
3656 relatively slow to moderate t1/2 (3.13 hours for single dose; 2.62 hours for multiple doses) and rapid
3657 absorption (1 hour for single dose; 0.5 hours for multiple doses) in geese following oral
3658 administration. Additionally, there was an accumulation ratio of 1.8 after multiple doses. However,
3659 it's noteworthy that an in-silico simulation of multiple dosing did not align with the results obtained
3660 from the in vivo multiple dosage study. Cloacal isolation allowed for the identification of various
3661 bacterial strains, all of which were commensal. Intriguingly, in both treatment groups, MIC values
3662 were remarkably high, indicating resistance (> 64 µg/ml) against tiamulin. This resistance was
3663 observed either prior to the initial administration for some strains or emerged shortly after the

initiation of treatment for others. These findings shed light on the complex dynamics of tiamulin pharmacokinetics and its implications in the context of resistance development in avian cloacal flora.

7- Metronidazole Pharmacokinetics in Geese (*Anser anser domesticus*) after Intravenous and Oral Administrations

Reference: **Fadel, C**., Łebkowska-Wieruszewska, B., Bourdo, K., Poapolathep, A., Hassoun, G., & Giorgi, M. (2023). Metronidazole pharmacokinetics in geese (Anser anser domesticus) after intravenous and oral administrations. Journal of veterinary pharmacology and therapeutics, 10.1111/jvp.13421. https://doi.org/10.1111/jvp.13421

Metronidazole (MTZ), a 5-nitroimidazole antimicrobial agent effective against both bacteria and protozoa, holds significant value in human and companion animal medicine where its usage is widespread. However, its application in farm animals is limited due to restrictions in various countries owing to insufficient data on nitroimidazoles.

The primary objective of this study was to evaluate the pharmacokinetics (PK) of MTZ in geese following single intravenous (IV) and oral (PO) administrations. The experiment involved eight healthy male geese aged fifteen months. Employing a two-phase, single-dose design (10 mg/kg IV, 50 mg/kg PO), the study incorporated a two-week washout period between the IV and PO phases. Blood samples were collected from the left wing vein at specified intervals (0, 0.085 [for IV only], 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 24, and 48 hours) and stored in heparinized tubes. Plasma MTZ concentrations were quantified using HPLC coupled to a UV detector. The obtained data were subjected to pharmacokinetic analysis utilizing PKanalix™ software, employing a non-compartmental approach. Notably, MTZ concentrations remained quantifiable and significantly above the LLOQ even at 24 hours post-administration for both IV and PO routes. Following IV administration, MTZ exhibited a t1/2 of 5.47 hours, a V_d of 767 mL/kg, and a total Cl of 96 mL/hr/kg. For the oral route, the bioavailability was high (85%), with a mean peak plasma concentration of 60.27 µg/mL observed at 1 hour. When normalized for the dose, no statistically significant differences

were observed in any of the PK parameters between the two routes of administration. These findings suggest that oral administration of MTZ holds promise in geese. However, it is imperative to conduct comprehensive research focusing on its pharmacodynamics and multiple-dose studies before considering its widespread adoption in geese. Further investigations are necessary to fully understand its efficacy and safety profile in this avian species.

Apart from the projects that have already been completed, a multitude of ongoing pharmacology initiatives are either currently underway or have been completed and are in the process of undergoing review before their anticipated publication. These upcoming studies hold the potential to enhance the progress of veterinary pharmacology and add to the expanding reservoir of knowledge dedicated to enhancing the health, veterinary pharmacotherapy, and well-being of animals.

8- The effect of butyric acid and nucleotides supplementation on broiler (Gallus gallus domesticus) growth performance, immune status, intestinal histology, and serum parameters

Reference: Aziz, A. A. A., Aziz, E. S. A. A., Khairy, M. H., **Fadel, C.**, Giorgi, M., & Abdelaziz, A. S. (2024). The effect of butyric acid and nucleotides supplementation on broiler (Gallus gallus domesticus) growth performance, immune status, intestinal histology, and serum parameters. *Open Veterinary Journal*, *14*(1), 324. https://doi.org/10.5455/OVJ.2024.v14.i1.29

Butyric acid and its derivatives have been shown to support the immune system, reduce inflammation, and alleviate oxidative stress in broilers, while also maintaining gut homeostasis and epithelial integrity. Additionally, the addition of nucleotides to the diet has been demonstrated to improve broiler performance.

The aim of this study was to investigate the effects of adding butyric acid and nucleotides to broiler feed on overall performance, immunity, levels of oxidant/antioxidant enzymes, intestinal histology, and hepatic functions.

Four experimental groups, each consisting of thirty chickens, were used. The control group received a normal diet without any additives. The other three groups received diets supplemented with butyric acid, nucleotides, or a combination of both. Necrotic enteritis was induced in a subset of birds from each group to evaluate the immune-modulatory effects of the supplements, while antioxidant status, intestinal histology, and liver functions were assessed in all experimental groups.

The results showed that the addition of butyric acid and nucleotides to the feed improved body weight, growth performance, hepatic functions, and antioxidant capabilities. In the BN group, histological analysis revealed significant improvement in gut health, characterized by enhanced proliferation in intestinal crypts and villus enterocytes, regardless of whether the birds were challenged with necrotic enteritis.

In conclusion, supplementing broiler feed with nucleotides and butyric acid can enhance growth and overall health.

9- A narrative review of the phenomenon of predatory journals to create awareness among researchers in veterinary medicine

Reference: **Fadel, C.,** Milanova, A., Suran, J., Sitovs, A., Kim, T. W., Bello, A., Abay, S. M., Horst, S., Mileva, R., Amadori, M., Oster, E., Re, G., Abul Kadir, A., Gambino, G., & Vercelli, C. (2024). A narrative review of the phenomenon of predatory journals to create awareness among researchers in veterinary medicine. *Journal of veterinary pharmacology and therapeutics*, 10.1111/jvp.13448. Advance online publication. https://doi.org/10.1111/jvp.13448

In recent years, especially in the wake of the COVID-19 pandemic, there has been a notable surge in the proliferation of predatory journals. These journals exploit the "open-access model" by resorting to deceptive practices, such as imposing exorbitant publication fees while failing to deliver the expected quality and often bypassing proper peer review procedures altogether. Such unethical

behaviors not only compromise the integrity of scientific research but also pose significant challenges for researchers in identifying trustworthy publication outlets. This is particularly concerning for early-career researchers who may struggle to discern and adhere to the appropriate criteria for selecting reputable journals. Moreover, publishing in journals that do not uphold the standards of scientific integrity raises serious ethical concerns.

This review endeavors to provide a comprehensive understanding of predatory journals by delineating their defining characteristics and elucidating the distinctions between reliable and predatory publications. Furthermore, it delves into the underlying motivations driving researchers to opt for predatory journals, while also scrutinizing the adverse ramifications of such publications on the scientific community at large. Additionally, the review explores prospective avenues for addressing this issue and offers insights into mitigating strategies.

In particular, the authors underscore the importance of informed decision-making when selecting journals for publication, especially for early-career researchers. They discuss the pivotal role of metrics, databases, and emerging technologies like artificial intelligence in guiding manuscript preparation and emphasize their relevance within the context of veterinary medicine. By empowering researchers with pertinent knowledge and tools, this review aims to foster a culture of academic integrity and promote the dissemination of high-quality research in the field.

10- Comparative pharmacokinetics of intravenous and subcutaneous pantoprazole in sheep and goats

Reference: **Fadel, C.,** Łebkowska-Wieruszewskac, B., Serih, F., Lisowski, A., Poapolathep, A., & Giorgi, M. (2024). Comparative pharmacokinetics of intravenous and subcutaneous pantoprazole in sheep and goats. The Veterinary Journal, 106138. https://doi.org/10.1016/j.tvjl.2024.106138

Abomasal ulcers pose a considerable challenge in intensive animal farming, exerting detrimental effects on both animal health and productivity. Although proton pump inhibitors (PPIs) like pantoprazole (PTZ) hold promise for treating these ulcers, there exists a dearth of comprehensive pharmacokinetic (PK) data regarding PTZ in adult goats and sheep. This study endeavors to address this gap by undertaking a thorough investigation and comparison of PTZ's PK profile in these species following single intravenous (IV) and subcutaneous (SC) administrations.

Five healthy male goats and sheep were enlisted for the study, and PTZ concentrations in plasma samples were meticulously determined using a validated analytical method. Non-compartmental analysis was conducted, and robust statistical comparisons were drawn between IV and SC administrations, as well as between species.

Interestingly, sheep and goats exhibited similar systemic exposure levels regardless of the administration route. However, sheep displayed a shorter half-life (t1/2) attributed to a higher volume of distribution (Vd) in comparison to goats. Clearance (Cl) values were comparable in both species, with low extraction ratio values. Notably, there were no significant differences observed in maximum concentration (Cmax) and time to reach maximum concentration (Tmax) between the two species following SC administration, indicating complete bioavailability. Moreover, the mean absorption time (MAT) exceeded the t1/2 in both species, suggesting a potential flip-flop phenomenon.

With the area under the curve (AUC) serving as a predictor for drug efficacy, and considering the absence of significant differences in systemic exposure between sheep and goats for any route of administration, dosage adjustment between the two species may not be warranted. Furthermore, based on previous studies, the administered doses might provide therapeutic effects and clinical efficacy in sheep and goats. In practical field settings, SC administration emerges as a more feasible option, offering not only complete bioavailability but also a prolonged half-life compared to IV administration. However, further studies are imperative to delve into the

pharmacokinetic/pharmacodynamic (PK/PD) interplay of PTZ in small ruminants afflicted with abomasal ulcers, thereby elucidating its therapeutic efficacy in such clinical scenarios.

11- Disposition Kinetics and Tissue Residues of Tilmicosin Following Intravenous, Subcutaneous, Single and Multiple Oral Dosing in Geese (Anser Anser domesticus).

Reference: Bourdo C., **Fadel C.,** Giorgi M., Šitovs A., Poapolathep A.; & Łebkowska-Wieruszewska B.

Tilmicosin (TMC), a semi-synthetic macrolide antibiotic known for its broad-spectrum bacteriostatic properties, is extensively utilized in veterinary medicine, particularly in various bird species. While its usage is prevalent in birds, it is often employed off-label in geese as well. This study aimed to explore the pharmacokinetics and tissue residues of TMC in geese through in vivo experiments.

Fifteen healthy adult male geese were subjected to longitudinal open studies, divided into three phases with one-month washout periods in between. TMC was administered to the geese through intravenous (IV, 5 mg/kg), subcutaneous (SC, 10 mg/kg), and oral (PO, 25 mg/kg for five consecutive days) routes, with blood samples collected at predetermined intervals. Tissue samples were also obtained for subsequent analysis at pre-determined times.

The concentration of TMC in goose plasma was measured using a fully validated HPLC method. Plasma concentrations were assessed for up to 4 hours for the PO and IV routes and up to 10 hours for the SC route. The study revealed a significant difference in bioavailability between subcutaneous (SC) and oral (PO) routes in geese, with SC administration showing 87% bioavailability compared to only 4% for PO administration. Due to the low absolute bioavailability and high individual variability observed with the oral route, its recommendation in geese may be discouraged at a population level. Factors such as gastrointestinal physiology, gastric emptying rates, enzymatic activity, product formulation, and feeding state may contribute to the limited oral bioavailability of tilmicosin (TMC). This finding contradicts the widely accepted presumption of high oral

bioavailability for TMC/macrolides, highlighting the need for further evaluation in other animal species.

In previous literature, TMC has shown rapid absorption after oral or subcutaneous injection, with a short oral Tmax observed in broiler chickens. However, the study observed a notably short Tmax of 0.5 hr after extra-vascular administrations in geese, potentially indicating a flip-flop phenomenon. The study also found that geese exhibited a relatively low elimination rate (Ebody) for TMC, suggesting limited metabolism and predominantly passive excretion. Furthermore, the study highlighted species-specific differences in plasma half-life values, underscoring the importance of considering inter-species variability in pharmacokinetic parameters. Notably, TMC exhibited extensive tissue distribution due to its high lipophilicity and low plasma protein binding, resulting in poor correlation between plasma concentrations and clinical effects. In geese, elevated TMC levels were observed in the liver, kidneys, and muscles, with prolonged tissue residence observed up to 120 hr. This prolonged tissue residence may be attributed to factors such as tissue binding, sequestration, and slow release, indicating a distinct and preferential distribution to specific target organs rather than plasma accumulation with repeated doses.

Regarding the multiple PO doses, provisional withdrawal times of 6, 7.5, and 8 days were recommended for the liver, muscles, and kidneys, respectively, based on the Maximum Residue Limits (MRL) set for these matrices in chickens by the European Medicines Agency (EMA). Although multiple oral doses did not lead to plasma accumulation, tissue data indicated extensive distribution and prolonged residence of TMC for up to 120 hours, implying a sustained therapeutic effect despite the brief plasma half-life.

Plasma TMC levels are inadequate indicators of total body TMC or effective therapeutic levels, underlining the need to evaluate tissue concentrations. Furthermore, existing literature consistently emphasizes the relevance of AUC/MIC and Cmax/MIC as key PK/PD indices for predicting TMC's antimicrobial efficacy. Considering the MIC range against Mycoplasma gallisepticum and the

substantial exceedance of thresholds set by these indices, preliminary assessments suggest effective tissue concentrations for Mycoplasma treatment with current dosing regimens (PO and SC), even with conservative assumptions regarding minimal lung tissue concentrations. However, further in vivo PK/PD studies and investigation of tissue protein bindings are essential to fully understand the intricate relationship between drug exposure and antimicrobial activity.

In conclusion, while oral administration of TMC is discouraged at the population level due to practical limitations, subcutaneous administration may be deemed suitable for geese, although it may not be practical for flock therapy.

CURRENTLY ONGOING PROJECTS IN 2024

Numerous additional projects are in progress, expanding beyond our primary focus on pain management strategies across various animal species. These endeavors aim to unravel the complexities of pharmacology in veterinary medicine, with the ultimate goal of developing tailored drugs specific to each species. These projects involve the examination of **omeprazole, metronidazole, clindamycin, hydroxyzine, cetirizine and torasemide in both sheep and goats.** Comparative pharmacology studies are being conducted between these two species to gain deeper insights into their physiological differences and how these variances influence the behavior of drugs within the body. Additionally, studies are underway on **imipramine, montelukast, aprepitant, and alprazolam in dogs**, considering both fasted and fed states. **Colistin and lincomycin** investigations are ongoing in **geese**. Furthermore, a new avenue of research is being pursued involving snails, highlighting the significance of pharmacokinetic and tissue residues studies, given the widespread consumption and industrialization of snails and their by-products across many countries.

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