KETOROLAC PHARMACOKINETICS AND EFFECTS ON NEUTROPHIL FUNCTION AND PROSTAGLANDIN E₂ CONCENTRATIONS IN DOGS

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by
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In partial fulfilment of requirements
for the degree of
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ABSTRACT

KETOROLAC PHARMACOKINETICS AND EFFECTS ON NEUTROPHIL FUNCTION AND PROSTAGLANDIN E₂ CONCENTRATIONS IN DOGS

Kirby Shawn Pasloske, B.Sc., D.V.M.  
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Three experiments were performed to analyze the pharmacokinetics and pharmacodynamics of the non-steroidal anti-inflammatory drug ketorolac tromethamine (Toradol™) in healthy dogs. The first experiment determined both intravenous (IV) and oral (PO) pharmacokinetics of the drug using high performance liquid chromatography (HPLC); the second assessed the effect of single IV and PO doses of the drug on peripheral neutrophil reactive oxygen species (ROS) production through time-dependent isolation, in vitro stimulation, and measurement of chemiluminescence; the final experiment investigated the effect of single IV and PO doses of the drug on endogenous plasma prostaglandin (PG) E₂ concentrations using enzyme immunoassay (EIA) measurement of a stable metabolite, bicyclo prostaglandin E₂.

Administration of 0.5 mg/kg of ketorolac tromethamine (0.34 mg/kg ketorolac) IV to healthy dogs produced an average peak plasma concentration (C₀) of 8.26 μg/mL, a steady state volume of distribution (Vss) of 0.33 L/kg, a plasma clearance (Clp) of 1.25 mL/min/kg and a plasma elimination half-life (t₁/₂p) of 4.55 hours. Four
dogs in the IV study exhibited biexponential decay of the drug and the remaining two dogs exhibited triexponential decay.

The peak absorption time \((t_{\text{max}})\) of ketorolac tromethamine following PO administration averaged 0.85 hours. The average peak plasma concentration \((C_{\text{max}})\) was 1.6 \(\mu\)g/mL. The peak was followed by an biexponential decay in all dogs and the \(t_{1/2}\) averaged 4.07 hours. The mean systemic bioavailability of the PO dose was 100.9%.

When a single 0.5 mg/kg IV dose of ketorolac tromethamine was administered to healthy dogs it suppressed neutrophil superoxide anion \((O_2^-)\) production, but this effect was not statistically significant. The suppression was observed when either phorbol myristate acetate (PMA) or formyl-methionine-leucine-phenylalanine (FMLP) was used as pharmacological stimulus.

Finally, ketorolac significantly \((p \leq 0.01)\) decreased the concentration of endogenous PGE\(_2\) in the plasma of healthy dogs when given as a single IV or PO dose of 0.5 mg/kg. Plasma PGE\(_2\) was significantly suppressed within 1 hour of both IV and PO treatment and normalized within 96 hours.
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The high performance liquid chromatography performed by Mr. Rick Renaud was invaluable to my project. Technical support provided by Ms. Catherine Gatere and Ms. Catherine Miller was greatly appreciated.

Finally, I would like to sincerely thank my parents for their love, support and continual guidance over my thirty two years. But most of all I need to thank my wife and dear friend Susie who's selfless love, tremendous courage, limitless patience and infinite support has made this work possible. She is, and will always be, the product of wonderful parents.
DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in this thesis was performed by me.

The dogs were cared for by the staff of the Central Animal Facility, University of Guelph during the experiment.

Blood samples were collected and processed with the help of Mr. John Burger, Ms. Catherine Gatere and Ms. Catherine Miller of the Department of Biomedical Sciences. Sample analyses for ketorolac using high performance liquid chromatography were performed with the help of Mr. Rick Renaud.

Mr. John Burger assisted in canine neutrophil isolation and chemiluminescence. He also assisted in plate preparation for the analyses of prostaglandin E₂ samples by enzyme immunoassay.
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INTRODUCTION

After surgery, trauma, or severe inflammation, pain control is of paramount importance. Incomplete knowledge about mechanisms regulating pain, a paucity of studies on appropriate drugs for animals, and concern over toxicity of unproven drugs have hindered veterinarians in managing pain (Hansen, 1994). Although 36 systemic anti-inflammatory and/or analgesic drugs are approved in Canada for humans (Krough, 1995), only six are approved for dogs (phenylbutazone, ketoprofen, dipyrone, fentanyl/droperidol, butorphanol and xylazine) (Bennet, 1995). Management of pain and inflammation requires that a veterinarian have rational treatment options; however, he or she must understand the drug's pharmacokinetics and its effects on cellular and soluble mediators of inflammation and pain.

Until recently, non-steroidal anti-inflammatory drugs (NSAIDs) were regarded as weak analgesic agents with a potent antiplatelet effect that severely limited their perioperative usefulness. However, the recent development of injectable NSAIDs has stimulated a re-evaluation of the potential role of this class of drugs in postoperative pain management. A potent injectable NSAID that warrants investigation in companion animals is ketorolac tromethamine (Toradol™). The drug has been used empirically in dogs at the human dose (especially in the Intensive Care Unit at the Ontario Veterinary College) with
encouraging results (Mathews et al., 1996). In humans, this newer NSAID has demonstrated analgesic efficacy equal to, or surpassing, some of the opioids, and its pharmacokinetics, safety and usefulness have been documented in detail (Buckley and Brogden, 1990). Despite this, some animals develop signs of toxicity that may be dose and/or frequency-dependent and almost nothing is known about its pharmacokinetics and mechanism of action in dogs.

**INFLAMMATION**

In 35 A.D., Celcus described the cardinal signs of inflammation—*rubor et tumor cum calore et dolore*—with Rudolph Virchow’s 19th century addition of *functio laesa*. These signs (of redness, swelling, heat, pain and loss of function) represent the outward signs of complex vascular, immunological and cellular reactions involving many soluble mediators of inflammation. This pathological phenomenon is essentially beneficial since it is the body’s response to insult or injury and it serves to minimize the deleterious effects of a threatening agent by diluting, localizing, destroying and, if possible, removing it (Higgins and Lees, 1984).

The process of inflammation can be initiated in any vascularized part of the body. It is a series of events, rather than a single occurrence, and it follows a course that is generally uniform in its sequence; but it may vary in intensity and duration, depending on the type and degree of initial stimulus. Inflammation is mediated by vasoactive amines (e.g. histamine and serotonin), cytokines, leukocyte products (e.g. enzymes and reactive oxygen species [ROS]), and the
products of arachidonic acid metabolism (eicosanoids), all of which are of cellular origin. Inflammation is also regulated by the kinins, complement and clotting systems found in plasma (Higgins and Lees, 1984).

**Pain**

Pain is an aversive sensory experience which elicits protective motor actions, results in learned avoidance and may modify species-specific behaviour (Zimmerman, 1986). Pain processing involves the perception and integration of noxious stimuli at specialized peripheral nerve endings called nociceptors. Once stimulated in the periphery, these specialized receptors transmit signals via the spinal cord to the somatosensory cortex of the brain for interpretation of the aversive stimulus.

Tissue injury leads to nociception by direct mechanical and thermal damage to nerve endings, to inflammation by the release of chemicals and enzymes from nerves and damaged tissue and, to pain generated by algogenic substances and 'sprouting' of damaged nerves into the injured tissue (Koch-Weser, 1980; Cousins, 1989). The same tissue injury promotes the release of substance P from nerve endings, resulting in vasodilation and an increase in vascular permeability (Dahl and Kehlet, 1991). This causes local edema and, in combination with the release of algogenic substances (e.g. prostaglandins, leukotrienes, bradykinin, serotonin, histamine and ROS), leads to inflammation and sensitization of nociceptors, resulting in pain. Thus, the primary afferent neuron (PAN) serves a dual function: (a) transmission of neural stimuli and, (b)
peripheral release of inflammatory mediators at the site of trauma (Levine et al., 1988).

Primary afferent nociceptors are generally categorized as either myelinated (A-fibres) or unmyelinated (C-fibres). Normally, myelinated A-fibres respond to noxious mechanical and thermal stimuli with a conduction velocity of 5-55 m/s and are responsible for initial stabbing-like pain. Unmyelinated C-fibres, having a slower conduction velocity of 0.8 m/s, respond to a wider range of noxious stimuli including mechanical, thermal, and chemical. These afferent nociceptors are responsible for the chronic pain sensation, referred to as burning or aching pain (Torebjork and Ochoa, 1980; Raja et al., 1988).

It is likely that in the vast majority of pain conditions, whether inflammatory or neuropathic, there is an associated phase of inflammation in which a variety of chemical mediators are able to alter the functions of PAN. Although the majority of nociceptors respond to thermal and mechanical stimulation, chemical signaling is likely to be the most common and diverse form of signal generation in all types of fine afferent fibres (Dray, 1995). Importantly, the lowering of PAN threshold depends on the presence of other cells and tissues such as leukocytes and sympathetic postganglionic neurons (Levine et al., 1993).

Several important pathophysiologic features are associated with inflammation besides the stimulation of peripheral nerve fibres to induce pain. There are changes in local blood flow and vascular permeability, activation and
migration of immune cells and changes in the release of growth and trophic factors from surrounding tissue. These events represent an orchestrated series of responses in which afferent nerves play an important role (Dray, 1994; Ray et al., 1994). In theory, the afferent nerve terminal may be exposed to a great many substances during tissue injury and inflammation, but there is little information about the composition or concentration of various components. However, these can produce manifold and complex changes in afferent fibres ranging from overt activation, or sensitization to other stimuli, to alterations in the phenotype and structure of sensory nerves (Levine et al., 1993; Dray, 1994; Ray et al., 1994; Woolf and Doubell, 1994). Enormous potential thus exists for interactions between different substances and between neural (sensory and sympathetic nerves) and non-neural systems (sensory nerves and immune cells).

**Neutrophils and Reactive Oxygen Species (ROS)**

Reactive oxygen species (ROS) or free radicals are normal products of cellular electron transfer reactions which are important for the regulation of a number of gene transcription activities (Dray, 1995). Normally, the production of ROS is finely controlled by the anti-oxidative activity of superoxide dismutase and catalase. However, during ischemia, which follows the rapid vasoconstriction response due to tissue injury, concentrations of reactive oxygen species decrease to subnormal levels and thereby reduce antioxidant activity. Then, tissue reperfusion creates an oxidative stress in which oxygen and
nitrogen species are produced in abundance which leads to the induction of a number of factors such as the expression of the immediate early gene (IEG) c-fos (Zhang et al., 1994).

The induction of c-fos and other genes by ROS produce a second wave of gene products encoding enzymes with free radical scavenging abilities (e.g. catalase) and tissue repair activity (e.g. collagenase, stromelysin), as well as the production of cytokines, cell surface receptors, adhesion molecules and growth factors (Dray, 1995).

Although important in the transcription of many genes and their products, ROS have received relatively little attention with respect to afferent fibre activation during tissue injury, inflammation and subsequent pain. These small molecular weight, highly cytotoxic molecules, contribute to the ‘cocktail’ of mediators thought to be responsible for nociceptor activation. Specifically, the ROS produced by neutrophils and other cell types may be central to the amplification of pain through inflammation. The major pathologic roles of ROS include increased endothelial permeability, proinflammatory activity and cytotoxicity through membrane unsaturated fatty acid damage (Hitt, 1988).

Reactive oxygen species are well recognized as important mediators of inflammation; however, evidence for their nociceptor activity is sparse. Hydrogen peroxide has been shown to enhance the effects of other inflammatory mediators, including bradykinin and prostaglandin (PG) E₂, while nitric oxide (NO), another oxygen containing reactive molecule, induces a burning feeling.
upon intradermal injection (Holthusen and Arndt, 1994).

At the inflammatory site, phagocytosing neutrophils produce ROS which are released into the external environment (Smith, 1994). These molecules are cytotoxic to surrounding cells and further the process of inflammation by acting as potent chemoattractants for additional phagocytes (McCord, 1983). Reactive oxygen species also help create edema by increasing endothelial and capillary permeability (Roisman et al., 1983; Halliwell, 1984). Local PG production increases because ROS increase conversion of arachidonic acid to proinflammatory PGs and thromboxane (TX) A₂ production. This favours platelet activation, microvascular stasis, and hypoxia (Schlafer et al., 1982). Finally, in the process of PG production superoxide anion (O₂⁻) is produced, thus amplifying the inflammatory process even further (Higgins, 1985).

**PROSTAGLANDIN E₂: HOMEOSTASIS, PAIN and INFLAMMATION**

Prostaglandins are members of a large family of arachidonic acid oxygenation products. Eicosanoids (20 carbon fatty acids) are derived from naturally occurring eicosapolyenoic acids, of which arachidonic acid is the most common in domestic animals (Higgins, 1985). Arachidonic acid, which is either obtained from the diet or synthesized from linoleic acid, is widely distributed in the body and is usually stored covalently bound in its esterified form, in the phospholipid fraction of the cell membranes of most body cells.

The initiation of PG synthesis occurs when cells are stimulated to release arachidonic acid by the triggering of specific receptors or by less specific
activation elicited by mechanical stress. This, in turn, increases intracellular calcium levels which facilitates the translocation of the enzyme phospholipase A2 or C from the cytoplasm to binding sites on cell membranes (Higgins, 1985; Conlon, 1988). After release, arachidonic acid is then re-esterified or metabolized by cyclo-oxygenase (COX) enzyme to produce oxygenated products such as PGs and TXs (Samuelsson, 1987). Cyclo-oxygenase products include PGD₂, PGE₂, PGF₂α, PGI₂ and TXA₂.

Prostaglandin E₂ has received a great deal of attention in recent years because of the many effects it exerts on the physiology of mammals. A large body of evidence now points to the existence of high affinity PGE-specific binding sites in a wide range of tissues. Such binding sites have been identified in adipocytes, endothelium, bronchioles, bone, adrenal gland, corpora lutea, myometrium, kidney, intestinal epithelium, liver, heart, brain and platelets (Coleman et al., 1990).

In common with the vast majority of hormones, neurotransmitters and autocrine and paracrine regulatory factors, prostanoids are coupled to their effector mechanisms by guanosine triphosphate (GTP)-dependent G proteins. To date, 15 immunologically distinct G proteins have been identified (Davies and Maclntyre, 1992) and pharmacological evidence supports the existence of three PGE-specific binding receptor subtypes, termed EP1, EP2 and EP3. The EP1 receptors are coupled to phospholipase-C-catalyzed inositol lipid hydrolysis (e.g. adrenal chromaffin cell function and osteoblast activity) (Negishi et al., 1989;
Morita et al., 1990). The EP2 receptors are coupled via a stimulatory G protein (Gs) and adenylate cyclase. Examples of EP2 biological responses include bronchodilation, vasodilation and gastric nonparietal cell secretion (Armstrong et al., 1990). The EP3 receptors have been linked to either: (a) inhibition of adenylate cyclase via inhibitory G protein (Gi) resulting in renal medullary blood flow regulation, collecting tubule responses to vasopressin, sodium and chloride reabsorption in the loop of Henle, and gastric parietal cell secretion, or (b) phospholipase-C-catalyzed inositol lipid hydrolysis causing inhibition of transmitter release from neurons and the augmentation of platelet aggregation (Armstrong et al., 1990; Gardiner, 1990; Watson and Abbot, 1992).

Not only is PGE$_2$ a participant in numerous homeostatic responses, but as a prostanoid it plays an important role in both acute and chronic inflammation (Lewis, 1983; Coleman et al., 1990). Prostaglandin E$_2$ is produced in large quantities in inflammatory conditions, whether these are induced by mechanical trauma, corrosive chemicals, burning, radiation, antigen-antibody reactions or immune-mediated responses (Lewis, 1983). Prostaglandin E$_2$ may be generated from a variety of inflammatory cells, (e.g. neutrophils and eosinophils), but macrophages appear to be the richest source (Coleman et al., 1990).

Prostaglandin E$_2$ is a potent vasodilator and high local concentrations of PGE$_2$ in inflammatory conditions are sufficient to induce local hyperemia through vasodilation (Williams, 1979). On its own, PGE$_2$ is a poor inducer of edema, but
it can enhance the edema-producing properties of agents such as bradykinin and histamine (Williams and Morley, 1973).

In 1972, Ferreira demonstrated that the E series PGs have hyperalgesic activity (Ferreira, 1972). Thus, while they are not pain-producing in their own right, they sensitize pain receptors to stimulation by agents such as bradykinin and serotonin (Ferreira et al., 1973; Thomas and West, 1973; Juan and Lembeck, 1974). It is this ability of PGs to amplify the pain-producing effects of other agents which explains the analgesic action of the non-steroidal anti-inflammatory drugs (NSAIDs). There is also growing evidence that the site of action of endogenous PGs in their hyperalgesic effects is not entirely peripheral; they may also have a central site of action (Ferreira, 1983). Recently, Malmberg and Yaksh were able to demonstrate the production of PGE$_2$ following both NMDA and substance P-induced activation of spinal neurons in the rat (Malmberg and Yaksh, 1992). Thus, peripheral administration of an inflammatory agent can cause prostanoid release both locally and centrally, and both may contribute to the associated hyperalgesia.

**NON-Steroidal ANTI-INFLAMMATORY DRUGS (NSAIDs)**

**Mechanisms of Action**

The oxygenation of polyunsaturated fatty acids such as arachidonic acid, by the cyclo-oxygenase (COX) enzyme or lipoxygenase enzyme systems leads to the formation of several important biologically active lipids of the inflammatory process, including the prostaglandins (PGs), thromboxane (TX) and prostacyclin
(Samuelsson et al., 1978) and leukotrienes (Samuelsson et al., 1980). These compounds, known collectively as the eicosanoids, have a vital role in the initiation and maintenance of several components of inflammation including, but not limited to, vasodilation, increased vascular permeability, leukotaxis, pain, bronchoconstriction and platelet aggregation.

In 1971, John Vane and associates Smith and Willis discovered that aspirin and indomethacin inhibited the biosynthesis and hence the release of stable PGs from tissue homogenates (Vane et al., 1971). The finding that PG production was suppressed or abolished while the release of the upstream phospholipase enzyme was unaffected implied that NSAIDs acted by preventing the conversion of arachidonic acid to PG (Smith and Willis, 1971). Vane, in 1976, subsequently proposed that COX inhibition may be the common denominator for all therapeutic and side-effects of NSAIDs.

Presently, two isoforms of COX are known to be present in eukaryotic organisms: COX-1 and COX-2 (Hayllar and Bjarnason, 1995). Non-steroidal anti-inflammatory drugs have an affinity for the ubiquitous COX-1 enzyme which is expressed in most tissue types other than mature erythrocytes. Cyclooxygenase-1 is involved in cellular homeostasis (Whittle et al., 1980; Xie et al., 1992) since it synthesizes PGs in response to physiological stimuli at a rate proportional to the availability of its substrate, arachidonic acid (Vane, 1994). The COX-1 enzyme’s active site is a long hydrophobic channel that recently was discovered through x-ray crystallography (Loll et al., 1995). It is likely that
aspirin-like drugs inhibit PG formation by excluding arachidonic acid from the upper portion of the channel. Cyclo-oxygenase-2 which shares approximately 60% homology with COX-1 (Xie et al., 1992) is expressed or induced at sites of inflammation through the action of cytokines, growth factors, tumour promoters and bacterial lipopolysaccharide (Boyce et al., 1994; Mitchell et al., 1994; Seibert et al., 1994). Macrophages and other migratory inflammatory cells have abundant COX-2 activity (Lee et al., 1992).

Evidence for the Suppression of Neutrophil Reactive Oxygen Species (ROS) Production By NSAIDs

The role of the COX-2 enzyme and its pathophysiological effects has been largely substantiated and answers most of the question: how do anti-inflammatory agents achieve their effects? It also helps ascribe a role to stable PGs as mediators of pain and inflammation. However, while this hypothesis may explain the reduction of PG-mediated fever, pain and swelling by NSAIDs, several unexplained problems have become apparent.

First, some stable PGs and their analogs can be anti-inflammatory since they can inhibit the activation of inflammatory cells in vitro (Orange et al., 1971; Henney et al., 1972; Zurier et al., 1974) and, in addition, they can ameliorate experimental adjuvant arthritis (Zurier and Quagliata, 1971) and immune complex nephritis (Zurier et al., 1978). Furthermore, PGs have been used effectively as immunosuppressants in the treatment of acute renal transplant rejection (Moran et al., 1990) and fulminant hepatic failure (Sinclair et al., 1989).
Misoprostol is a synthetic PGE₁ analog commercially available for use in the prevention of NSAID induced gastric ulcers. In addition, it has been shown to paradoxically augment the inhibitory effects of NSAIDs on neutrophil activation (Kitsis et al., 1991).

Second, sodium salicylate, the oldest of the NSAIDs, shares many of the properties of aspirin (acetylsalicylic acid) but it does not inhibit PG biosynthesis in disrupted cell preparations at therapeutically effective plasma concentrations (Abramson et al., 1985). Similarly, the widely used analgesic and antipyretic drug acetaminophen does not inhibit PG synthesis, nor is it anti-inflammatory (Weissman, 1987). Based on the clinical utility of these two related drugs, it appears doubtful that the only mechanism of action of NSAIDs is to inhibit synthesis of PGs.

Third, whereas low doses of aspirin and other NSAIDs markedly inhibit synthesis of PGs in vitro (Vane, 1971; Humes et al., 1981) and in vivo (Pedersen, 1984), higher doses are required for a clinical anti-inflammatory effect (Flowers et al., 1980). These observations would suggest that, at higher concentrations, NSAIDs exert an anti-inflammatory effect independent of COX inhibition.

Based on the previous discussion, an alternative mechanism can be proposed to help fully explain the anti-inflammatory and analgesic actions of NSAIDs. During acute inflammation, inflammatory cells (notably neutrophils) are activated, diapedes out of the circulation (by chemoattraction) and proceed to
sites affected by any number of noxious stimuli such as infectious organisms, trauma, foreign bodies and immune-mediated disease. It is at these sites where neutrophils initiate phagocytosis and killing, but may also injure host tissue through the release of three classes of cytotoxic substances: reactive oxygen species (ROS), such as hydrogen peroxide (H\(_2\)O\(_2\)), superoxide anion (O\(_2^\cdot\)), singlet oxygen (\(1^\circ\)O\(_2\)) and the hydroxyl radical (OH\(^-\)); lysozomal enzymes, such as lysozyme, myeloperoxidase, collagenase and elastase; and the lipoxygenase products of arachidonic acid metabolism such as leukotriene (LT) B\(_4\).

Research over the last three decades has demonstrated that high (antirheumatic) concentrations of NSAIDs inhibit human neutrophil activation (Walker et al., 1976; Simchowitz et al., 1979; Ford-Hutchinson, 1983; Hopkins et al., 1983; Perianin et al., 1983; Kaplan et al., 1984; Perianin et al., 1984; Van Epps et al., 1986; Hyers et al., 1992). More specifically, certain NSAIDs can decrease the production of ROS by neutrophils. For example, millimolar concentrations of indomethacin, ibuprofen and phenylbutazone (Simchowitz et al., 1979), piroxicam, aspirin (Kaplan et al., 1984) and ketorolac (Hyers et al., 1992) inhibit the O\(_2^\cdot\) production of human neutrophils stimulated with the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (FMLP), both in vitro and in vivo. In addition, piroxicam inhibits human neutrophil O\(_2^\cdot\) production in response to the tumour promoting agent phorbol 12-myristate 13-acetate (PMA) in vitro (Kaplan et al., 1984) and neutrophils derived from the synovial fluid of rheumatoid arthritis patients produce less O\(_2^\cdot\) following 10 days of therapy with
The 'Respiratory Burst' and Native Granulocyte Chemiluminescence

The ROS produced by neutrophils have been implicated in the pathophysiology of numerous disorders of cellular metabolism. During the neutrophil's respiratory burst, the ROS generated include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH•) and singlet oxygen (¹O$_2$). Hydrogen peroxide is also a substrate for myeloperoxidase, a potent microbicidal haloperoxidase of polymorphonuclear leukocytes and monocytes capable of broad spectrum oxygenation activity (Allen et al., 1972).

This 'respiratory burst' activity, characterized by increased glucose dehydrogenation via the hexose monophosphate shunt and increased non-mitochondrial O$_2$ consumption, is necessary for effective microbicidal action (Allen, 1984). The enzyme responsible for the production of these molecules is nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase in its reduced form. Nicotinamide adenosine dinucleotide phosphate is the primary electron donor for the reduction of oxygen during the respiratory burst. The NADPH oxidase enzyme is present in the membrane of azurophilic granules and also free in the plasma membranes of neutrophils. Upon activation of the neutrophil, the cytosolic components of the enzyme translocate to the plasma membrane to associate with cytochrome b$_{558}$. This biologically active enzyme complex provides for controlled univalent reduction by keeping electrons bound to the active site and not allowing O$_2^-$ into free solution (Wymann et al., 1987).
The NADPH oxidase system is shared by other phagocytes. With most stimuli, the respiratory burst of eosinophils is greater than that of equivalent numbers of neutrophils comparably stimulated. In addition, monocytes respond to stimulation with a brisk respiratory burst, although its magnitude is less than that of equivalent numbers of neutrophils comparably stimulated (Klebanoff, 1992).

The oxygenation of biological substrates by activated phagocytes can yield electronically excited products capable of relaxing to ground state by photoemission. This activity is responsible for the native luminescence associated with phagocyte microbicidal function (Allen et al., 1972; Allen, 1979). Neutrophil chemiluminescence is O$_2$ - dependent/mitochondrial-independent and correlates with hexose monophosphate activity (Allen, 1984).

This native chemiluminescence has been successfully used for the analysis of phagocyte function. However, as an analytical technique, it lacks sensitivity. Native substrate oxygenations are typically low quantum yield reactions; that is, the yield of photons per given number of substrate oxygenations is relatively low. These limitations are obviated by chemiluminigenic probing. A chemiluminigenic probe is an organic substrate whose oxygenation results in a relatively high yield of excited products (Allen, 1984). As such, the sensitivity for detecting oxygenation activity is increased in proportion to the increase in quantum yield by native substrates. The chemiluminigenic probe also needs to be chemically compatible with the
biological system and non-toxic at the concentration employed. The use of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) or lucigenin (10,10'-dimethyl-9,9'-biacridinium dinitrate; DBA) fulfills these criteria.

**Stimulus-Response Coupling and the Onset of the Respiratory Burst in the Neutrophil**

The main function of the neutrophil is to phagocytize and kill invading bacteria. The killing depends largely on the activation of the respiratory burst and subsequent formation of $O_2^-$, $H_2O_2$, and other reactive oxygen metabolites. In addition, when human neutrophils are challenged in vitro with a variety of particulate and non-particulate agents, they demonstrate the same burst of oxidative activity. Some of the more common stimuli include the chemotactic peptide FMLP, the anaphylatoxin C5a, platelet activating factor (PAF), leukotriene B4 (LTB₄), phorbol myristate acetate (PMA), and calcium ionophores (Wymann et al., 1987). The biochemical basis of the transduction of extracellular signals into intracellular events and cellular proliferation has long been a subject of great interest. Activation of the neutrophil in response to inflammatory stimulus follows the general outlines of stimulus-response coupling in all secretory cells (Day, 1988). Within 5 to 10 seconds after engaging ligands that provoke inflammation, the neutrophil is transformed into a secretory cell capable of provoking tissue injury. This process is shared not only with other inflammatory cells such as macrophages and lymphocytes, but also with salivary gland cells, thyroid cells, and neurons; indeed, with all eukaryotic cells capable
of transducing signals from the environment (Abramson and Weissman, 1989).

A complex sequence of events follows the generation of a signal at the plasmalemma. Ligands such as FMLP, lectins such as concanavalin A, soluble pro-inflammatory mediators such as LTB₄ and PAF and immune complexes engage appropriate receptors at the external face of the plasmalemma and launch stimulus-response coupling in the neutrophil (Smith et al., 1983; Berridge and Irvine, 1984; Korchak and Rutherford et al., 1984; Nishizuka, 1984). The binding of these diverse ligands leads to the hydrolysis of a membrane phosphatidylinositol 4,5-biphosphate (PIP₂) by phospholipase C, in a reaction regulated by at least one guanosine trisphosphate (GTP) G protein (Ford-Hutchinson, 1983). The products of this hydrolysis are inositol 1,4,5-triphosphate (IP₃) and sn-1,2-diacylglycerol (DAG). These two molecules serve synergistically as 'twin signals' to activate the neutrophil (Korchak and Vienne et al., 1984).

It is hypothesized that, subsequent to PIP₂ hydrolysis, stimulus-response coupling proceeds as follows. The water soluble IP₃ enters the cytoplasm, where it engages receptors on the endoplasmic reticulum (or possibly in distinct calcium containing organelles [calciosomes]) to cause calcium release. Directly thereafter, levels of intracellular calcium increase to tenfold higher levels. In contrast, DAG remains associated with the plasma membrane where it activates protein kinase C, which catalyzes phosphorylation of the discrete cellular proteins that regulate cell activation (Dewald et al., 1988).
The effects of the 'twin signals' can be mimicked (in the absence of ligand-receptor interactions) by fungal products, such as the ionophores of divalent cations, A23187 and ionomycin, which can promote calcium movement bidirectionally across the plasmalemma and raise cytosolic calcium levels. In complementary fashion, the action of PMA also bypasses the mechanism of phosphoinositide hydrolysis by directly activating protein kinase C (Abramson et al., 1991). Raised levels of intracellular calcium and activated protein kinase C are synergistic in action. Inositol 1, 4, 5 trisphosphate facilitates the action of DAG, since increased cytosolic calcium is required for the translocation of the C kinase from the cytoplasm to the membrane.

Both PMA and ionophores are considered 'incomplete secretagogues' since neither alone can elicit the release of azurophilic granule contents from the cell. The chemotactic peptide FMLP, however, is a 'complete secretagogue' since its binding to the neutrophil receptor promotes the release of both intracellular messengers (Kaplan et al., 1984).

**Proposed Site of Action of NSAIDS in the Neutrophil**

The disruption of neutrophil oxygen radical production by sodium salicylate and other NSAIDs is considered one of many non-PG dependent properties of this group of drugs. Other examples of non-PG dependent mechanisms include: inhibition of mononuclear cell phospholipase C activity (Moran et al., 1990), disruption of the 12-hydroperoxyeicosatetraenoic acid peroxidase of the lipoxygenase pathway in platelets (Siegel et al., 1979),
uncoupling of oxidative phosphorylation in mitochondrial membranes (Miyahara and Karler, 1965), and alteration of the uptake and insertion of arachidonic acid into the membrane of cultured human monocytes (Bomalaski et al., 1987). Finally, salicylates inhibit anion transport across a variety of cell membranes, including human erythrocytes (Lucas-Heron and Fontenaille, 1979) rabbit choroid plexus (Kim et al., 1983) and renal tubular epithelium (Snow and Maass, 1979).

As a class, NSAIDs are planar, anionic molecules that partition into lipid environments, such as the lipid bilayers of plasma membranes; the more acidic the pH, as at inflammatory sites, the greater the lipophilicity (Lombardino et al., 1975). In addition, NSAIDs sequester into inflammatory exudate most likely as a result of high protein binding (Lees, 1997). It is not too surprising, then, that NSAIDs have been found to inhibit a variety of membrane-associated processes. Sodium salicylate and other NSAIDs significantly inhibit the binding of FMLP to the human neutrophil and, therefore, prevent its activation (Ford-Hutchinson, 1983; Minta and Williams, 1985; Perez et al., 1987).

It is clear, however, that isolated effects of NSAIDs on the binding of FMLP are not sufficient to explain all their effects on neutrophils. For example, preliminary research has shown that canine neutrophils may lack FMLP receptors (Redl et al., 1983; Strom and Thomsen, 1990) or that these receptors may not be expressed under certain experimental conditions. Furthermore, Hatch et al. (1978) compared the FMLP response of equal numbers of
neutrophils from different species. Human neutrophil superoxide anion production was twenty times higher than that of guinea pigs and 60 thousand times that of rabbits.

Non-steroidal anti-inflammatory drugs also inhibit activation in response to other ligands, such as C5a, PAF, LTB4 (Hopkins et al., 1983; Abramson et al., 1985) and sodium fluoride (Abramson et al., 1990); the last agent is purported to activate directly the plasmalemma G protein. As well, the NSAID piroxicam inhibits \( \text{O}_2^- \) production of human neutrophils activated by PMA (Kaplan et al., 1984). Finally, Perez and co-workers have shown that the NSAID meclofenamic acid inhibits C5a-induced neutrophil functions, while not inhibiting the binding of radiolabeled C5a (Perez et al., 1987). Therefore, NSAIDs must exert actions other than simply affecting the binding of one or another chemoattractant.

Signaling through the plasma membrane is probably regulated by one of the family of G-proteins (Yuli et al., 1982). Data compiled by Abramson et al. (1991) indicate that salicylates and other NSAIDs interact with a G protein in the neutrophil plasmalemma and thereby uncouple post-receptor signaling events. In this study, both salicylate and piroxicam interfered with the capacity of pertussis toxin to inhibit neutrophil activation. Pertussis toxin can ribosylate the alpha subunit of the neutrophil G protein; thus, NSAIDs may interfere with some G protein event (Figure I).

Most evidence would place the action of NSAIDs distal to receptor-ligand engagement, but proximal to the activation of protein kinase C or the rise of
cytosolic calcium. Non steroidal anti-inflammatory drugs share a phenol structure and, as mentioned earlier, are acidic, lipophilic molecules that would be expected to partition into a lipid environment, such as exists within the neutrophil plasmalemma. Assigning each NSAID to only one mechanism of action, however, is likely too simplistic considering their differing effects upon ROS production in the neutrophil.

**THE NSAID KETOROLAC TROMETHAMINE (TORADOL™)**

**Parenteral NSAIDs**

Until recently, NSAIDs were regarded as weak analgesics with a potent antiplatelet effect that severely limited their peri- and postoperative usefulness. However, the introduction of parenteral NSAIDs in both human and veterinary medicine has prompted their use for the treatment of postoperative pain. Ketorolac (commercially available as the tromethamine salt form to improve stability and oral bioavailability) and indomethacin are products marketed in North America for humans, while diclofenac is available in the European Community. Injectable indomethacin has been marketed in the USA since 1985, but is only licensed for the treatment of patent ductus arteriosus in newborn infants (Abramowicz, 1990). Phenylbutazone is commercially available as a parenteral formulation to veterinarians in North America while dipyrrone, another injectable NSAID, is only available in Canada. They are licensed for the treatment of arthritis and allied disorders in dogs (Bennet, 1995). Ketoprofen, an NSAID prescribed by general practitioners for a number of years, has been
recently introduced into the North American veterinary market. It is available in both oral and injectable form and is licensed for the treatment of inflammation and pain in companion animals associated with musculoskeletal disorders, fever and postoperative pain (Bennet, 1995).

**Ketorolac Tromethamine**

Since its introduction into the Canadian market in April, 1991, much attention has been given to ketorolac as an adequate or even preferred parenteral analgesic for the treatment of postoperative pain in humans. More specifically, the parenteral and oral formulations of ketorolac have been studied extensively in randomized double-blind clinical trials that have involved placebo and active controls. Ketorolac seems to have an efficacy similar to opioid-related drugs in humans (Yee et al., 1986; O'Hara et al., 1987; Stanski et al., 1990) as the sole agent for the treatment of moderate to severe postoperative pain. As well, the dose of opioids may be reduced when used concurrently with ketorolac (Gillies et al., 1987; Forbes et al., 1990).

Ketorolac's analgesic benefits have not been restricted to people. A recent randomized double-blind pain study using dogs demonstrated that ketorolac alone was equivalent to or better than the narcotics butorphanol and oxymorphone and the NSAID flunixin meglumine in relieving pain as a result of shoulder arthrotomy, and laparotomy (Mathews et al., 1996). In murine studies, ketorolac produced up to 800 times the analgesic effect of acetylsalicylic acid (Rooks et al., 1982).
The analgesic activity of ketorolac has been related to its PG synthesis-inhibiting activity (Rooks et al., 1982; Rauk and Laifer, 1993); however, other possible mechanisms have been studied to explain its potency and efficacy. It has been demonstrated that ketorolac is unable to bind to mu, kappa and delta opioid receptors (Lopez-Munoz et al., 1987). However, Uphouse et al. (1993) reported that nor-binaltorphimine (a kappa antagonist) can antagonize the analgesic effect of ketorolac. Domer (1990) suggested that endogenous opioid release could be involved in the analgesic effect of this drug. The exact mechanism(s) of action involved in the production of the analgesic effect of ketorolac, however, remains to be elucidated.

**Ketorolac Tromethamine Chemistry**

Ketorolac is a member of the pyrrolo-pyrrole group of NSAIDs. The chemical name for the drug is (±)-5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid, 2-amino-2-(hydroxymethyl)-1,3-propanediol. Ketorolac is a mixture of R-(+)- and S-(−)-ketorolac. As with most NSAIDs, the biological effect is associated with the S form of the drug (Hayball et al., 1993). Ketorolac has a pKa of 3.5, thus allowing it to be absorbed rapidly in an acidic environment such as the stomach. Once absorbed into the circulation (pH 7.4), the majority of the drug is in the ionized form. Ketorolac has an n-octanol/water partition coefficient of 0.26 suggesting that it is minimally distributed throughout the body after equilibration. The molecular weight of ketorolac tromethamine is 376.4 (Fleeger, 1996).
Tromethamine, in addition to being a chemical component of Toradol™ (TRIS, THAM), has been used successfully for the treatment of acidosis in dogs (Moon et al., 1997) and humans (Arieff, 1993). Whether tromethamine has an effect on neutrophil ROS production or endogenous PGE2 concentrations is unknown and would require specific experiments using clinical in vivo or in vitro concentrations of the drug.

**Ketorolac Pharmacokinetics in Humans and Animals**

Pharmacokinetics describes the liberation, absorption, distribution, metabolism (biotransformation), and excretion of a drug over time. It is a mathematical description of concentration changes of drugs within the body. An understanding of pharmacokinetics is essential to interpret clinical drug literature and to understand dosage regimens, particularly for the application of dosing regimens in diseased patients.

Pharmacokinetic modelling is the science of collecting drug concentration over time data, breaking it down into best fit exponential decay curves using statistical methods and then calculating meaningful pharmacokinetic parameters. A common approach to studying the pharmacokinetic behaviour of drugs is to depict the body as a series of compartments. In many instances these compartments, which are mathematical entities only, have no anatomical basis, but are useful in describing the disposition pharmacokinetics of a drug. Blood or plasma concentrations are usually measured when pharmacokinetic studies are performed because direct tissue measurement is usually not feasible or
practical.

Multiple pharmacokinetic studies of ketorolac have been carried out on healthy human volunteers (Mroszczak et al., 1985; Mroszczak et al., 1987; Jung et al., 1988). In all of these studies, ketorolac and a fluorinated internal standard were extracted from acidified plasma with a volume to volume (v/v) mixture of ethyl acetate/hexane (30:70). The organic phase then was evaporated and purified further by partitioning between hexane and methanol (v/v)(9:1). Methanol was then evaporated, the residue reconstituted in methanol and a portion subjected to reverse phase high performance liquid chromatography (HPLC). The mobile phase of the C\textsubscript{18} chromatography column was a mixture of acetonitrile and acidic water (v/v)(34:66) at pH 2.75. Detection of ketorolac was at an ultra violet wavelength of 313 nm. The limit of detection of most assays was as low as 5 ng of ketorolac per mL of plasma. Standard curves were linear over a range of 10 to 5000 ng per mL.

Jung et al. (1988) demonstrated a number of important pharmacokinetic facts about single dose IV and PO ketorolac in humans. After IV administration or absorption, ketorolac tromethamine dissociates into the anion form of ketorolac at physiological pH. The decline in plasma ketorolac levels following IV administration is best described by either two- or three- compartment models with elimination from the central compartment. The plasma half life of the drug is quite short (5-6 h) when using an average dose of 0.2 mg/kg. The steady state volume of distribution of 0.111 L/kg is a result of extensive plasma protein
binding (99.2%), drug ionization and polarity (Mroszczak et al., 1987) and suggests little distribution of ketorolac to the tissues. The multicompartmental character of the disposition of ketorolac is indicated by the steady state volume of distribution \( (V_{ss}) \), which was approximately 35% smaller than the volume of distribution during drug elimination \( (V_p) \). Plasma clearance \( (Cl_p) \) follows first order kinetics at a rate of 0.35 mL/min/kg. The absorption of ketorolac following PO administration is rapid (i.e. time to reach maximum plasma concentration \( (t_{max}) \) was less than one hour) the drugs PO bioavailability \( (F) \) complete (100%) and the drugs systemic decay mirrors the IV route.

The pharmacokinetics of ketorolac has been studied in species other than humans. Intravenous, PO and IM ketorolac tromethamine has been administered to cynomolgus monkeys, rabbits, rats, and mice (Mroszczak et al., 1987). Kotorolac was absorbed rapidly \( (t_{max} < 1.0 \text{ h}) \) and efficiently \( (> 87\%) \) following PO and IM doses in all species. The plasma half-life of ketorolac ranged from 1.1 h (rabbits) to 3.8 h (mice) with no consistent changes in half-life with dose or route of administration. The total plasma clearance ranged from 0.43 mL/min/kg (mice) to 2.44 mL/min/kg (rats) following IV administration. The protein binding of ketorolac ranged from 72% (mice) to 98.3% (monkeys). The steady state volume of distribution ranged from 0.07 L/kg (monkeys) to 0.38 L/kg (rats) following the IV dose. Linear pharmacokinetics of ketorolac were observed (as demonstrated by proportional increases in area under the curve \([AUC]\) and maximum plasma drug concentration \([C_{max}]\)) in the mouse after single
PO dose incremental increases of ketorolac ranging from 0.25 to 16 mg/kg.

**Ketorolac Metabolism**

In the same multiple species experiment just described, \(^{14}\)C-labeled ketorolac was administered IV, PO and IM in solutions to humans, cynomolgus monkeys, rabbits, rats and mice (Mroszczak et al., 1987). Radioactivity was excreted predominantly in the urine, ranging from 78.9% (mice) to 92% (humans) to 100% (monkeys) following IV dosing. The rest of the drug was recovered in the feces. The dose excreted into urine was primarily as ketorolac conjugates, ketorolac and para-hydroxy-ketorolac in humans. The major metabolite of ketorolac, the acyl-glucuronide, appears to account for 72-77% of the IV dose in humans. The other major metabolite of ketorolac (para-hydroxy-ketorolac) represents up to 12% of the drug excreted into the urine. Para-hydroxy-ketorolac appears to have 20% of the anti-inflammatory activity and 1% of the analgesic activity of ketorolac (Mroszczak et al., 1990).

As with the parent drug, the pharmacokinetics of para-hydroxy-ketorolac are linear following single IM dose of 60-90 mg ketorolac (Jung et al., 1989). The monkey was similar to humans with respect to kinetics, but did not form the para-hydroxy-ketorolac metabolite. The rabbit was unusual in that it exhibited substantial presystemic metabolism (50%). The rat excreted a much higher percentage of radioactivity into the feces and formed an additional unidentified metabolite. Metabolically, the mouse was most similar to humans.

Two other features of ketorolac metabolism are worth noting. Firstly, the
possibility of renal glucuronidation has been suggested in order to explain the apparently high urinary recovery of the acyl-glucuronide metabolite. This suggestion was based on in vitro studies using microsomes of renal origin (obtained from rabbits), which possessed six-fold more activity than microsomes of hepatic origin (Brocks and Jamali, 1992). Unfortunately, details of this experiment were not published. Considerable renal glucuronidation has been seen with other NSAIDs (ibuprofen) in the perfused rat kidney (Ahn et al., 1991).

Secondly, the extraction ratio of ketorolac is low (Mroszczak et al., 1987; Jung et al., 1988) and, therefore, the elimination of ketorolac is highly dependent on intrinsic clearance through metabolism of the unbound fraction in plasma, and not dependent on hepatic blood flow.

**Relationship Between Pharmacokinetics and the Analgesic Effect of Ketorolac**

Information on the relationship between analgesic effect and plasma levels of an analgesic is scant. Dahlstrom et al. (1978) proposed a model that related the analgesic effect of morphine to mathematically derived effector compartments in the rat. Bhargava et al. (1991) reported that morphine has a linear relationship between the cumulative area under the functionality index against time curve (AUCₑ) against the cumulative area under the curve (AUC) for morphine concentration versus time.

In the case of NSAIDs, information about the relationship between plasma levels and analgesic effect is limited and inconclusive. In addition, plasma
concentrations of NSAID do not appear to correlate well with action in tissue. For example, the duration of action of many NSAIDs is longer than the presence of drug in the plasma (Lees, 1997). This appears to be caused by the local action of NSAIDs, sequestration of NSAIDs in inflamed tissues, and possibly residual effects of NSAIDs on pain and the inflammatory process. Walker and Kasmerski, (1988) reported on the pharmacokinetic and pharmacodynamic evaluation of diflunisal in the rat; however, they did not find a model that related the plasma concentration of diflunisal with its analgesic effect. Granados-Sotos et al. (1992) however, reported a model that related acetaminophen’s plasma concentrations with its analgesic effect in the pain-induced functional impairment model (PIFIR).

In a 1995 study, these same researchers performed PIFIR using PO ketorolac tromethamine and found that ketorolac produced a dose-dependent analgesic effect, measured as the recovery of the functionality in an injured limb (Granados-Sotos et al., 1995). Recovery of functionality of the injured limb reached its maximum effect at a dose of 3.2 mg/kg or higher. When the functionality index was plotted against ketorolac blood concentrations, a direct relationship was observed that was well described by the sigmoidal maximal effect model. In dogs, Mathews et al. (1996) have observed (using a 0-10 pain scoring scale) that, at a dose of 0.5 mg/kg IM, ketorolac was an effective analgesic when administered to dogs who had undergone shoulder arthroscopy or laparotomy. More specifically, when completely recovered from anesthesia
(i.e. 12 h after extubation), 100 percent of dogs receiving shoulder arthrotomy were weightbearing on the operated limb.

In a single dose experiment of ketorolac in humans who had dental surgery to remove at least one third molar, Forbes et al. (1990) administered 10 mg of PO ketorolac to patients at the postoperative onset of moderate to severe pain. The mean maximum pain relief was achieved three hours after drug administration. This is in contrast to pharmacokinetic data of ketorolac in healthy volunteers in which the time to reach maximum drug concentration in the plasma (t\text{max}) is approximately one hour or less. In another study involving patients who had undergone major surgery (Stanski et al., 1990) and received an IM dose of ketorolac (30-90 mg), the time-effect curve of pain relief also indicated a somewhat delayed onset (60-120 min) compared with the t\text{max} obtained from pharmacokinetic studies of healthy volunteers (37-58 min). The only published ketorolac therapeutic response values are by Benet et al. (1996) indicating effective analgesic plasma concentration in 50% of the human population (EC\text{50}) to be 0.1 to 0.3 μg/mL.

**Ketorolac Gastrointestinal Toxicity**

Patients receiving NSAIDs are at risk for upper gastrointestinal tract bleeding (Carson et al., 1987). In general, people using NSAIDs have a three-fold increased probability of developing a serious gastrointestinal adverse event than non-NSAID users (Gabriel et al., 1991). Several published reports describe gastrointestinal ulceration associated with the use of ketorolac (Erstad and
Neumayer, 1992; Estes et al., 1993; Fuller and Kalekas, 1993; Wiedrick et al., 1994; Wolfe et al., 1994). From 1990 to 1993, approximately 26 million patients received ketorolac, and, according to Hoffman La-Roche, 73 deaths were possibly associated with the drug (Choo, 1993). This prompted the company to reduce the initial parenteral dose from 30 to 10 mg and the maximum duration of parenteral treatment from five to two days (Committee on safety of medicines, 1993).

The mechanisms by which NSAIDs induce gastrointestinal damage are complex and not yet fully understood. At present, it is proposed that there are three possible ways by which the potentially damaging NSAID and/or its metabolite(s) gain access to the gastric mucosa; firstly a topical effect; secondly a systemic due to homeostatic prostaglandin (PG) inhibition; and thirdly, by enterohepatic circulation in the bile and possible intestinal reflux into the stomach (Price and Fletcher, 1990).

**Topical Gastrointestinal Toxicity**

Non-steroidal anti-inflammatory drugs are weak organic acids. When administered orally they damage the mucosal barrier of the stomach as a consequence of becoming concentrated within the mucosal cell. Most NSAIDs have the common physicochemical properties of high lipid solubility and low pKa which encourages concentration within mucosal cells. The high concentration of NSAID in mucosal cells can lead to uncoupling of oxidative phosphorylation in cell mitochondria leading to decreased adenosine triphosphate (ATP) formation.
This causes the cell membrane permeability to change, thus allowing back diffusion of hydrogen ions and subsequent mucosal damage (Schoen and Vender, 1989).

**Secondary Gastrointestinal Toxicity: The Role of Prostaglandin Inhibition**

It is well established that PGs have important physiological roles in the gastrointestinal tract (Rask-Madsen et al., 1990), including regulation of blood flow, stimulation of mucous production and facilitation of cell division (Robert, 1979; Cassidy and Lightfoot, 1980; Granger et al., 1980; Lewis, 1983; Levi et al., 1990; Wallace et al., 1992). As a group, NSAIDs inhibit both the constitutively expressed (cytoprotective) cyclo-oxygenase-1 (COX-1) enzyme and the inducible (inflammatory) COX-2 enzyme. Not surprisingly, therefore, inhibition by NSAIDs of ‘cytoprotective’ PGs has long been regarded as a major factor in the development of gastric ulceration and hemorrhage. The role of PG inhibition in mediating NSAID-induced injury has been supported by studies showing that gastric damage occurs with parenteral as well as PO administration of NSAIDs (Grossman et al., 1961; Main and Whittle, 1975).

The issue as to whether NSAID induced PG inhibition, specifically PGE₂, on its own is enough to initiate intestinal damage is clearly contentious. Redfern and Feldman (1989) produced intestinal lesions by raising antibodies toward PGs. Other data, however, suggest that there is no convincing temporal relationship between inhibition of COX and intestinal macroscopic damage (Whittle, 1981). There also appears to be a lack of relationship between the
severity of injury and the reduction of mucosal PGs; a 95% inhibition of PG synthesis can be achieved without apparent damage (Ligumski et al., 1983; Levine et al., 1988). Rainsford and Willis (1982) suggest that gastric damage induced by acidic NSAIDs is primarily due to their effects in altering membrane permeability, and that their inhibitory effect on PG synthesis, although additive, is only secondary.

**Ketorolac Renal Toxicity**

There have been several case reports of renal failure following the use of ketorolac tromethamine (Boras-Uber and Brackett, 1992; Schoch et al., 1992; Fong and Gora, 1993; O'Leary et al., 1993; Pearce et al., 1993; Smith et al., 1993). The patients in these reports all had known risk factors for the development of NSAID-induced renal failure and some had prolonged administration of ketorolac.

The kidney, especially the renal medulla, is one of the most active PG producing tissues (Folkert et al., 1984). It is also known that most nephron segments from the glomerulus to the collecting duct are capable of synthesizing PGs (Grenier et al., 1981), although the exact pattern of PG production within the kidney varies along the nephron segment accruing to the degree of stimulation present. In addition, renal interstitial cells may produce PGs and the COX enzyme has been identified in the afferent arteriole (Zusman and Keiser, 1972).
The most important role of PGs in the modulation of renal function is in control of blood flow and the glomerular filtration rate (GFR). Animal and human studies have shown that renal vasodilating PGs play a minor role in maintaining renal perfusion in normal individuals (Schnerman et al., 1984). In addition, they control the state of contraction of the mesangial cell which support the capillary loops in the renal glomeruli (Scharschmidt et al., 1983). Despite this, there is no good evidence that PGs are essential to maintain renal function in the normal unstressed kidney. The majority of clinical studies have demonstrated that inhibition of the COX system with NSAIDs does not have a significant effect on renal hemodynamics in normal kidneys (Lifschitz, 1983).

However, in patients with altered renal hemodynamics, such as those with decreased actual or effective circulating blood volume, renal PGs are critical in maintaining renal perfusion (Harris, 1992). Patients with diminished circulating volume respond with increased secretion of catecholamines and activation of the renin-angiotensin system (i.e. the production of angiotensin II), which leads to vasoconstriction. Other noted renal vasoconstrictors in pathological conditions include other eicosanoids such as leukotrienes or TXA₂, arginine vasopressin and sympathetic nerve activation. Upon activation of one or more of these systems the kidney responds with local release of vasodilating PGs to maintain local renal perfusion.

Non-steroidal anti-inflammatory drugs, by decreasing the synthesis of PGs (either PGE₂ or PGI₂), allow unopposed vasoconstriction. For example,
30% blood loss in the dog, which activates both the renal sympathetic nerves and angiotensin II vasoconstrictor systems, produces a significant decrease in renal blood flow and GFR only when the COX system has been inhibited by pretreatment with indomethacin (Henrich et al., 1978). Patients with decreased blood volume, cardiac disease, renal insufficiency and dehydration, as well as the elderly, are therefore potentially susceptible to acute NSAID-induced renal insufficiency (Koren et al., 1980; Blackshear et al., 1983; Perazella and Buller, 1993; Corwin and Bonventre, 1984; Poirer, 1984; Toto et al., 1986; Whelton et al., 1990).
Figure I. Stimulus response coupling in the neutrophil. X represents the insertion into the lipid bilayer and the potential uncoupling of protein-protein interactions by ketorolac. G = G protein; FMLP = formyl-methionine-leucine-phenylalanine; PMA = phorbol myristate acetate; PLC = phospholipase C; PIP2 = phosphatidylinositol 4, 5-bisphosphate; IP3 = inositol 1, 4, 5-triphosphate; DAG = sn-1, 2-diacylglycerol; PKC = protein kinase C; GTP = guanosine triphosphate; ROS = reactive oxygen species; Ca = calcium; -ve = inhibitory effect; Rap1 = low-molecular-weight G protein; NADPH oxidase: nicotinamide adenosine dinucleotide phosphate oxidase (reduced formed). Diagram adapted from Abramson et al., 1989.
RATIONALE

Ketorolac tromethamine is a unique non-narcotic, non-steroidal anti-inflammatory drug (NSAID) that has demonstrated analgesic efficacy equal to, or surpassing, some of the opioids, and its pharmacokinetics, safety and usefulness in human beings have been documented in detail (Buckley and Brogden, 1990). The drug has also been used successfully in dogs for the treatment of postoperative pain at an extrapolated dose of 0.5 mg/kg (Mathews et al., 1996). Despite this, some animals develop signs of toxicity that may be dose and/or frequency dependent and almost nothing is known about its pharmacokinetics and mechanisms of action. Therefore, the purpose of the literature review was to comprehensively document three important ideas:

(1) the importance of the E series prostaglandins (PG) in cellular homeostasis, pain and inflammation and their relationship to the (NSAIDs) including ketorolac tromethamine.

(2) the effect of ketorolac and the (NSAIDs) on the production of neutrophil reactive oxygen species (ROS) as a 'possible' analgesic mechanism of action for the drug.

(3) the pharmacokinetics of ketorolac in animals and people; the relationship of plasma concentration to analgesic effect; and, ketorolac specific gastric and renal toxicity as it relates to the inhibition of prostanoid production.
A comprehensive discussion of these ideas has lead to the establishment of the following investigational objectives:

(1) to determine the pharmacokinetics of ketorolac following intravenous (IV) and oral (PO) administration of ketorolac tromethamine to healthy dogs.

(2) to calculate pharmacologically-sound IV and PO ketorolac tromethamine doses for dogs by correlating our pharmacokinetic findings with reported therapeutic plasma levels in human beings and other animals.

(3) to establish whether endogenous PGE$_2$ exists in the plasma of healthy dogs.

(4) to determine if, and to what magnitude, ketorolac alters endogenous PGE$_2$ levels in the plasma of healthy dogs after single dose IV and PO administration of ketorolac tromethamine.

(5) to determine the effect of single dose IV and PO ketorolac tromethamine on neutrophil reactive oxygen species (ROS) production in healthy dogs.
CHAPTER II
PHARMACOKINETICS OF KETOROLAC TROMETHAMINE (TORADOL™)
AFTER INTRAVENOUS AND ORAL SINGLE DOSE ADMINISTRATION IN DOGS

INTRODUCTION

Despite public expectations and practitioners' desires to provide the best care for their patients, postoperative pain in veterinary medicine is often unrecognized and poorly treated (Kitchen et al., 1987; Bonica, 1992). The recognition and alleviation of such pain are essential in the management of veterinary surgical patients because of the deleterious physiological effects of postoperative pain and the need to address humane and ethical concerns. Traditionally, the opioids such as morphine, oxymorphone, meperidine, pentazocine, fentanyl and butorphanol have been used to treat the pain associated with surgery. However, veterinary clinicians may be reluctant to use schedule "N" opioids in their practice, citing concerns over Drug Enforcement Administration regulations and opioid side effects, including respiratory and cardiovascular suppression, sedation and euphoria (Hansen, 1994). Other currently available useful analgesics include α₂-adrenergic agonists and non-steroidal anti-inflammatory drugs (NSAIDs).

The side effects of bradycardia and hypotension have precluded the routine use of the α₂-adrenergic agonists in systemically ill animals in the past; however, newer members of this group (e.g. medetomidine) may be more useful. Among the NSAIDs, phenylbutazone, dipyrone and flunixin meglumine are available in
parenteral formulations for veterinary use, yet none are approved for the treatment of severe postoperative pain in dogs. Parenteral and oral ketoprofen has recently been approved in Canada for the treatment of postoperative pain in companion animals, but thorough documentation of its clinical usefulness is lacking at this time.

Ketorolac tromethamine (Toradol™) is a relatively new potent, non-narcotic analgesic agent with cyclo-oxygenase (COX) inhibitory activity which is approved for use in humans (Muchowski, 1982; Rooks et al., 1982; Rooks et al., 1985; Laneuville et al., 1994). It is commercially available in both oral and injectable formulations. The drug is classified as an NSAID with the chemical name (±)-5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid, 2-amino-2-(hydroxymethyl)-1,3-propanediol. It has been approved by the United States Food and Drug Administration since 1988, and the Canadian Health Protection Branch since 1991, for the short-term treatment of moderate to severe acute pain in humans (Hoffman-La Roche Ltd., 1996). Ketorolac tromethamine has been shown to be superior to both aspirin (Bloomfield et al., 1984) and an aspirin-codeine combination (Forbes et al., 1990) for the treatment of moderate to severe post-operative pain in humans. In addition, ketorolac has similar analgesic efficacy to opioid-related drugs in humans (Yee et al., 1986; O'Hara et al., 1987; Stanski et al., 1990), and has been used concurrently with opioids for its narcotic-sparing effects (Gillies et al., 1987; Forbes et al., 1990).

Although ketorolac is not approved for use in animals, a recent pain study (Mathews et al., 1996) documented that ketorolac at a dose of 0.5 mg/kg IM every
six hours for three treatments is equivalent, or better than, the narcotics butorphanol and oxymorphone and the NSAID flunixin meglumine in relieving pain following shoulder arthrotomy or laparotomy in dogs. The dose chosen and its frequency of administration in that study were extrapolated from the human literature since there have been no pharmacokinetic data regarding ketorolac reported for the dog. An assumed maximum dose and frequency was selected in order to examine possible gastrointestinal or renal lesions associated with ketorolac use in dogs.

The objective of this study was to determine the pharmacokinetics of single dose (0.5 mg/kg) intravenous (IV) and oral (PO) ketorolac administration in dogs and to calculate a pharmacologically-sound dosing regimen based on previously reported therapeutic plasma concentrations in humans and other animals. Ketorolac tromethamine is not currently approved for IV administration; however, it has been successfully administered by this route to humans in clinical situations (Jung et al., 1988; Peirce et al., 1990; Larsen et al., 1993) and, therefore, it was used by this route in dogs.

MATERIALS AND METHODS

Experimental Design and Pharmacokinetic Study

Six mixed breed pre-conditioned dogs 1-5 years old were used in this study. All dogs were treated according to the Guide to the Care and Use of Experimental Animals, Volumes 1 and 2 (Olfert et al., 1993). The mean weight (± SD) of the dogs was 27.9 (6.5) kg. There were four intact females and two intact males in the study. The dogs were determined to be healthy by physical examination, complete blood
were fasted the night before drug administration and then fed their normal volume of adult maintenance diet eight hours after drug administration. Dogs had access to water at all times during the study.

The study was performed using a randomized crossover design with each dog initially assigned to one of two groups (IV or PO). All dogs were allowed a seven day washout period before the administration of the drug by the other route and the blood sampling was then repeated. Each group of three dogs received either IV ketorolac tromethamine administered into the cephalic vein or the PO capsule at the clinically effective analgesic dose currently employed by the Ontario Veterinary College Intensive Care Unit (0.5 mg/kg). Venous blood samples (8.5 mL) were taken from the dogs through pre-placed 19 gauge x 12 inch jugular catheters (Intracath™, Becton Dickinson, Trudell Medical, London, Ontario, Canada) at 0 (before treatment), 3, 5, 10, 15, 20, 30, 45, 60, and 90 minutes, and at 2, 3, 4, 6, 8, 10, 12, 24, 48 and 96 hours following treatment. The blood samples were collected in heparinized vacutainers (Becton Dickinson, Rutherford, New Jersey, USA), promptly centrifuged (room temperature, 10 min, 1000 x g) and the plasma frozen in 2 mL sterile Cryotubes™ (Nalgene Company, Rochester, New York, USA) at -70°C until analyzed.
Preparation of Spiking Solutions and Calibration Curve Standards

A 100 μg/mL stock solution of ketorolac was prepared by adding 1.47 mg of ketorolac tromethamine (a gift from Hoffman-La Roche Ltd., Mississauga, Ontario, Canada) to 10 mL HPLC grade methanol (Fisher Scientific, Toronto, Ontario, Canada). Spiking solutions of 0.1, 0.2, 0.4, 1, 2, 10, 20, and 30 μg/mL were then prepared by serial dilution of the stock solution with methanol/water (9:1) (Mili-Q™ Water, Millipore Systems Inc., Mississauga, Ontario, Canada). A 2 μg/mL spiking solution of flourinated ketorolac (a gift from Hoffman-La Roche Ltd., Palo Alto, California, USA) was prepared by diluting 2 mL of a 100 μg/mL stock solution with 98 mL methanol:water (9:1).

Spiking solutions were then diluted 1:10 with untreated canine plasma (OVC Teaching Hospital) to produce eight, 1 mL standards, containing 10, 20, 40, 100, 200, 1000, 2000, and 3000 ng/mL ketorolac tromethamine. To each one mL standard was added 0.1 mL of the 2 μg/mL (200 ng) flourinated ketorolac spiking solution.

Ketorolac Extraction and Analysis

The ketorolac assay was performed using reverse phase high performance liquid chromatography (HPLC) (Wu et al., 1986). The extraction of ketorolac from plasma was from a method developed in our laboratory. On the day of analysis, 2 mL plasma samples were thawed at room temperature, vortexed briefly, and centrifuged for 5 min at 1000 X g. One mL aliquots were spiked with 200 ng of the internal standard, flouro ketorolac. The samples were then acidified with 100 μl of 0.5 M
sodium acetate (adjusted to pH 3.0 with glacial acetic acid, Fisher Scientific, Toronto, Ontario, Canada), vortexed and then diluted with 2 mL acidic water (adjusted to pH 2.75 with phosphoric acid, Fisher Scientific, Toronto, Ontario, Canada) before application to Waters Sep-Pak™ C₁₈ cartridges (Waters Corp., Milford, MA, USA) which had been previously activated with 5 mL 100% methanol and washed with 10 mL acidic water. Samples were percolated through the Sep-Pak™ and washed with 2 mL acidic water followed by 5 mL hexane (Fisher Scientific, Toronto, Ontario, Canada). Extracts were eluted with 8 mL diethyl ether (Fisher Scientific, Toronto, Ontario, Canada) and evaporated under nitrogen at 35°C. The dried residue was redissolved in 100 μL of 50% acetonitrile (Fisher Scientific, Toronto, Ontario, Canada) in acidic water (pH 2.75), briefly vortexed, and 10 μL was injected into the chromatographic system.

A Waters HPLC (Waters Corp., Milford, MA, USA) equipped with a Model 6000A pump, a Model 441 UV absorbance detector, a WISP 710B autosampler and a Nova-Pak™ C₁₈ column (100 mm x 8 mm, 4 μm) was used for the chromatography analysis. The mobile phase consisted of acetonitrile/acidic water (34/66, v/v, pH 2.75), and the flow rate was 2 mL/min. The absorption wavelength was set at 313 nm. Waters Baseline 810 Chromatography software was used to record chromatograms, integrate peaks and plot a standard curve using internal standard correction.
Pharmacokinetic and Statistical Analysis of Individual Curves and Mean Data

The individual plasma concentration-time data were fit to monoexponential, biexponential, and triexponential equations. Initial estimates for each equation were determined by a curve stripping computer program (Slidewrite Plus™, 1990). The IV and PO plasma ketorolac versus time profiles were then analyzed by iterative nonlinear least squares regression analysis with equal weighting of the data using commercial software (PCNONLIN™, 1992). The number of exponential terms required to describe the data for each individual dog was determined by application of the F test criterion ($p \leq 0.05$) (Ludden et al., 1994). Standard pharmacokinetic equations were used to describe the disposition parameters of a 0.34 mg/kg dose of the parent drug, ketorolac (Baggot, 1977; Gibaldi and Perrier, 1982; Rowland and Tozer, 1989). By means of a statistical software package (SAS™ For Windows, 1996) pharmacokinetic parameters were then compared with respect to route of administration using analysis of variance (ANOVA). A 95% confidence level ($p \leq 0.05$) was used in all tests.

Two of the dogs in the IV study were best described by the triexponential equation: $C_{p,t} = Pe^{-\alpha t} + Ae^{-\beta t} + Be^{-\gamma t}$ where $C_{p,t}$ is the plasma concentration at any time $t$, $P$ is the y-intercept of the initial distribution phase, $A$ is the y-intercept for the second distribution phase, $B$ is the y-intercept for the elimination phase, $e$ is the base of the natural logarithm ($2.71828$), $\alpha$ is the slope of the initial distribution phase, $\beta$ is the slope of the second distribution phase, and $\gamma$ is the slope of the elimination phase. Drug distribution and elimination for the remaining dogs in the IV...
study, and all dogs in the PO study, were best described by the biexponential equation: $C_p(t) = Ae^{-\alpha t} + Be^{-\beta t}$.

The elimination half-life ($t_{1/2\beta}$) was calculated by $t_{1/2\beta} = 0.693/\beta$. The area under the plasma concentration versus time curves for both the IV ($\text{AUC}_{IV(0-t\infty)}$) and the PO ($\text{AUC}_{PO(0-t\infty)}$) study were calculated by the method of trapezoids (Rowland and Tozer, 1989). The area under the first moment curve ($\text{AUMC}_{(0-t\infty)}$) was calculated in a similar fashion but the product of time and drug concentration vs. time was integrated rather than drug concentration vs. time.

The total plasma clearance ($Cl_p$) was calculated from $Cl_p = \text{dose}/\text{AUC}_{IV}$. The volume of distribution during the terminal elimination phase ($V_p$) was calculated by the ratio $Cl_p/\beta$. The apparent steady state volume of distribution ($V_{ss}$) was calculated using $V_{ss} = (\text{Dose}_{IV})(\text{AUMC})/\text{AUC}_{IV}^2$. The absolute bioavailability (F) was determined as the ratio (%) of the area under the curve (AUC) after PO dosing to that after IV dosing. Ketorolac peak plasma concentrations ($C_o$) in the IV study were calculated using $C_o = P + A + B$. Peak plasma concentrations ($C_{max}$) of ketorolac and times to reach peak concentration ($t_{max}$) for the PO study were determined from the individual plasma concentration-time curves. Average steady state concentrations ($Cav_{ss}$) for ketorolac after repeated IV and PO dosing of 0.5 mg/kg were determined by $Cav_{ss} = F \cdot \text{total dose}/Cl_p \cdot \tau$ where $\tau$ is the frequency of dosing.

**RESULTS**

Calibration curves using spiked plasma were calculated daily with the coefficient of variation ($r$) being no less than 0.9996 on any given day. None of the intra-assay
and inter-assay coefficients of variations were greater than 3.9%. The limit of detection was 5 ng of ketorolac per mL of plasma and was determined by establishing background noise at 0.02 Absorbance Units Full Scale (AUFS). The assay was linear over the range of 0.01 μg to 3.00 μg of ketorolac per aliquot of plasma using 1.0 mL of plasma for analysis. Recovery of ketorolac (n=6) was 92.8 ± 1.5%, while recovery of flouro ketorolac (n=6) was 92.3 ± 3.8%.

A semi-logarithmic plot of the mean plasma ketorolac concentrations following 0.5 mg/kg IV administration is shown in Figure 1. Intravenous raw data appear in Appendix I. A summary of the IV pharmacokinetic parameters are listed in Table I. The mean pharmacokinetic parameters of IV ketorolac were characterized by a maximum plasma concentration (Cₚ) of 8.26 μg/mL, a terminal half-life (t₁/₂ₚ) of 4.55 h, a plasma clearance (Clₚ) of 1.25 mL/kg/min, a steady state volume of distribution (Vss) of 0.33 L/kg, an elimination phase volume of distribution (V₁) of 0.60 L/kg and an area under the curve (AUC₀⁻⁻¹ₐₚ) of 7.25 μg *h/mL.

Figure 2 is a semi-logarithmic scatter plot of the raw IV data of the two dogs whose data best fit a triexponential function. The mean triexponential equation Cpₜ = 16.37e⁻⁷⁰.⁰⁰⁰ + 2.6655e⁻¹.⁷₇₅₅₅ + 0.1989e⁻⁰.₇₅₉₁₇ has been superimposed over the raw data as a measure of goodness of fit. Figure 3 is a plot of the mean IV data of the remaining four dogs in the study whose raw data best fit a biexponential function. The mean biexponential equation of the line Cpₜ = 1.6875e⁻².⁵₆₁₄₆ + 1.2430e⁻⁰.₁₃₈₉₀ has been superimposed over the data similar to that of Figure 2. The slopes and intercepts for the equations in Figures 2 and 3 were taken from Table I.
Figure 1 also includes a semi-logarithmic plot of the mean plasma ketorolac concentrations following 0.5 mg/kg PO administration. Raw PO data appear in Appendix II. Table II summarizes the PO pharmacokinetic parameters. The mean pharmacokinetic parameters of PO ketorolac were characterized by time to reach maximum plasma concentration ($t_{\text{max}}$) = 51.2 min, $C_{\text{max}}$ = 1.60 $\mu$g/mL, $t_{1/2\beta}$ = 4.07 h, $\text{AUC}_{\text{po}}(0-36\text{h}) = 7.38$ $\mu$g*h/mL and PO bioavailability (F) = 100.9%.

Figure 4 is a semi-logarithmic plot of the mean PO data of all six dogs in the study. The individual drug distribution and elimination data from each dog were best described by biexponential equation with the mean equation : $C_p = 9.533^{(-1.514)} + 1.4672^{(0.172)}$. The drug elimination portion of the equation of the line has been superimposed over the mean PO decay data as a measure of goodness of fit. The slope and intercepts for this equation were taken from the pharmacokinetic parameters listed in Table II.

Figure 1 also illustrates the similarities between the disposition kinetics of IV and PO ketorolac tromethamine at a dose of 0.5 mg/kg. The mean PO data can almost be directly transposed on the mean IV data. However, when the mean IV and PO data are graphed in a simple linear manner, AUC and plasma decay differences can be detected as illustrated in Figure 5.

Statistical analyses of mean pharmacokinetic parameters with respect to route of administration showed no significant differences ($p<0.05$).
DISCUSSION

The decay of plasma ketorolac following IV administration in this study was best described by either a two or three compartment model depending on the individual animal. These results are similar to the findings of Jung et al. (1988) who found that from a group of 15 healthy young human volunteers receiving IV ketorolac at a dose of 0.14 ± 0.03 mg/kg, a certain number fit a three compartment model significantly better than a two compartment model.

Although Jung's data, like ours, received iterative nonlinear regression analyses using PCNONLIN™ software, there was no discussion of the criteria used for model selection, nor was there any attempt to construct or scrutinize exponential equations using the mean pharmacokinetic parameters. The semi-logarithmic scatter plot in Figure 2 of our results section is an attempt to demonstrate the strength of our pharmacokinetic modelling in the two dogs whose IV data best fit a three compartmental model based on the F test criteria. Up to 12 h post-dosing, the triexponential equation of the line fits well. However, since there is only one data point past 12 h, the mean terminal decay becomes statistically less credible because the terminal decay in the one dog did not contain a 24 h observation.

Examination of the biexponential equation and mean data points of Figure 3 indicates that the mean IV data points past 12 h are less reliable indicators of the terminal decay since the 24 h time point represents only three dogs and the 48 h time point represents only one dog. In other words, the terminal decay of IV
ketorolac past 12 h may have appeared different if the drug had been measurable in more dogs past this point.

In addition to some of the discrepancies seen with the terminal decay of Figure 3, critical evaluation of the mean IV data points from 4 to 8 h shows a rise and fall which may be indicative of recycling of drug from a peripheral compartment. The pharmacological significance of recycling for a drug (e.g. enterohepatic recycling) usually depends on the fraction of the dose excreted in the bile. When the amount is substantial, the effects of a single dose, and more importantly multiple doses, will be prolonged and reflected in the overall elimination rate constant (Baggott, 1977).

An evaluation of the decay component of the PO data in Figure 4 re-emphasizes the limitations of computer modelling. After 12 h, the elimination curve poorly fits the last two mean data points. This is because the means at 24 and 48 h do not contain a complete set of data. Observations below the limit of detection were not included in the mean and, therefore, the points shown are higher than would be expected were data below the range of detectability included. However, since all decay parameters in this study were given equal weight, the final mean of the decay data suffers from the same limitations as the IV data; insufficient data past the 12 h time in a number of animals.

The use of power transformation or weighting of the data to stabilize variance is not an uncommon practice. Since pharmacokinetic modelling relies on non-linear data, small variances at low concentrations demonstrate inflated variability when graphed on a logarithmic axis (Kuehl, 1994). In our study, we believed that
transforming the data was unnecessary since: 1) the F test criteria is useful for selecting models with up to two or three multiexponential terms, provided that the exponential constants are significantly different; 2) the sensitivity of the assay is linear over the concentration range used in the assay, and 3) the intra-assay and inter-assay coefficients of variation are low (Ludden et al., 1994).

The plasma elimination half-life ($t_{1/2}$) of ketorolac in dogs by either route of administration was fairly similar to that reported in humans. Plasma elimination half-lives in humans have been reported as low as 4.5 h (Jallad et al., 1990) and as high as 6.0 h (Mroszczak et al., 1987). In these and similar studies (Jung et al., 1988), a range of dosages anywhere from 0.14 to 1.7 mg/kg was administered by IV, IM and PO routes confirming that, in this range of dosages, first order pharmacokinetic principles apply. Our study used a dose of 0.5 mg/kg administered by the IV and PO route, and not surprisingly, first order pharmacokinetics applied to these data as well.

Another interesting feature of the plasma disposition characteristics for ketorolac shows that, between 1 and 6 hours after drug administration, the average PO levels are higher than the average IV levels (Figure 5). Therefore, assuming that the patient has a normally functioning gastrointestinal tract, switching to PO administration after the first IV dose appears to be a legitimate alternative.

Previous studies in humans have shown a steady state volume of distribution ($V_{ss}$) of 0.11 L/kg (Jung et al., 1988) and a terminal volume of distribution ($V_t$) no larger than 0.25 L/kg (Brocks and Jamali, 1992). The low $V_{ss}$ is most likely a result
of extensive plasma protein binding which is greater than 99% (Mroszczak et al., 1987), and is suggestive of little tissue distribution. The larger $V_\beta$ in these studies (approximately 40%) is suggestive of the multicompartamental character of the disposition of ketorolac, whereby drug leaves the non-vascular compartments to the vascular compartment upon excretion. Our study demonstrates similar multicompartamental behaviour of ketorolac in the dog in that the $V_{ss}$ of 0.33 L/kg is only 55.0% of the $V_\beta$.

Although multicompartamental behaviour of ketorolac appears to take place in the dog as it does in humans, the mean values of the volumes of distribution are noticeably larger in the dog. A possible explanation for this difference may be lower plasma protein binding. While human beings have an exceptionally high degree of binding (>99%), the mouse, for example, has considerably less (72%) (Mroszczak et al., 1987). If plasma protein binding of ketorolac in the dog was similar to the mouse, there would be considerably more free drug in the vasculature for distribution into peripheral tissue. The percentage of ketorolac bound to plasma protein in the dog has yet to be determined.

Pharmacokinetic principles state that, if $V_\beta$ increases and the slope of the terminal decay ($\beta$) remains constant, the clearance rate of a drug must increase (i.e. $Cl = V_\beta \cdot \beta$) (Baggott, 1977). The clearance rate of 1.25 mL/min/kg in this study was greater than that reported in a human study, which ranged from 0.3-0.7 mL/min/kg (Brocks and Jamali, 1992). Clearance of ketorolac in humans is primarily renal (92%), with only 6% of the drug being eliminated in the feces, thus suggesting either
limited biliary excretion or extensive enterohepatic recycling (Brocks and Jamali, 1992). However, in the rat, up to 26% of ketorolac is recovered in the feces and total plasma clearance is reported to be as high as 2.44 mL/min/kg following IV dosing (Mroszczak et al., 1987). If the dog, like the rat, were to have a similar rate of clearance of ketorolac from the plasma (regardless of the major organ of clearance), then this would explain its ability to clear the parent drug or its metabolites from a large volume of distribution and still maintain an elimination constant (β) similar to humans.

The mean peak absorption (t_{max}) of ketorolac tromethamine following PO administration was rapid and similar to human studies which ranges from 0.33 h (Jallad et al., 1990) to 0.83 h (Jung et al., 1988). In animal studies, the t_{max} of ketorolac tromethamine after PO administration has been reported to occur at as low as 0.08 h in the rat (Mroszczak et al., 1987). Ketorolac tromethamine liberates the anion ketorolac in the stomach of all monogastric species. Its pKa of 3.5 means that in a strongly acidic environment the major portion of the drug is in the nonionized form and this passes rapidly into the circulation where it is trapped in the ionized form. Species differences in gastric pH, motility, mucosa type and area, may explain differences seen in the t_{max} amongst species.

The bioavailability of ketorolac tromethamine following PO administration was highly variable in this study and ranged from 43.1 to 161.6%, with a mean of 100.9% (Table II). In addition, the time to reach maximum plasma concentration (t_{max}) after PO dosing was highly variable (17.6 to 128.4 min). One possible explanation for
these variabilities may be technical error associated with the reformulation of the commercially available tablet into fixed dose gelatin capsules. Capsules were used in the study to facilitate better drug handling and to try and ensure that the dogs received the full complement of drug when dosed. Fixed dose studies in other species have been conducted with oral solution (Mroszczak et al., 1987) and whole commercial tablets (Jung et al., 1988); however, to our knowledge, this is the first study to use gelatin capsules filled with ketorolac tromethamine and lactose. The proportion of lactose was not consistent within capsules since it served as a filler and, therefore, each animal's proportional dose of ketorolac tromethamine and lactose was not identical. This could affect dissolution of the drug and rate and extent of absorption.

Studies in humans have demonstrated variability in PO bioavailability as well, with means of 75 to 100% being reported (Brocks and Jamali, 1992). Mean PO bioavailabilities in animal studies have varied even more with a low of 41% reported in rabbits and a high of 115% reported in monkeys (Mroszczak et al., 1987). One possible explanation for these differing bioavailability results is that some studies, such as Mroszczak et al. (1987), used as few as four subjects (human or animal). The wide range of observed values suggests that too few subjects were used to obtain an accurate measure of the extent of absorption.

For an analgesic such as ketorolac the desired pharmacodynamic effect is the removal of pain without adverse side effects (i.e. gastric ulceration or renal compromise). The relationship between the pharmacokinetics and the analgesic
effect of ketorolac has been studied in animals (Granados-Sotos et al., 1995; Mathews et al., 1996) and humans (Forbes et al., 1990; Stanski et al., 1990) using pain scoring systems. In all cases, an analgesic effect was observed no earlier than 0.75 h and no later than 7 h after administration of IM ketorolac tromethamine. Doses have varied in animal studies from 0.5 mg/kg in the dog (Mathews et al., 1996) to 3.2 mg/kg in the rat (Granados-Sotos et al., 1995), and in humans from 0.15 (Forbes et al., 1990) to 1.5 mg/kg (Stanski et al., 1990). In rats, a peak plasma concentration of 4.8 μg/mL correlated with the mean maximal analgesic effect in the formalin pain-induced functional impairment model. In humans, a steady state EC50 (effective analgesic plasma concentration in 50% of the population) of 0.1-0.3 μg/mL is reported (Benet et al., 1996).

Correlation of our pharmacokinetic study with the only documented pain control study of ketorolac tromethamine in dogs (Mathews et al., 1996) suggests that for effective pain control, while minimizing the potential for gastrointestinal or renal lesions, a dosing schedule of 0.5 mg/kg every eight or more hours is rational pharmacotherapy. In quantitative terms, repeated doses of 0.5 mg/kg every eight hours for a maximum of three treatments by either route should yield an average steady state concentration (Cavss) of 0.57 μg/mL. Administering the drug every 12 hours at the same dose should yield a Cavss of 0.38 μg/mL.

The rationale for an 8-12 h dosing regimen is based on Mathews et al. (1996) study evaluating the efficacy and safety of ketorolac. While the dosing regimen was every six hours in that study, it was noted that the six hour frequency was excessive.
In addition, based on clinical experience in acute pain management, it is advised that a parenteral or PO dose of 0.5 mg/kg ketorolac tromethamine not be given for more than two treatments. This is particularly true if the true elimination $t_{1/2b}$ of the drug is longer than this single dose study has demonstrated. Finally, there are specific indications, contraindications and guidelines for pain management in dogs receiving ketorolac tromethamine which should be consulted prior to use to avoid gastric ulceration and hemorrhage and renal failure (Mathews, 1996).
TABLE I. Ketorolac pharmacokinetic decay parameters\(^{†}\) in dogs following IV administration of 0.34 mg/kg\(\|\).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Car</th>
<th>Cas</th>
<th>Cha</th>
<th>Dap</th>
<th>Gra</th>
<th>Jaq</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(C_0) (µg/mL)</td>
<td>18.87</td>
<td>3.19</td>
<td>19.59</td>
<td>1.24</td>
<td>3.58</td>
<td>3.10</td>
<td>8.26</td>
<td>8.5</td>
</tr>
<tr>
<td>(P) (µg/mL)</td>
<td>16.24</td>
<td>NA</td>
<td>16.50</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>16.37</td>
<td>0.18</td>
</tr>
<tr>
<td>(\pi) (1/h)</td>
<td>69.79</td>
<td>NA</td>
<td>70.21</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>70.00</td>
<td>0.30</td>
</tr>
<tr>
<td>(A) (µg/mL)</td>
<td>2.417</td>
<td>2.820</td>
<td>2.914</td>
<td>0.5220</td>
<td>1.951</td>
<td>1.457</td>
<td>1.6875</td>
<td>0.96</td>
</tr>
<tr>
<td>(\alpha) (1/h)</td>
<td>2.156</td>
<td>3.384</td>
<td>1.395</td>
<td>0.8387</td>
<td>2.943</td>
<td>1.880</td>
<td>2.2614</td>
<td>1.14</td>
</tr>
<tr>
<td>(B) (µg/mL)</td>
<td>0.2175</td>
<td>0.9719</td>
<td>0.1803</td>
<td>0.7210</td>
<td>1.633</td>
<td>1.646</td>
<td>1.2430</td>
<td>0.47</td>
</tr>
<tr>
<td>(\beta) (1/h)</td>
<td>0.2809</td>
<td>0.0620</td>
<td>0.0781</td>
<td>0.0385</td>
<td>0.2725</td>
<td>0.1826</td>
<td>0.1524</td>
<td>0.11</td>
</tr>
<tr>
<td>(t_{1/2B}) (h)</td>
<td>2.47</td>
<td>11.17</td>
<td>8.87</td>
<td>18.00</td>
<td>2.54</td>
<td>3.80</td>
<td>4.55(\dagger)</td>
<td>-</td>
</tr>
<tr>
<td>(Cl_p) (mL/kg/min)</td>
<td>3.39</td>
<td>0.52</td>
<td>1.58</td>
<td>0.51</td>
<td>0.97</td>
<td>0.55</td>
<td>1.25</td>
<td>1.13</td>
</tr>
<tr>
<td>(V_{ss}) (L/kg)</td>
<td>0.33</td>
<td>0.35</td>
<td>0.52</td>
<td>0.31</td>
<td>0.22</td>
<td>0.27</td>
<td>0.33</td>
<td>0.10</td>
</tr>
<tr>
<td>(V_b) (L/kg)</td>
<td>0.72</td>
<td>0.50</td>
<td>1.21</td>
<td>0.79</td>
<td>0.21</td>
<td>0.18</td>
<td>0.60</td>
<td>0.39</td>
</tr>
<tr>
<td>(AUMC) (µg·h(^2)/mL)</td>
<td>2.68</td>
<td>123.46</td>
<td>19.83</td>
<td>115.49</td>
<td>21.54</td>
<td>82.10</td>
<td>60.85</td>
<td>52.9</td>
</tr>
<tr>
<td>(AUC_{0-48h}) (µg·h/mL)</td>
<td>1.67</td>
<td>11.00</td>
<td>3.59</td>
<td>11.19</td>
<td>5.82</td>
<td>10.25</td>
<td>7.25</td>
<td>4.13</td>
</tr>
</tbody>
</table>

\(^{†}\) = Definitions for pharmacokinetic parameters are presented in Appendix III
\(^{\|}\) = a 0.5 mg/kg dose of ketorolac tromethamine equals 0.34 mg/kg ketorolac
\(^{\dagger}\) = Harmonic mean
NA = not applicable
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Car</th>
<th>Cas</th>
<th>Cha</th>
<th>Dap</th>
<th>Gra</th>
<th>Jaq</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{\text{max}}$ (min)</td>
<td>30.6</td>
<td>128.4</td>
<td>60.0</td>
<td>17.6</td>
<td>44.1</td>
<td>26.6</td>
<td>51.2</td>
<td>40.6</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>1.6</td>
<td>1.6</td>
<td>1.3</td>
<td>2.0</td>
<td>1.6</td>
<td>1.3</td>
<td>1.6</td>
<td>0.26</td>
</tr>
<tr>
<td>$\alpha$ (1/h)</td>
<td>3.682</td>
<td>0.472</td>
<td>2.153</td>
<td>0.360</td>
<td>1.617</td>
<td>0.798</td>
<td>1.514</td>
<td>1.27</td>
</tr>
<tr>
<td>B (µg/mL)</td>
<td>1.4690</td>
<td>1.5748</td>
<td>1.0743</td>
<td>1.8557</td>
<td>1.6117</td>
<td>1.2122</td>
<td>1.4672</td>
<td>0.28</td>
</tr>
<tr>
<td>$\beta$ (1/h)</td>
<td>0.4649</td>
<td>0.0488</td>
<td>0.1144</td>
<td>0.0430</td>
<td>0.2744</td>
<td>0.0757</td>
<td>0.1702</td>
<td>0.17</td>
</tr>
<tr>
<td>$t_{1/2\beta}$ (h)</td>
<td>1.49</td>
<td>14.2</td>
<td>6.06</td>
<td>16.1</td>
<td>2.53</td>
<td>9.15</td>
<td>4.07†</td>
<td>-</td>
</tr>
<tr>
<td>$\text{AUC}_{0-0.5h}$ (µg•h/mL)</td>
<td>1.44</td>
<td>13.98</td>
<td>4.83</td>
<td>18.08</td>
<td>3.25</td>
<td>4.42</td>
<td>7.67</td>
<td>6.71</td>
</tr>
<tr>
<td>F (%)</td>
<td>86.2</td>
<td>127.1</td>
<td>131.5</td>
<td>161.6</td>
<td>55.8</td>
<td>43.1</td>
<td>100.9</td>
<td>46.7</td>
</tr>
</tbody>
</table>

† = Definitions for pharmacokinetic parameters are presented in Appendix III
‡ = a 0.5 mg/kg dose of ketorolac tromethamine equals 0.34 mg/kg ketorolac
†† = Harmonic mean
Figure 1. Semilogarithmic plot of mean (+ SD) plasma ketorolac concentration versus time after IV and PO administration of 0.5 mg/kg ketorolac tromethamine in dogs (n = 6).
Figure 2. Semilogarithmic scatter plot of ketorolac concentrations versus time after IV administration of 0.5 mg/kg of the drug in 2 dogs. The mean triexponential equation of the line $C_p(t) = 16.37e^{(-70.00)} + 2.6655e^{(-1.7755t)} + 0.1989e^{(-0.1798t)}$ has been superimposed over the raw data as a measure of goodness of fit.
Figure 3. Semilogarithmic plot of mean (+SD) ketorolac concentrations versus time after IV administration of 0.5 mg/kg of the drug. Each mean point represents 4 dogs unless indicated by parentheses. The mean biexponential equation of the line $C_p(t) = 1.6875e^{-2.2614t} + 1.2430e^{0.1389t}$ has been superimposed over the mean data as a measure of goodness of fit.
Figure 4. Semilogarithmic plot of mean (+SD) ketorolac concentrations versus time after PO administration of 0.5 mg/kg of the drug. Each mean point represents 6 dogs unless indicated by parentheses. The terminal equation of the line $C_p = 1.4672e^{(-0.1702)}$ has been superimposed over the mean data as a measure of goodness of fit.
Figure 5. Linear plot of mean plasma (+SD) ketorolac concentration versus time after IV and PO administration of 0.5 mg/kg ketorolac tromethamine in dogs (n = 6).
CHAPTER III

EFFECTS OF KETOROLAC (TORADOL™) ON CANINE NEUTROPHIL SUPEROXIDE ANION AND SECONDARY REACTIVE OXYGEN SPECIES PRODUCTION

INTRODUCTION

Ketorolac tromethamine (Toradol™) is a non-steroidal anti-inflammatory drug (NSAID) effective in the short term management of pain in humans and it is the only human NSAID in Canada available in both a parenteral and an oral (PO) form. It is a member of the pyrrolo-pyrrole group of NSAIDs with the chemical name (±)-5-benzoyl-2,3-dihydro-1H-pyrrolizine-1-carboxylic acid, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

It is now generally accepted that NSAIDs, including ketorolac, exert their anti-inflammatory, antipyretic and antinociceptive effects by inhibition of the cyclo-oxygenase (COX) isoenzymes 1 and 2 (Vane, 1971; Laneuville et al., 1994). Specifically, the NSAIDs inhibiting the COX-2 isoenzyme appear to have the most clinical value since it is the products of this inducible enzyme, the proinflammatory prostanoids, that produce inflammation and, indirectly, pain (Hayllar and Bjarnason, 1995).

Ketorolac's product monograph (Hoffman-La Roche Ltd., 1996) describes it is an NSAID, but it differs from other NSAIDs in that its analgesic property far exceeds its anti-inflammatory one. In humans, the analgesic potency of ketorolac is significantly greater than that of other NSAIDs (Buckley and Brogden, 1990;
Forbes et al., 1990). In experimental animal studies, ketorolac has produced up to 800 times the analgesic effect of acetylsalicylic acid (Rooks et al., 1982) and appears at least as or more effective than flunixin meglumine, butorphanol and oxymorphone for postoperative pain relief in dogs following arthrotomy or laparotomy (Mathews et al., 1996).

The mechanisms by which ketorolac produces its profound analgesic effect are still contentious. Previously, it was believed that the drug's primary mechanism of antinociception was a peripheral antiinflammatory effect; however, recent studies have since demonstrated a possible central action related to modulation of opioid receptor function through prostaglandin (PG) synthesis or via excitatory amino acid (e.g. glutamate) or neuropeptide (e.g. substance P) actions (Malmberg and Yaksh, 1992; Malmberg and Yaksh, 1993). These results are overlaid by the fact that ketorolac appears to exert an antinociceptive response especially in situations where inflammation is present (Rooks et al., 1982).

The reactive oxygen species (ROS), which include superoxide anion (O$_2^-$) and the secondary reactive oxygen species (SROS) including hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH•) and singlet oxygen (¹O$_2$), have received little attention with respect to afferent fibre activation during tissue injury, inflammation and subsequent pain. However, these small molecular weight highly reactive molecules may be central to the amplification of pain through inflammation. They are cytotoxic to surrounding cells and chemoattractive to
phagocytes (McCord, 1983). They increase conversion of arachidonic acid to the proinflammatory prostanoids and increase production of further \( \text{O}_2^- \) (Higgins, 1985). Hydrogen peroxide has been shown to enhance the effects of other inflammatory mediators, including bradykinin and \( \text{PGE}_2 \), while nitric oxide, another reactive oxygen containing molecule, induces a burning feeling upon intradermal injection (Holthusen and Arndt, 1994).

Previous research has demonstrated that NSAIDs such as indomethacin, ibuprofen, piroxicam, aspirin, phenylbutazone (Simchowitz et al., 1979; Kaplan et al., 1984) and ketorolac (Hyers et al., 1992) will inhibit pharmacologically-stimulated human neutrophil \( \text{O}_2^- \) production in a dose-dependent manner. We, therefore, hypothesized that canine neutrophil \( \text{O}_2^- \) and SROS production would be decreased after in vivo exposure to a therapeutically proven analgesic dose of ketorolac tromethamine (Mathews et al., 1996) The objective of this study was to evaluate whether clinically effective intravenous (IV) or oral (PO) doses of ketorolac tromethamine administered to dogs would alter neutrophil \( \text{O}_2^- \) and/or SROS production measured ex vivo.

**MATERIALS AND METHODS**

**Experimental Design**

The experimental design of this study was identical to that in Chapter II except that blood (8.5 mL) was only collected at 0 (before treatment), 1, 24 and 96 h following drug treatment.
Canine Neutrophil Isolation

Canine neutrophil isolation was by modification of a previously described method (Thomsen and Ahnfelt-Ronne, 1989). Blood samples (8.5 mL) were collected in vacutainers (Becton Dickinson, Rutherford, New Jersey, USA) containing 1.5 mL ACD (acid-citrate-dextrose). The samples were then transferred to 15 mL sterile polypropylene centrifuge tubes (Sarstedt, St. Laurent, Quebec, Canada) containing 4 mL 6% (w/v) dextran (MW= 200,000-300,000) (ICN Biomedicals Inc., Aurora, Ohio, USA) in 0.9% saline. The tubes were capped, inverted five times and the blood left to sediment (room temperature, 60 min). The plasma was then transferred to 50 mL sterile, polypropylene centrifuge tubes (Fisher Scientific, Toronto, Ontario, Canada), and residual red blood cells were lysed with 20 mL hypotonic saline for 30 seconds, with isotonicity being restored with 10 mL of hypertonic saline. This fluid was centrifuged (room temperature, 10 min, 400 x g) and the resulting cell pellet was resuspended in 0.6 mL of calcium-magnesium-free Hanks' balanced salt solution (HBSS; pH 7.4), layered over 2 mL of 30% (wt/vol) bovine serum albumin (Fraction V Powder, Sigma Chemical Co., St. Louis, Missouri, USA) in 0.9% saline and centrifuged (room temperature, 10 minutes, 400 x g). After centrifugation, the supernatant was discarded and the cell pellet resuspended in 1 mL of HBSS. An aliquot of cells was counted with a hemocytometer and then the total cells were diluted with HBSS to 2x10^6 viable neutrophils/mL and placed on ice. Mean (± SD) neutrophil purity (n=48) was 85.5 ± (5.5)%. The majority of
the contaminating cells after isolation were eosinophils, but a small number of the mononuclear cell series were also present. Wright's stained blood smears were prepared for differential and morphological examination. Viability of the neutrophils was estimated to be greater than 98% by trypan blue exclusion (Merchant et al., 1965).

**Preparation of Drugs and Reagents**

Ketorolac tromethamine (Toradol™, Hoffman-La Roche, Mississauga, Ontario, Canada; 10 mg/mL injectable solution) was purchased from the Ontario Veterinary College Hospital Pharmacy. The same pharmacy also prepared gelatin capsules containing ketorolac (ground from commercial 10 mg tablets) for each dog according to its weight. Stock solutions of 10⁻² M phorbol myristate acetate (PMA) and N-formyl-methionyl-leucyl-phenylalanine (FMLP) (both from Sigma Chemical Co., St. Louis, Missouri, USA) were prepared in dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, Missouri, USA) and frozen at -70°C in 50 μL aliquots. Working solutions of 10⁻⁵ M PMA and 10⁻⁶ M FMLP were prepared by diluting the stock solutions with HBSS. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, Missouri, USA) was prepared once weekly by diluting 2.6 mg in 7.5 mL of DMSO to a stock solution of 1.9 mM. Lucigenin (bis-N-methylacridinium nitrate; Sigma Chemical Co., St. Louis, Missouri, USA) in a 1.9 mM stock solution was prepared by diluting 5.0 mg in 5 mL HBSS. Working solutions (3 x 10⁻⁵ M) of both luminol and lucigenin were prepared by taking 1 mL of each stock solution and adding 18 mL
Luminol and Lucigenin-Dependent Chemiluminescence Assay

Chemiluminescence assays were performed within two hours of neutrophil isolation using a two-channelled lumi-aggregometer (Model 460-VS, Chrono-Log Corporation, Havertown, PA, USA) in glass cuvettes (7 x 52 mm) (Biopool Canada Inc., Burlington, Ontario, Canada). Each cuvette contained 100 μL of neutrophil suspension (200,000 cells) and 300 μL HBSS, and was pre-incubated at 4°C prior to chemiluminescence assay. Duplicate samples were used for each assay, and to each of these was added one of four possible combinations of a pharmacological stimulus (50 μL) and a chemiluminigenic probe (50 μL): luminol/PMA, luminol/FMLP, lucigenin/PMA, or lucigenin/FMLP. Phorbol myristate acetate or FMLP were always added last. A magnetic stir bar was placed in each cuvette to ensure a uniform suspension of neutrophils. Each cuvette was then placed in one of the two temperature controlled (37°C) chambers of the integrating lumi-aggregometer.

Data Analysis

The lumi-aggregometer was connected to a two channel chart recorder (Model SE120, BBC, Goerz Metrawatt, Fisher Scientific, Toronto, Ontario, Canada). The recorder channels were set to 100 mV maximum and paper speed at 30 cm/h. The dependent variable time, in minutes, was measured as the time from the moment the cuvettes were placed in the instrument until maximum chemiluminescence production (peak height) was attained. Peak
height in millivolts (mV) was measured from the baseline. In order to estimate total chemiluminescence production, peak time and peak height were multiplied and expressed as area under the curve (AUC; mV x min). Typical examples of canine neutrophil chemiluminescent response curves (pre-ketorolac tromethamine administration) using different combinations of chemiluminigenic probes and pharmacological stimuli are illustrated in Figure 6.

By means of a statistical software package (SAS™ For Windows, 1996) the absolute data were analyzed by an analysis of variance (ANOVA) for repeated measures. Data was analyzed for repeated measures over a 24 h period and then again over a 96 h period. Independent variables examined statistically were route of drug administration and time before and after drug administration. When the ANOVA indicated differences among treatments (p ≤ 0.05), multiple range tests (Bonferroni) between means were conducted. A 99% confidence level (p ≤ 0.01) was used when making multiple comparisons of four treatments (i.e. the 96 h period) and 98% (p ≤ 0.02) when comparing three treatments (i.e. the 24 h period).

In order to evaluate any changes in canine neutrophil reactive oxygen species (ROS) production after ketorolac tromethamine treatment relative to control values, the 0 h (pre-drug treatment) absolute values (mV x min) for each dog were converted to 100%. The absolute values of the treated samples (1, 24 and 96 h) were then divided into their corresponding absolute pre-drug treatment values (Appendices X-XIII) and the relative mean responses calculated.
RESULTS

Phorbol Myristate Acetate Neutrophil Stimulation

The absolute PMA-dependent chemiluminigenic responses of canine neutrophils using luminol as the chemiluminigenic probe are shown in Figure 7. There was no significant difference in secondary reactive oxygen species (SROS) production between treated neutrophils and pre-treatment neutrophils, nor among treatment groups over time. There was also no statistically significant difference between routes of administration.

The results in Figure 8 show absolute neutrophil chemiluminescent responses when lucigenin was used as the probe. There were no statistically significant differences in superoxide anion (O$_2^-$) production between treated neutrophils and pre-treatment neutrophils, nor among treatment groups over time. There were also no statistically significant differences in O$_2^-$ production between drug routes.

Figure 9 shows mean PMA-luminol-dependent neutrophil responses relative to the control (pre-drug treatment) response (100%). There appears to be no obvious differences in treated responses when compared to the control response, nor among treatment groups with respect to time and drug route.

Figure 10 shows mean PMA-lucigenin dependent neutrophil responses relative to the control response. Neutrophils isolated from dogs 24 h post IV ketorolac tromethamine administration produced noticeably less O$_2^-$ (41.0%) than did neutrophils isolated from the dogs pre-drug administration and than
those isolated 1 h post-treatment (38.4% less). When drug routes are compared 24 h post-drug administration, neutrophils isolated from the IV route produced 46.7% less O$_2^-$ than neutrophils isolated from the PO route. At 96 h post-drug administration, neutrophils isolated from the IV route produced 46.6% less O$_2^-$ than neutrophils isolated from the PO route. However, these differences were not significant.

**N-Formyl-Methionine-Leucine-Phenylalanine Neutrophil Stimulation**

The results of Figure 11 show absolute neutrophil chemiluminescent responses using luminol as the probe. There were no significant differences in secondary reactive oxygen species (SROS) production between treated neutrophils and pre-treatment neutrophils, nor among treatment groups over time. There was also no statistically significant difference between drug routes.

The absolute FMLP-dependent chemiluminogenic responses of canine neutrophils using lucigenin as the probe are shown in Figure 12. There were no significant differences in O$_2^-$ production between treated neutrophils and pre-treatment neutrophils, nor among treatment groups over time. In addition, there were no statistically significant differences in O$_2^-$ production between drug routes.

Figure 13 shows mean FMLP-luminol-dependent neutrophil responses relative to the control (pre-drug treatment) response (100%). There are no obvious differences in treated responses when compared to the control response nor among treatment groups with respect to time and drug route.
Figure 14 shows mean FMLP-lucigenin-dependent neutrophil responses relative to the control response. Neutrophils isolated from dogs at 24 and 96 h post IV ketorolac administration produced 59.9% and 53.9% less \( \text{O}_2^\cdot \) respectively, than did neutrophils isolated from dogs pre-drug administration. In addition, neutrophils isolated 24 and 96 h post IV ketorolac tromethamine administration produced 53.7% and 47.7% less \( \text{O}_2^\cdot \) than neutrophils isolated 1 h post-drug treatment, respectively. When drug routes are compared 24 h post-drug administration, neutrophils isolated from the IV route produced 51.8% less \( \text{O}_2^\cdot \) than neutrophils isolated from the PO route. However, these differences were not significant.

**DISCUSSION**

Evaluation of the data from this study shows that a single 0.5 mg/kg dose of ketorolac tromethamine administered IV or PO produced no significant effect on canine neutrophil ROS production over time and with respect to route. However, this study does suggest that ketorolac may have a biological effect on canine neutrophil \( \text{O}_2^\cdot \) production. When canine peripheral neutrophils were treated with this drug, and then pharmacologically stimulated with PMA, \( \text{O}_2^\cdot \) production was reduced 24 h post IV drug administration when compared with the pre-drug response. Superoxide anion production was also noticeably decreased at 24 and 96 h post IV drug administration when FMLP was the cell stimulus. Hyers et al. (1992) also demonstrated that ketorolac reduced neutrophil \( \text{O}_2^\cdot \) production after using FMLP as the pharmacological stimulus;
however, the concentration of ketorolac was one hundred times higher than the clinically effective plasma concentration of 0.1-0.3 μg/mL seen in humans (Benet et al., 1996), the neutrophils were from human subjects and the studies were conducted in vitro.

Our study is unique in that canine neutrophils were isolated, purified, and pharmacologically stimulated with both a complete secretagogue (FMLP) and an incomplete secretagogue (PMA) after in vivo incubation with ketorolac. The pharmacokinetic study reported in Chapter II of this thesis shows that, when ketorolac tromethamine is given IV to live animals at the clinically effective analgesic dose of 0.5 mg/kg, the mean maximum plasma concentration of IV ketorolac is 2.6 μg/mL (Appendix I). If the drug were given at a clinical dose and frequency of 0.5 mg/kg every eight hours for a minimum of 24 h, the average steady state plasma concentration would be 0.57 μg/mL. It can, therefore, be concluded that: 1) the effect of ketorolac on neutrophil O$_2^-$ production in this study occurred at a much lower concentration than in Hyer’s et al. 1992 study (3 μg/mL vs. 30 μg/mL); 2) the effect of ketorolac was at a therapeutic concentration; and 3) since the drug effect occurred in vivo, results may be more relevant to the clinical situation.

The oxygenation of biological substrates by activated neutrophils can yield electronically excited products capable of relaxing to ground state by photo emission. This native luminescence of the neutrophil can be quantified with the use of chemiluminigenic probes. Luminol and lucigenin react with certain

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reactive oxygen species and enhance light transmission. It has been found that luminol reacts with hydrogen peroxide (H₂O₂), probably with singlet oxygen (¹⁰O₂) but unlikely the hydroxyl radical (OH•). Lucigenin seems to be able to discriminate and only react with O₂⁻ and, therefore, appears to be a superior probe since it detects only O₂⁻ and is independent from the main hydrogen peroxide catalysts (e.g. myeloperoxidase) that form the remainder of the secondary reactive oxygen species (SROS) (Muller-Peddinghaus, 1984).

Both chemiluminigenic probes were used in this study, but only lucigenin-amplified chemiluminescence (i.e. O₂⁻ production) was suppressed by ketorolac when the effect of time after drug administration was considered. This finding suggests that: 1) ketorolac suppresses SROS production but luminol-dependent chemiluminescence is not sensitive enough to detect these changes; 2) ketorolac does not suppress the production of the SROS which include H₂O₂, ¹⁰O₂ and OH•; 3) ketorolac suppresses SROS production, but their production may be temporally limited by the action of superoxide dismutase, myeloperoxidase and glutathione peroxidase in the neutrophil; or 4) any combination of statements 1, 2 and 3 may be true.

Our work suggests that ketorolac may have two sites of action since it suppressed both FMLP- and PMA-stimulated canine neutrophil O₂⁻ production (Figure I - Literature Review). The bacterial chemoattractant and complete secretagogue FMLP acts by binding appropriate transmembrane receptors on the neutrophil which are coupled to guanosine triphosphate (GTP)-dependent
trimeric G_i protein. This in turn activates membrane associated phospholipase C which then cleaves phosphatidyl inositol-bisphosphate into the 'twin signals' inositol trisphosphate and diacylglycerol (Potter, 1992). Abramson and Leszczynska et al. (1991) suggest that the inhibition of neutrophil function by NSAIDs, which is independent of the inhibition of prostaglandin synthesis, is due to the disruption of G_i protein-dependent events. They were able to demonstrate that the NSAIDs salicylate and piroxicam prevent adenosine diphosphate (ADP)-ribosylation of the \( \alpha_i \)-subunit of the plasmalemmal G_i protein by autoradiographic analysis.

Concordant with this observation, and most likely as a consequence, both salicylate and piroxicam interfered with the capacity of pertussis toxin to inhibit neutrophil activation (pertussis toxin catalyzes the ADP ribosylation of the \( \alpha_i \) subunit of G_i protein). The most logical explanation of these observations is that ketorolac, like most NSAIDs, is a lipophilic anion which can insert into the lipid bilayer and alter membrane viscosity and influence ligand (e.g. FMLP) binding (Karnovsky et al., 1982; Valentino et al., 1987; Abramson et al., 1990). Thus, intercalation of ketorolac into the neutrophil membrane may prevent interaction of G_i protein with the phospholipase catalytic site and ultimately decrease \( \mathrm{O}_2^- \) production.

Work by Strom and Thomsen (1990) concluded that canine neutrophils lack FMLP receptors because they do not aggregate or migrate when exposed to FMLP at a concentration of \( 10^{-6} \) or \( 10^{-7} \) M. We, however, in almost every
assay (Appendix XIII) were able to stimulate canine neutrophils to produce $O_2^-$ and SROS using FMLP at a concentration of $10^{-7}$ M. It may be that higher concentrations of the chemotactic peptide are needed to produce aggregation and chemotaxis or that the FMLP receptor is inducible and is “switched on” or “left on” during isolation. Our isolation method differed from Strom and Thomsen and, therefore, this may be of importance.

If ketorolac inhibits $O_2^-$ production after stimulation by PMA, it is likely at a site of action ‘downstream’ from G-protein (Figure I - Literature Review). Phorbol myristate acetate acts by binding directly to the cytosolic Ca$^{2+}$-dependent protein kinase C enzyme and activating it (Nishizuka, 1984). It is possible that ketorolac prevents the association of protein kinase C with intracellular Ca$^{2+}$ and/or phosphatidyl serine. The two latter molecules react with protein kinase C at the inner membrane and participate in its activation. A second possibility is that ketorolac may prevent low-molecular-mass GTP binding proteins present in neutrophils from carrying out their functional roles. The Rap 1 protein has been demonstrated to be present in neutrophils (Bokoch and Quilliam, 1990), to be associated with NADPH-oxidase complex (Rotrosen et al., 1992), and to be phosphorylated by protein kinase C (Bokoch and Quilliam et al., 1990). Thus, ketorolac may disturb neutrophil protein kinase C activation of subcellular components like Rap 1 as well as membrane fluidity across the entire plasmalemma.
The results of this study may also suggest that, because ketorolac appears to decrease neutrophil $O_2^-$ production both at 24 and 96 h post IV administration: 1) disturbances in neutrophil plasmalemma fluidity and/or activation of subcellular components are not immediate after in vivo drug exposure; and/or 2) it is the ‘newly’ formed neutrophil population that is most affected by ketorolac or ketorolac affects neutrophils while in the bone marrow.

Finally, when time-dependent comparisons are observed more closely, one of the most interesting results of the study is that PO ketorolac administration did not have a suppressive effect on $O_2^-$ production. In other words, only in circumstances where the drug was given IV did the mean neutrophil $O_2^-$ production decrease between pre-treatment and post-treatment neutrophils. This may be due to the significant difference in maximum ketorolac plasma concentrations (Appendices I and II) between the two routes of drug administration. When the dogs received ketorolac tromethamine by the IV route, neutrophils were initially exposed to almost twice the maximum concentration of drug than when given the drug by the PO route (2.6 µg/mL vs. 1.6 µg/mL). Drug translocation across membranes is governed by concentration gradients and, therefore, if any drug effects are to be observed they should be observed in studies using IV ketorolac tromethamine.

The critical drug concentration argument is further supported by the fact that when the experimental data are examined for route-dependent percentage differences in neutrophil $O_2^-$ production after drug administration, neutrophils
isolated from dogs 24 h after IV drug administration produced almost 50% less PMA-dependent $O_2^-$ than neutrophils isolated from the PO route (Figure 10). In addition, 96 h post-drug administration, neutrophils isolated from the IV route produced almost 50% less $O_2^-$ than did neutrophils isolated from the PO route. Furthermore, neutrophils isolated from dogs 24 h after IV drug administration produced greater than 50% less FMLP-dependent $O_2^-$ than did neutrophils isolated from the PO route (Figure 14).

One final consideration in this discussion is that the experimental design of this study made it very difficult to document statistically significant suppression between treatments. Repeated measures studies are beneficial because measurements on the same unit tend to be less variable than measurements on different units and precision should increase. However, because of the intrinsic variability associated with the day to day ex vitro measurement of the neutrophil oxidative burst both between and within dogs, any in vitro effects of the drug on neutrophil ROS production would have had to be substantial since, when using multiple comparisons among treatments, the level of evidence against the null hypothesis (i.e. the P value) decreases as the number of comparisons increase (Kuehl, 1994).

Of further interest is that the circulating half-life of the normal mammalian neutrophil is 10-12 h (Golde and Balwin, 1992). Therefore, one could argue that the experimental unit of measurement (i.e. neutrophil population) is constantly changing over time within the dog causing concerns over the validity of using a
repeated measures design. A better approach may have been to isolate and culture neutrophils in vitro with ketorolac over time and use a simple Student’s t test (p ≤ 0.05) to test for differences in the production of ROS. Indeed, we have conducted multiple Student’s t tests on our experimental data and statistically significant difference among treatments can be found.

In conclusion, this study demonstrated that when ketorolac tromethamine was administered to dogs by the IV route at the effective postoperative analgesic dose of 0.5 mg/kg, it appeared to have a suppressive effect on the relative production of $O_2^-$ when compared to untreated neutrophils. Unfortunately, the suppression was not statistically significant and any inferences regarding drug effect are made with caution. Secondly, the use of two secretagogues (PMA and FMLP) in this study allowed us to speculate on plausible mechanisms of action for the drug.
Figure 6. Typical reactive oxygen species (ROS) response curves characterized by secretagogue and chemiluminigenic probe. Each curve represents the response of $2 \times 10^5$ untreated canine neutrophils. PMA = phorbol myristate acetate; FMLP = formyl-methionine-leucine-phenylalanine.
Figure 7. Mean (+SE) PMA-luminol-dependent neutrophil secondary reactive oxygen species (SROS) production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).
Figure 8. Mean (+SE) PMA-lucigenin-dependent neutrophil superoxide anion production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).
Figure 9. Mean (+SE) relative PMA-luminol-dependent neutrophil secondary reactive oxygen species (SROS) production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).
Figure 10. Mean (+SE) relative PMA-lucigenin-dependent neutrophil superoxide anion production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).
Figure 11. Mean (+ SE) FMLP-luminol-dependent neutrophil secondary reactive oxygen species (SROS) production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).
Figure 12. Mean (+ SE) FMLP-lucigenin-dependent neutrophil superoxide anion production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).
Figure 13. Mean (+SE) relative FMLP-luminol-dependent neutrophil secondary reactive oxygen species (SROS) production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).
Figure 14. Mean (+ SE) relative FMLP-lucigenin-dependent neutrophil superoxide anion production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).
CHAPTER IV

PLASMA PROSTAGLANDIN E₂ CONCENTRATIONS AFTER SINGLE DOSE ADMINISTRATION OF KETOROLAC TROMETHAMINE (TORADOL™) IN DOGS

INTRODUCTION

Prostaglandin E₂ (PGE₂) is an arachidonic acid oxygenation product. Other products of arachidonic acid oxygenation (eicosanoids) include PGD₂, PGF₂α, PGI₂ and thromboxane A₂ (TXA₂). The formation of PGE₂ and the other eicosanoids is through the action of the cyclo-oxygenase (COX) isoenzymes: COX-1 and COX-2.

Pain and the inflammatory process are mediated by a large number of soluble agents, including PGE₂. Prostaglandin E₂ is a vasodilator, platelet aggregator, and a potent pyretic and sensitizer of small diameter pain fibres (Higgins, 1985; Conlon, 1988; Davies and MacIntyre, 1992). However, the role played by PGE₂ and other eicosanoids in the inflammatory process is not as well defined as once supposed. In vitro, PGI₂ and PGs of the E series have demonstrated anti-inflammatory activity by inhibiting the activation of neutrophils, platelets and mononuclear cells (Weissman et al., 1971; Tateson et al., 1977; Weksler et al., 1977). In vivo, PGE suppresses the development of adjuvant arthritis in rats (Zurier, 1971), prevents the development of glomerulonephritis in NZB/NZW F₁ hybrid mice (Zurier et al., 1977) and inhibits the reverse passive Arthus reaction in mice (Kunkel et al., 1979). Prostaglandin
E₂ maintains microvascular integrity, regulation of cell proliferation and mucus production in the gastrointestinal tract (Robert, 1979; Cassidy and Lightfoot, 1980; Granger et al., 1980, Lewis, 1983; Levi et al., 1990; Wallace and Granger 1992). It also is important in renal hemodynamics, especially in the kidney receiving less than normal blood flow (Henrich et al., 1978).

Clinically, PGs have been used effectively as immunosuppressants in the treatment of acute renal transplant rejection (Moran et al., 1990) and hepatic failure (Sinclair et al., 1989). Commercially, the PGE₁ analog drug, misoprostol, is available for the prevention of non-steroidal anti-inflammatory drug (NSAID) induced gastritis.

As previously mentioned, the formation of PGE₂ and the other eicosanoids is through the action of the COX isoenzymes. Cyclooxygenase-2, is undetectable in most tissues under normal physiological conditions and, therefore, COX-2 production of PGE₂ is negligible. The COX-2 isoenzyme is inducible at sites of inflammation through the action of cytokines and endotoxins, with macrophages appearing to have the most abundant COX-2 activity (Hayllar and Bjarnason, 1995). Cyclo-oxygenase 1, by contrast is expressed in most tissues and is involved in cellular homeostasis, synthesizing PGs in response to physiological stimuli at a rate proportional to the availability of arachidonic acid.

Ketorolac tromethamine (Toradol™) and other NSAIDs inhibit endogenous PG secretion based on their affinity for the COX-1 isoenzyme (Vane, 1994). Numerous case reports of gastrointestinal ulceration (Ernstad and
Neumayer, 1992; Estes et al., 1993; Fuller and Kalekas, 1993; Wiedrick et al., 1994; Wolfe et al., 1994) and renal failure (Boras-Uber and Brackett, 1992; Schoch et al., 1992; Fong and Gora, 1993; O'Leary et al., 1993; Pearce et al., 1993; Perazella and Buller, 1993; Smith et al., 1993) have been reported in systemically ill hospitalized human patients receiving ketorolac tromethamine. Ketorolac tromethamine has been used successfully for the treatment of postoperative pain in young, healthy, well hydrated dogs using a parenteral dose of 0.5 mg/kg every six hours for three treatments and is currently recommended for postoperative orthopedic pain in dogs at a dose of 0.3 to 0.5 mg/kg every 8 - 12 h for one or two treatments (Mathews et al., 1996). However, dosing beyond this frequency is not recommended for postoperative pain management as gastrointestinal and renal toxicity may occur (Mathews, 1996). Therefore, the objectives of this study were to determine: 1) baseline plasma PG\(_E\_2\) in dogs and; 2) if, and to what magnitude, intravenous (IV) and oral (PO) ketorolac tromethamine, administered at a clinically effective single dose of 0.5 mg/kg (Mathews et al., 1996) could alter endogenous PG\(_E\_2\) plasma levels in dogs.

**MATERIALS AND METHODS**

The experimental design of this study was identical to Chapter II except that plasma was only harvested from blood samples (8.5 mL) collected at 0 (before treatment), 1, 24 and 96 h following drug treatment. The plasma was frozen in 2 mL sterile Cryotubes™(Nalgene Company, Rochester, New York, USA) at -70°C until analyzed.
Extraction of PGE$_2$ from Plasma

The extraction of PGE$_2$ from plasma was by a previously developed method (Caymen Chemical Company, Ann Arbor, MI, USA). On the day of analysis, 2 mL plasma samples were thawed to room temperature, vortexed for 30 sec, and centrifuged at room temperature for 5 min at 1000 X g. Aliquots of the samples (1.5 mL) were placed in 10 mL glass tubes (Fisher Scientific, Toronto, Ontario, Canada). To each sample, 7 picograms (pg) of tritium labeled 5,6,8,11,12,14,15-$^3$H(N)-PGE$_2$ (NEN Life Science Products, Boston, MA, USA) were added. Absolute ethanol (2 mL; USP; Commercial Alcohols Inc., Brampton, Ontario, Canada) was added to each sample and vortexed for 1 min. Samples were allowed to stand at 4°C for 5 min and then centrifuged (4°C, 10 min, 1500 X g) to remove precipitated proteins. The supernatant was decanted into 10 mL glass tubes (Fisher Scientific, Toronto, Ontario, Canada). Next, 8 mL of pH 4 adjusted (10 N HCl; Fisher Scientific, Toronto, Ontario, Canada) 0.1 M phosphate buffer (Caymen Chemicals, Ann Arbor, MI, USA) were added to each sample and vortexed for 30 sec. The samples were applied to Waters Sep-Pak$^\text{TM}$ C$_{18}$ cartridges (Waters Corp., Milford, MA, USA). Sep-Pak$^\text{TM}$ cartridges were first activated with 5 mL absolute ethanol followed by 5 mL of pure water (Milli-Q$^\text{TM}$ Water, Millipore Systems Inc., Mississauga, Ontario, Canada) before application of the sample. Samples were percolated through the Sep-Paks$^\text{TM}$ and washed with 5 mL water followed by 5 mL HPLC grade hexane (Fisher Scientific, Toronto, Ontario, Canada) and evaporated under nitrogen at 35°C.
The samples were then covered with Parafilm™ (Sigma Chemical Co., St. Louis, Missouri, USA) and placed in a -20°C freezer until further analysis.

**Prostaglandin E₂ Extraction Coefficient Calculations**

On the day of analysis, extracted samples were taken from the freezer and reconstituted with 1 mL EIA buffer (Caymen Chemical Company, Ann Arbor, MI, USA), sonicated (30 sec) and vortexed (30 sec). Each sample (0.5 mL) was added to 4 mL scintillation fluid (Ecolite™, ICN Biomedicals, Aurora, OH, USA) and counted in a liquid scintillation counter (Tracor Analytic Delta 300, N.C.S. Instrumentation Inc., Mississauga, Ontario, Canada). The remaining fluid (500 μL) was used for EIA analysis.

**Derivitization of Standards and Samples to Bicyclo PGE₂**

Prostaglandin E₂ is rapidly converted in vivo to its 13,14-dihydro-15-keto metabolite (Appendix XIV) with more than 90% of its metabolism occurring in the lungs. Unfortunately, this metabolite is not chemically stable and undergoes a variable amount of degradation to PGA products (Granstrom et al., 1987). For this reason, blood, urine or other samples from animals or human beings often contain very little intact PGE₂ and measurement of the metabolites is necessary to provide a reliable estimate of actual PGE₂ production. Therefore, extracted plasma samples and PGE₂ standards (Caymen Chemical Company, Ann Arbor, MI, USA) were incubated overnight with 150 μL of 1 M bicarbonate buffer to convert any PGE₂ to the stable derivative, bicyclo PGE₂.
**Bicyclo PGE₂ Assay**

Bicyclo PGE₂ concentrations were determined using a commercially available enzyme immunoassay (EIA) kit (Bicyclo PGE₂ Enzyme Immunoassay Kit [catalogue # 514531, 96 well] Caymen Chemical Company, Ann Arbor, MI, USA). The assay is based upon the competition between free bicyclo PGE₂ and bicyclo PGE₂ tracer for a limited number of bicyclo PGE₂-specific antiserum binding sites. The bicyclo PGE₂ is linked to an acetylcholinesterase molecule. When Ellman's reagent (which contains the substrate to acetylcholinesterase) is added to a well containing acetylcholinesterase, a reaction product is formed which absorbs strongly at 412 nm. A standard curve was generated based on the ratio of a particular standard well (B) to that of a maximum binding well (Bₐ). The initial concentration for each sample was calculated by identifying the percent B/Bₐ on the standard curve and reading the corresponding x-axis (Appendix XV). Samples having values of greater than 80% or less than 20% were not included in the final PGE₂ calculations since they fell out of the linear range of the curve. The final corrected PGE₂ concentration for each sample was calculated using the formula in Appendix XVI.

**Data Analysis**

The 96 well EIA plates were read at 412 nm using a plate reader (Microplate Autoreader, Model EL311, Biotek Instruments Inc., Highland Park, Winooski, Vermont, USA). Absorbance data were quantified using commercial software (Biotek™ Application Software 1987, Highland Park, Winooski, USA).
Vermont, USA). All PGE_{2} samples were read at three different dilutions. From these separate readings individual PGE_{2} concentrations were calculated and the mean was used in the final calculations.

By means of a statistical software package (SAS™ For Windows, 1996) the absolute data were analyzed by analysis of variance (ANOVA) for repeated measures. Independent variables examined statistically were route of drug administration and time before or after drug administration. When the ANOVA indicated differences among treatments (p<0.05), multiple range tests (Bonferroni) between means were conducted. A 99% confidence level (p<0.01) was used for all multiple range tests.

In order to evaluate plasma PGE_{2} changes after ketorolac tromethamine treatment relative to control values (i.e. similar to the evaluation of relative neutrophil ROS production before and after drug treatment; Chapter III), the 0 h (pre-drug treatment) absolute values (pg/mL) for each dog were converted to 100%. The absolute values of the treated samples (1, 24 and 96 h) were then divided into their corresponding absolute pre-drug treatment values (Appendices XVIIa and XVIIb) and the relative mean responses calculated.

RESULTS

The mean (± SD) PGE_{2} recovery from plasma samples (n = 48) in this study was 70.1 (± 3.0)%. The intra-assay coefficient of variation (CV) for extraction was 4.3%. The formula used to calculate PGE_{2} recovery from a sample (i.e. the extraction coefficient) is shown in Appendix XVI. The intra- and
inter-assay coefficient of variation for the enzyme immunoassay (EIA) was < 10%. The bicyclo PGE₂ detection limit was 1.5 pg/mL of plasma.

The mean plasma PGE₂ concentrations with respect to time after ketorolac tromethamine administration are shown in Figure 16. After IV administration of 0.5 mg/kg ketorolac tromethamine, 1 and 24 h plasma samples contained significantly less (p≤0.01) PGE₂ than did plasma samples collected from dogs pre-drug treatment. The 1 and 24 h plasma samples contained 45.6 pg/mL and 40.0 pg/mL, respectively, less PGE₂ than did pre-drug plasma samples. After PO administration of 0.5 mg/kg ketorolac tromethamine, 1 h plasma samples contained 37.4 pg/mL less PGE₂ than did pre-treatment plasma samples. This decrease was statistically significant (p≤0.01). In addition, 24 h post-drug administration samples contained 52.2 pg/mL less plasma PGE₂ than the 96 h plasma samples. This decrease was also statistically significant (p≤0.01). When the data were analyzed by ANOVA using drug route as the dependent variable, no statistically significant differences were detected among treatments (p≤0.05).

A comparison of the mean percent changes in plasma PGE₂ concentrations with respect to time after ketorolac tromethamine administration are shown in Figure 17. After IV administration of 0.5 mg/kg ketorolac tromethamine, 1 and 24 h plasma samples contained 53.3% and 47.3%, respectively, less PGE₂ than pre-drug samples. After PO administration of 0.5 mg/kg ketorolac tromethamine, 1 h plasma samples contained 50.1% less PGE₂
than did pre-treatment plasma samples, and 24 h plasma samples contained 74.7% less PGE2 than 96 h plasma samples.

**DISCUSSION**

The cyclo-oxygenase 1 (COX-1) dependent formation of the eicosanoids, and specifically PGE2, is determined by the physiological status of the host and the availability of arachidonic acid. Results of this study suggest that: 1) healthy dogs produce basal amounts of the prostanoid PGE2 at detectable levels; and 2) the endogenous arachidonic acid-dependent synthesis of PGE2 in healthy dogs, as represented by plasma PGE2 concentration, is reversibly decreased after treatment with a single dose of the NSAID ketorolac tromethamine by either the IV or PO route.

The measurement of PGE2 in dog plasma has been reported previously. Knapp et al. (1994) collected plasma from 10 healthy dogs in tubes containing ethylenediaminetetraacetic acid (EDTA) and the COX inhibitor, aspirin. The average plasma PGE2 concentration reported in their study was 35.0 ± 17.0 pg/mL, with a CV of 49.0%. The researchers used a radioimmunoassay method to detect bicyclo PGE2. In another study using venous blood drained from the pancreaticoduodenal vein of isolated and perfused canine pancreases, Kuroda et al. (1990) reported the mean endogenous plasma PGE2 in 11 healthy mongrel dogs to be 214.0 ± 46.0 pg/mL (CV = 21.0%) using high performance liquid chromatography as the quantitative assay. When the plasma was collected in
EDTA and the COX inhibitor indomethacin added, the mean PGE₂ concentration was 71.0 ± 20.0 pg/mL (CV = 28.0%).

In our study, blood was collected in heparinized containers without a COX inhibitor. The plasma PGE₂ concentration was moderately higher than in the aspirin treated blood (74.8 vs. 35.0 pg/mL) in Knapp’s study, and similar to indomethacin treated blood (74.8 vs. 71.0 pg/mL), but lower than untreated blood (74.8 vs. 214.0 pg/mL), in Kuroda’s study. The CV for our study averaged 33.0% using an enzyme immunoassay for the detection of bicyclic PGE₂. Therefore, it would appear that the collection of canine blood in heparinized tubes, rapid plasma isolation and freezing (-70°C), and subsequent enzyme immunoassay for bicyclic PGE₂ resulted in assay sensitivities (pg/mL) and CVs(%) similar to that reported from previous studies.

The vast majority of NSAIDs, including ketorolac tromethamine, are reported to serve as reversible, competitive inhibitors of COX activity, whereas the prototypical NSAID aspirin irreversibly inhibits COX activity. Thus, the effects of aspirin are related to the turnover rate of COX enzyme in different target tissue. For example, platelets are especially sensitive to the effects of aspirin since they have little or no capacity for protein biosynthesis and the production of COX enzyme. In practical terms, this means that a single dose of aspirin will inhibit the platelet COX for the life of the platelet (8 to 11 days), as is seen in humans (Lecompte et al., 1994). In this study, endogenous PGE₂ production was lowest 1 h after administration of ketorolac tromethamine by
either route. However, PGE$_2$ production at 24 h was greater than in the 1 h samples and was fully recovered, or greater (see PO results), by 96 h. Therefore, the activity of ketorolac appears to be reversible and is further supported by the Pallapies et al. (1994) study which demonstrated that a 30 mg parenteral dose of ketorolac tromethamine in humans significantly suppressed thromboxane B$_2$ (TXB$_2$) synthesis in vitro for only 24 h.

Although COX suppression by ketorolac tromethamine appears reversible, chronic suppression of COX-1-dependent PGE$_2$ by the drug may be associated with gastrointestinal ulceration in people and dogs. Prolonged administration of ketorolac tromethamine has also been associated with renal failure in hemodynamically compromised people. Therefore, suppression of endogenous plasma PGE$_2$ concentration in dogs given the drug by the IV or PO routes may be important if it is administered in multiple doses. Our work suggests that a single IV or PO dose of 0.5 mg/kg ketorolac tromethamine significantly decreases endogenous PGE$_2$ for a period of up to 24 h, with recovery occurring by 96 h. The same dose administered every eight hours by either the IV or PO route for a period of 24 h would result in steady state plasma levels of 0.57 $\mu$g/mL (Results; Chapter II). Consequently, it would be expected that this concentration of drug would maintain the pharmacodynamic suppression of endogenous PGE$_2$ until it was discontinued. Thus, patient selection, dose of drug and duration of therapy become vitally important when administering ketorolac tromethamine in both people and dogs (Mathews, 1996).
Moreover, results of this study and the ketorolac pharmacokinetic study in Chapter II suggest that if ketorolac tromethamine were to be administered repeatedly at a dose of 0.5 mg/kg for at least 24 h (IV or PO), continuous endogenous PGE$_2$ suppression would be expected. Therefore, simultaneous administration of a PGE$_2$ analog (e.g. misoprostol) is advocated.

In conclusion, the purpose of this study was threefold: 1) to report a reliable and repeatable method for the measurement of PGE$_2$ in the plasma of dogs; 2) to investigate and report the endogenous plasma PGE$_2$ concentration in healthy dogs, and 3) to confirm the presumptive reversible suppression of endogenous plasma PGE$_2$ in healthy dogs treated with a single clinically effective IV or PO dose (0.5 mg/kg) of the NSAID ketorolac tromethamine. Results of this study satisfy all three of these objectives.
Figure 15. Mean (+SE) plasma PGE$_2$ levels before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6). Means sharing the same letter are significantly (p<0.01) different from each other by repeated measures ANOVA and Bonferroni's test.
Figure 16. Mean (+ SE) plasma PGE₂ concentrations (expressed as a percentage) before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).
CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

The objectives of this investigation were: 1) to determine the pharmacokinetics of intravenous (IV) and oral (PO) ketorolac after administration of 0.5 mg/kg of the drug in healthy dogs; 2) to calculate pharmacologically-sound and rational IV and PO ketorolac tromethamine doses for dogs by correlating our pharmacokinetic data with known therapeutic plasma levels in humans and other animals; 3) to determine the effect, if any, of a single 0.5 mg/kg IV and/or PO dose of ketorolac tromethamine on canine neutrophil reactive oxygen species (ROS) production (ex vivo); 4) to measure endogenous PGE$_2$ concentration in the plasma of healthy dogs using a commercially available enzyme immunoassay (EIA) kit and, 5) to determine whether a single 0.5 mg/kg IV and/or PO dose of ketorolac tromethamine reversibly suppresses endogenous PGE$_2$ concentrations in the plasma of healthy dogs and, if so, to determine the magnitude and duration of suppression.

The pharmacokinetic study revealed that ketorolac tromethamine administered at a single IV dose of 0.5 mg/kg is well tolerated and demonstrates first order disposition kinetics with an average elimination half-life ($t_{1/2p}$) of 4.55 h, steady state volume of distribution ($V_{ss}$) of 0.33 L/kg and a plasma clearance ($Cl_p$) of 1.25 mL/kg/min. A single PO dose of the drug at 0.5 mg/kg takes an average of 0.85 h to reach maximum plasma concentration ($t_{max}$), has a PO
bioavailability (F) of 100.9% and is eliminated by first order kinetics with a $t_{1/2}$ of 4.07 h.

Mathews et al. (1996) found that administering an IM dose of 0.5 mg/kg ketorolac tromethamine every six hours for three treatments to healthy, well hydrated dogs recovering from shoulder arthrotomy or laparotomy surgery provided effective analgesia. Their clinical impression of the drug was that a single 0.3 -0.5 mg/kg IM or IV dose is effective within 1 h and lasts as long as 12 h post administration (Mathews, 1996). Our data show mean plasma ketorolac levels to be approximately 1 µg/mL 0.75 h after drug administration by either route. The data also show mean plasma ketorolac levels to be approximately 0.15 µg/mL 12 h after drug administration by either route. Therefore, if plasma ketorolac concentrations were a guide for therapeutic drug monitoring, a reasonable goal would be to confine peak ketorolac levels to no more than 1.0 µg/mL 1 h after drug administration and trough levels to no more than 0.15 µg/mL 12 h after drug administration. However, these levels should only be maintained for a period of 24 h or less because of the potentially toxic effects of the drug (Mathews, 1996).

In humans, a steady state EC$_{50}$ (effective analgesic plasma concentration in 50% of the population) of 0.1-0.3 µg/mL is reported for ketorolac (Benet et al., 1996). Correlation of our pharmacokinetic data with the Mathews et al. (1996) postoperative pain study in dogs suggests an effective analgesic dose of 0.5 mg/kg administered by either the IV or PO route every 8-12 h for no more than
24 h (five half-lives) should yield an average steady state concentration ($C_{ave}$) of 0.57 μg/mL. Administering the drug every 12 h at the same dose IV or PO should yield a $C_{ave}$ of 0.38 μg/mL. These pharmacokinetic findings must be interpreted cautiously, however, since our study was carried out on healthy dogs and only a single dose of the drug was administered. In the future, a multiple dose study of ketorolac administered at 8-12 h intervals in healthy dogs should be conducted to validate the pharmacokinetic parameters found in this study and to look for evidence of accumulation of drug in the animal.

Neutrophil function studies revealed that chemiluminescence was a satisfactory assay for repeatedly measuring canine neutrophil production of superoxide anion ($O_2^-$) and secondary reactive oxygen species (SROS), including singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^•$). Both luminol and lucigenin were suitable chemiluminigenic probes, amplifying the native neutrophil chemiluminigenic response into a quantifiable millivolt (mV) response.

When ketorolac tromethamine was administered to dogs by the IV route at the effective postoperative analgesic dose of 0.5 mg/kg, it had a suppressive effect on the relative production of neutrophil $O_2^-$ when compared to control, although it was not statistically significant. Reactive oxygen species (ROS) are pro-inflammatory (Higgins, 1985) and since ketorolac appears to exert an antinociceptive response especially when inflammation is present (Rooks et al., 1982) suggests that the biological inhibition of $O_2^-$ by ketorolac may play a role in
the mechanisms by which the drug produces analgesia. These results also indicate that ketorolac tromethamine may also have therapeutic usefulness in a variety of conditions thought to be mediated by the canine neutrophil including pain, inflammation and sepsis.

When compared to the IV route, the biological inhibition of $O_2^\cdot$ did not occur when ketorolac tromethamine was administered by the PO route. This difference may be due to the fact that mean maximum ketorolac plasma concentrations for the PO route were less than for the IV route, thus suggesting that the pharmacodynamic effect of ketorolac may be concentration-dependent. Furthermore, the inhibition of $O_2^\cdot$ after IV ketorolac tromethamine administration was not immediate (i.e. 24 and 96 h), indicating that the biological effect of ketorolac may be dependent on the time that it takes to translocate across biological membranes (e.g. neutrophil plasmalemma).

The relative suppression of $O_2^\cdot$ production was observed when phorbol myristate acetate (PMA) was used as the pharmacological stimulus, thus suggesting ketorolac’s site of action may be: 1) the inner surface of the membrane where protein kinase C (PKC) is activated (Nishizuka, 1984); and/or 2) a PKC substrate such as the nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase associated rap 1 guanosine triphosphate (GTP) binding protein (Bokoch et al, 1990).

Superoxide anion suppression also appeared to be present when the secretagogue formyl-methionine-leucine-phenylalanine (FMLP) was used as the
cell stimulus. This supports: 1) a second possible site of action of ketorolac and other NSAIDs to be associated with the disruption of Gi protein-dependent events (Abramson et al., 1991); and 2) the expression of FMLP receptors on the cell surface of canine neutrophils. This observation must be treated with some degree of caution, however, since canine neutrophil stimulation with FMLP did not occur in every assay (Appendix XIII). Consequently, observations in these assays may not be truly \( O_2^- \) suppression, but a failure of FMLP to stimulate the cells. One possible reason for the discrepancy in neutrophil stimulation with FMLP is that FMLP receptors may not be constitutively expressed on the canine neutrophil and are dependent on some ill-defined induction mechanism.

The ability to directly manipulate neutrophil activity in vitro through the use of artificial stimulants such as PMA and FMLP is a subtle reminder of the many natural influences that this cell experiences in vivo. Therefore, to say that \( O_2^- \) suppression by ketorolac is strictly a direct effect may be an oversimplification. Neutrophil activity, both in vivo and in vitro, is influenced by many soluble mediators, including cytokines, hormones and bioactive lipids and ketorolac may influence their release and, subsequently, interfere with neutrophil ROS production. For example, ketorolac may interfere with the release of the monocyte dependent pyrogenic cytokines, interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-\( \alpha \)) and IL-6 that prime various pathways contributing to the activation of NADPH oxidase (Smith, 1994). The bioactive lipid leukotriene \( B_4 \) is a strong neutrophil chemoattractant released by both the endothelium and
neutrophil that serves as a potent neutrophil priming agent (Kubes, 1993). Ketorolac may influence its release and indirectly inhibit neutrophil activation. Finally, neuroendocrine hormones such as insulin-like growth factor 1 (IGF-1) (Fu et al., 1992), prolactin (Gala, 1991) and atrial natriuretic factor (ANF) (Wiedermann et al., 1992) appear to be strong neutrophil priming agents whose actions may be influenced by ketorolac.

The final study of this thesis was the measurement of plasma prostaglandin E$_2$ (PGE$_2$) before and after treatment of dogs with a single IV or PO dose of 0.5 mg/kg ketorolac tromethamine. Results showed that healthy dogs produce endogenous amounts of PGE$_2$ at detectable levels and that there was a significant ($p<0.01$) decrease in the absolute concentration of endogenous plasma PGE$_2$ at 1 h (IV and PO route) and 24 h (IV route) after drug administration. The inhibition of cyclo-oxygenase (COX) dependent PGE$_2$ production, however, appears to be reversible, with PGE$_2$ levels recovering to pre-treatment levels by 96 h after drug administration.

In both the IV and PO pharmacokinetic studies, mean plasma ketorolac concentrations 1 h after drug administration are approximately 1 μg/mL. At 24 h, the plasma concentrations in the IV and PO study were 0.055 μg/mL and 0.086 μg/mL, respectively (Appendices I and II). At 96 h there was no detectable drug in the plasma. These results suggest that inhibition of endogenous PGE$_2$ production is dependent on the plasma ketorolac concentration. In contrast, the effect of ketorolac in the neutrophil experiment was delayed and did not appear
to correlate with plasma concentrations. This temporal difference in drug effect may be due to the fact that COX is expressed in the most well perfused tissues of the body, including the lung, kidney, liver and stomach (O'Neill and Ford-Hutchinson, 1993); therefore, drug effect is not limited by drug distribution. However, the effect of ketorolac on neutrophil O$_2^-$ production may be dependent on the ability of the drug to translocate across the cell membrane; therefore, any drug effect may be delayed.

Finally, the inhibition of endogenous plasma PGE$_2$ production in normal dogs occurred with a therapeutic dose of ketorolac tromethamine. Prostaglandin E$_2$ has important physiological roles in the gastrointestinal tract (Rask-Madsen et al., 1990) and the vasodilating PGs (including PGE$_2$ and PGl$_2$) play a minor role in maintaining renal perfusion in healthy animals and human beings (Schnerman et al., 1984). However, whether NSAID-induced PGE$_2$ inhibition on its own is enough to initiate intestinal damage is contentious and the majority of clinical studies have demonstrated that inhibition of the COX system with NSAIDs does not have a significant effect on renal hemodynamics in normal kidneys (Lifschitz, 1983).

While the cytoprotective importance of PGE$_2$ in the healthy person or animal may be disputable, its role in the hemodynamically compromised or post-anesthetic patient may be critical. The interruption of normal blood flow to the gastrointestinal tract and/or kidneys may dramatically increase the cytoprotective role of PGE$_2$. Therefore, treatment of individuals with unopposed
vasoconstriction, or decreased actual or effective circulating blood volume, with ketorolac tromethamine and other potent COX inhibitors may increase the risk for gastric ulceration and/or renal failure.

**Future Research Considerations**

Our research has demonstrated that the pharmacokinetic profile of ketorolac tromethamine in dogs when administered at a single dose of 0.5 mg/kg by either the IV or PO route is fairly similar to that observed in humans (Jung et al., 1988). Also, the drug appears to have a suppressive effect on canine neutrophil superoxide anion (O$_2^-$) production, although using the present experimental design we were unable to demonstrate statistical significance. The great variability in canine neutrophil oxidative burst in both control and treated dogs may also have contributed to the lack of significant findings. Human neutrophils also show great variability in the magnitude of the oxidative burst when isolated from healthy subjects and stimulated in vitro (Smith and Weidemann, 1993). Therefore, future research should determine the normal range of the major canine neutrophil functions from a larger population of healthy dogs to better facilitate comparisons of the effects of treatments on neutrophils.

Finally, our research documented that a single 0.5 mg/kg IV or PO dose of ketorolac tromethamine has a profound effect on endogenous PGE$_2$ concentration in the plasma of healthy dogs. By 1 h after administration of the drug by either route there was a significant suppression of plasma levels
(p≤0.01). By 24 h the suppression was still significant in the IV group. This suppression was reversible, however, and plasma PGE$_2$ recovered to pre-drug treatment values by 96 h.

Although these results were informative, future studies are required to determine: 1) whether multiple doses of ketorolac tromethamine over a period of 24 h or more (5 half-lives) continuously suppresses endogenous PGE$_2$ in the plasma of healthy dogs; 2) whether the cytoprotective PGE$_1$ analog, misoprostol, administered simultaneously with ketorolac to dogs reduces the risk of developing gastric and/or renal toxicity; and 3) measure the relative selectivity of ketorolac for COX-1 and COX-2 inhibition since it is proposed that NSAIDs selective for COX-2 may be more therapeutic and less toxic (Vane, 1994). In addition to the above three areas of investigation, multiple dose pharmacokinetic studies of ketorolac at steady state are essential before sound recommendations for multiple dose regimens can be made.
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Appendix I. Ketorolac concentrations (μg/mL) in dog plasma following IV administration of a 0.34 mg/kg dose (n = 6).

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Appendix II. Ketorolac concentrations (µg/mL) in dog plasma following PO administration of a 0.34 mg/kg dose (n = 6).

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### Appendix III. Definitions of pharmacokinetic parameters

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<td>$t_{\text{max}}$ (min)</td>
<td>the time to reach peak drug concentration</td>
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<td>$C_{\text{max}}$</td>
<td>maximum concentration of a drug derived from the concentration vs. time curve</td>
</tr>
<tr>
<td>$C_0$</td>
<td>maximum concentration of a drug derived from the addition of a curves Y-intercept(s)</td>
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<td>$P$ ($\mu$g/mL)</td>
<td>amount of drug in the body at initial distributive phase in a triexponential model (Y-intercept)</td>
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<tr>
<td>$\pi$ (1/h)</td>
<td>slope of the initial distribution phase in a triexponential model</td>
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<td>amount of drug in the body at distributive phase in a biexponential model (Y-intercept)</td>
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<td>$\alpha$ (1/h)</td>
<td>Slope of the distribution phase in a biexponential model</td>
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<tr>
<td>$B$ ($\mu$g/mL)</td>
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<td>$\beta$ (1/h)</td>
<td>slope of the elimination phase</td>
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<td>$t_{1/2\beta}$ (h)</td>
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<td>$Cl_p$ (mL/kg/min)</td>
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<td>$AUMC_{0-96\text{h}}$ ($\mu g \cdot h^2/\text{mL}$)</td>
<td>area under the first moment curve from time = 0 to time = 96 h</td>
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<td>$AUC_{0-96\text{h}}$ ($\mu g \cdot h/\text{mL}$)</td>
<td>total area under the plasma drug concentration versus time curve from time = 0 to time = 96 h</td>
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<tr>
<td>$F_{\text{oral}}$ (%)</td>
<td>the bioavailability or fraction of an oral drug dose that reaches systemic circulation compared to the same IV dose</td>
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Appendix IV. Semilogarithmic plot of dog CAR plasma ketorolac concentration versus time after IV and PO administration of 0.5 mg/kg ketorolac tromethamine.
Appendix V. Semilogarithmic plot of dog CAS plasma ketorolac concentration versus time after IV and PO administration of 0.5 mg/kg ketorolac tromethamine
Appendix VI. Semilogarithmic plot of dog CHA plasma ketorolac concentration versus time after IV and PO administration of 0.5 mg/kg ketorolac tromethamine.
Appendix VII. Semilogarithmic plot of dog DAP plasma ketorolac concentration versus time after IV and PO administration of 0.5 mg/kg ketorolac tromethamine.
Appendix VIII. Semilogarithmic plot of dog GRA plasma ketorolac concentration versus time after IV and PO administration of 0.5 mg/kg ketorolac tromethamine.
Appendix IX. Semilogarithmic plot of dog JAQ plasma ketorolac concentration versus time after IV and PO administration of 0.5 mg/kg ketorolac tromethamine.
Appendix X. Absolute and relative PMA-luminol-dependent secondary reactive oxygen species (SROS) production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).

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NA = not applicable
Appendix XI. Absolute and relative PMA-lucigenin-dependent superoxide anion production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).

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NA = not applicable
Appendix XII. Absolute and relative FMLP-luminol-dependent secondary reactive oxygen species (SROS) production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).

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NA = not applicable
Appendix XIII. Absolute and relative FMLP-lucigenin-dependent superoxide anion production before and after treating dogs with 0.5 mg/kg ketorolac tromethamine (n = 6).

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<td>107.1</td>
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<td>7.8</td>
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</table>

NA = not applicable
Appendix XIV. Prostaglandin E₂ Metabolism.

Prostaglandin E₂

1½ - 3 minutes in plasma → 15-OH PGDHase → 15-keto Prostaglandin E₂

15-Oxoprostaglandin Δ⁹-reductase → 13,14-dihydro-15-keto Prostaglandin A₂ → Na₂CO₃ → Bicyclo Prostaglandin E₂ (stable derivative)

13,14-dihydro-15-keto Prostaglandin E₂ (unstable) → Na₂CO₃
Appendix XV. Typical standard curve generated using commercially supplied PGE$_2$ standards.
Appendix XVI. Formula used to calculate PGE$_2$ extraction coefficients (%) and corrected final concentrations (µg/mL) from plasma samples.

Recovery Factor = \( \frac{2 \times \text{cpm of sample}}{^{3}\text{H}-\text{13,14-dh-15-k PGE}_2 \text{ added to sample (cpm)}} \)

13,14-dh-15-k PGE$_2$ (pg) in extracted sample =
\[ \frac{\text{Value from EIA (pg/ml)}}{\text{Recovery Factor}} - \text{added } ^{3}\text{H}-\text{13,14-dh-15-k PGE}_2 \text{ (pg)} \]

Total 13,14-dh-15-k PGE$_2$ in sample (pg/ml) =
\[ \frac{13,14\text{-dh-15-k PGE}_2 \text{ (pg) in extracted sample}}{\text{Volume of sample used for purification (ml)}} \]
Appendix XVIIa. Absolute Plasma Prostaglandin E₂ Concentrations in Dogs Before and After IV and PO Single Dose Administration of 0.5 mg/kg Ketorolac Tromethamine (n = 6).

<table>
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<th>Cha</th>
<th>Cas</th>
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<th>Car</th>
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* = Means sharing the same letter are significantly different from each other (p ≤ 0.01) by repeated measures ANOVA and Bonferroni's test with respect to time after administration of ketorolac tromethamine.
Appendix XVIIb. Relative Plasma Prostaglandin E₂ Concentrations in Dogs Before and After Single Dose IV and PO Administration of 0.5 mg/kg Ketorolac Tromethamine (n = 6).

<table>
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<th>Gra</th>
<th>Cha</th>
<th>Cas</th>
<th>Jaq</th>
<th>Car</th>
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NA = not applicable.